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MEIOTIC ARREST IN OOCYTES REGULATED BY A *SPISULA* FACTOR

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ABSTRACT

Ovarian oocytes of the mouse, *Spisula* and *Chaetopterus* are arrested in the dictyate stage of meiotic prophase. Upon isolation, mouse and *Chaetopterus* oocytes undergo spontaneous maturation manifested by germinal vesicle breakdown (GVBD) while *Spisula* oocytes retain their germinal vesicles. The present report describes a substance isolated from *Spisula* with meiotic arresting activity. The substance was purified from *Spisula* tissues by 70% ethanol extraction, chromatography on a Dowex 1-×8 column, and reversed phase HPLC.

GVBD in *Spisula* oocytes can be induced by insemination (5×10^4 sperm/ml) or treatment with serotonin or KCl. A crude ethanolic extract of *Spisula* tissues blocked oocyte maturation induced with serotonin. Upon washing in natural seawater (NSW) the oocytes proceeded to undergo maturation. Forskolin and 3-isobutyl-1-methylxanthine (IBMX) blocked GVBD induced with sperm or serotonin. Dibutyryl cyclic adenosine 3′,5′-monophosphate (dbcAMP) and dibutyryl cyclic guanosine 3′,5′-monophosphate (dbcGMP) blocked GVBD induced by sperm, while higher concentrations of these nucleotides were required to block serotonin-induced GVBD. However, none of the compounds tested including the *Spisula* extract influenced KCl-induced GVBD.

Isolated *Chaetopterus* oocytes suspended in artificial seawater (ASW) retained their GV and underwent spontaneous GVBD when placed in NSW. *Spisula* extract, dbcAMP and dbcGMP inhibited maturation of *Chaetopterus* oocytes suspended in NSW. dbcGMP, however, was a more potent inhibitor than dbcAMP, suggesting that cGMP may be the factor that maintains meiotic arrest in *Chaetopterus* oocytes. Spontaneous maturation of mouse oocytes was blocked by HPLC-purified *Spisula* factor at a concentration of 5 μg/ml in combination with 50 μ*M* dbcAMP. We conclude that a substance found in *Spisula* tissues sustains meiotic arrest in mouse, *Spisula,* and *Chaetopterus* oocytes.

INTRODUCTION

Female gametes develop to a specific stage of meiosis and remain arrested at that stage while in the ovary and in some species even after spawning (Kanatani, 1973; Tsafriri, 1978; Masui and Clarke, 1979; Channing *et al.,* 1980; Meijer and Guerrier, 1984). The factor(s) that maintains oocytes in the arrested state of prophase I of meiosis has not been identified. One of the arresting factors may be intracellular cyclic nucleotides (Dekel and Beers, 1978). This hypothesis is based on the findings that compounds that increase intracellular cyclic adenosine 3′,5′-monophosphate (cAMP), *e.g.,* cholera toxin, forskolin, dibutyryl cAMP (dbcAMP), theophylline and 3-isobutyl-1-methylxanthine (IBMX), also inhibit spontaneous or hormone-induced maturation

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of isolated oocytes (Maller *et al.*, 1979; Hubbard and Terranova, 1982; Powers and Paleos, 1982; Tsafriri *et al.*, 1982; Sato and Koide, 1984a; Cho *et al.*, 1974; Nekola and Smith, 1975; Stern and Wassarman, 1975; Hillensjo, 1977; Hillensjo *et al.*, 1978; Schorderet-Slatkine *et al.*, 1978). We have demonstrated that bovine follicular fluid contains substances that sustain meiotic arrest (Sato and Koide, 1984b). Evidence will be presented showing that a substance isolated from *Spisula* sustains meiotic arrest in mouse, *Spisula,* and *Chaetopterus* oocytes.

Materials and Methods

The present investigation was conducted at the Marine Biological Laboratory, Woods Hole, Massachusetts, during July and August of 1983 and 1984. Specimens of *Spisula soldissima* and *Chaetopterus pergamentaceus* were obtained from the Department of Marine Resources. Prepubertal Swiss mice (Nelson-Collins strain) of approximnately 15–20 g body weight were obtained from the animal facility at The Rockefeller University, New York.

Chemicals

Forskolin (HL 362, lot no. RC 1622) was a gift of Hoechst-Roussel Pharmaceuticals Inc., New Jersey. IBMX, serotonin (5-hydroxytryptamine hydrochloride), dbcAMP, dibutyryl cyclic guanosine 3′,5′-monophosphate (dbcGMP), and other chemicals were purchased from Sigma Chemicals.

Preparation of oocytes and sperm

Ovaries from female *Spisula* and *Chaetopterus* were excised, minced in artificial seawater (ASW), and strained through a pad of cheesecloth into a large beaker containing ASW (Cavanaugh, 1974). The oocytes were allowed to settle by gravity and the suspension medium was aspirated off. This washing process was repeated at least three times. Suspensions of oocytes prepared in this manner were used within one hour after extirpation. Excised testes from *Spisula* were stored in the refrigerator until used. During the storage period, a milky seminal fluid containing sperm was collected. This sperm suspension was drawn into calibrated pipettes, to permit accurate measurement of sperm concentrations.

Mouse oocytes were obtained as described in a previous paper (Sato and Koide, 1984a).

Induction of germinal vesicle breakdown (GVBD) of Spisula *and* Chaetopterus *oocytes*

One drop of a suspension containing approximately 2000 *Spisula* oocytes was added to 5 ml of ASW contained in a Falcon tissue culture dish (55 mm in diameter). GVBD of the *Spisula* oocytes was induced by insemination (final concentration of sperm: 8×10^6/ml), or by treatment with KCl (35 mM) or serotonin (50 μM). GVBD of *Chaetopterus* oocytes was induced by transfer into NSW. After 30 min, oocytes were examined under a dissecting microscope to determine the presence or absence of germinal vesicles. At least 500 oocytes were scored for each determination.

Mouse oocytes

Oocytes were cultured immediately after recovery in an incubator continuously flushed with an atmosphere of 95% air and 5% CO_2 saturated with water at

37 ± 0.5°C. About 10 to 15 oocytes were pipetted into 0.2 ml of medium under light paraffin oil. After 3 h of incubation, the oocytes were scored for the presence or absence of intact germinal vesicles.

Purification of a meiosis arresting factor from Spisula *tissues*

Spisula tissues (muscles and gonads) were excised, cut into small pieces, suspended in 70% ethanol, and homogenized in a Waring blendor for 5 min. The resulting brei was left standing at 4°C overnight, and filtered. The ethanolic extract (500 ml) was concentrated in a rotary vacuum evaporator at 38°C. The concentrated extract was dialyzed against 1 liter of distilled water at 4°C overnight, twice. The diffusate was applied to a Dowex 1-×8 resin column (6 × 10 cm) (200–400 mesh, chloride form, Bio-Rad) and eluted in sequence with 500 ml each of H_2O, 0.005 *N* HCl and 750 ml of 0.1 *N* HCl. The pooled samples were concentrated to 50 ml and lyophilized. The lyophilized powder was dissolved in 50 ml of water, neutralized with 1 *N* NaOH to pH 6.5 and lyophilized. The dry powder was dissolved in culture medium and assayed for activity. The active fraction located within the 0.1 *N* HCl fraction was further purified by high performance liquid chromatography using a Waters Radial Pak column. The column was eluted with 0.05 *M* $NH_4H_2PO_4$, and 10% aqueous methanol, pH 5.0, at a flow rate of 2.0 ml/min.

Assay for maturation inhibitory activity

The crude ethanol extract of *Spisula* tissues, and the oviducts purified by chromatography on Dowex 1-×8 and reversed phase HPLC were tested for GVBD inhibitory activity with oocytes of *Spisula, Chaetopterus,* and the mouse. The test samples were added to the suspension medium at varying concentrations. The percent inhibition was calculated according to the following formula:

$$\% \text{ inhibition} = \frac{\% \text{ oocytes GVBD (control)} - \% \text{ oocytes GVBD (expt)}}{\% \text{ oocytes GVBD (control)}} \times 100.$$

RESULTS

Spisula *oocytes*

The ethanolic extract of *Spisula* tissues at concentrations of 1 mg/ml or higher inhibited oocyte maturation induced with serotonin (Fig. 1). However, KCl-induced maturation was not affected. It was further observed that *Spisula* muscle extract at 1 mg/ml or higher suppressed motility of *Spisula* sperm. Because of the inhibitory influence on sperm motility, we were unable to evaluate the effect of *Spisula* muscle extract on sperm-induced GVBD of *Spisula* oocytes.

Spisula oocytes, induced by serotonin (5 μM) underwent GVBD within 15 min. (Fig. 2). After 30 minutes a gradual increase in the frequency of GVBD had occurred and by 30 min about 78% of oocytes had undergone GVBD. When oocytes were incubated in a medium containing *Spisula* extract at a concentration of 3 mg/ml, marked inhibition of GVBD resulted, *i.e.,* 88% of oocytes retained intact GV after 30 min of incubation (Fig. 2). When the treated oocytes were transferred to a control medium, 70% underwent GVBD within 15 min (Fig. 2). Hence, the inhibition affected by *Spisula* extract is reversible.

As control substances, forskolin and IBMX were tested. At concentrations of 5 μg/ml and higher both compounds inhibited dramatically the maturation of oocytes

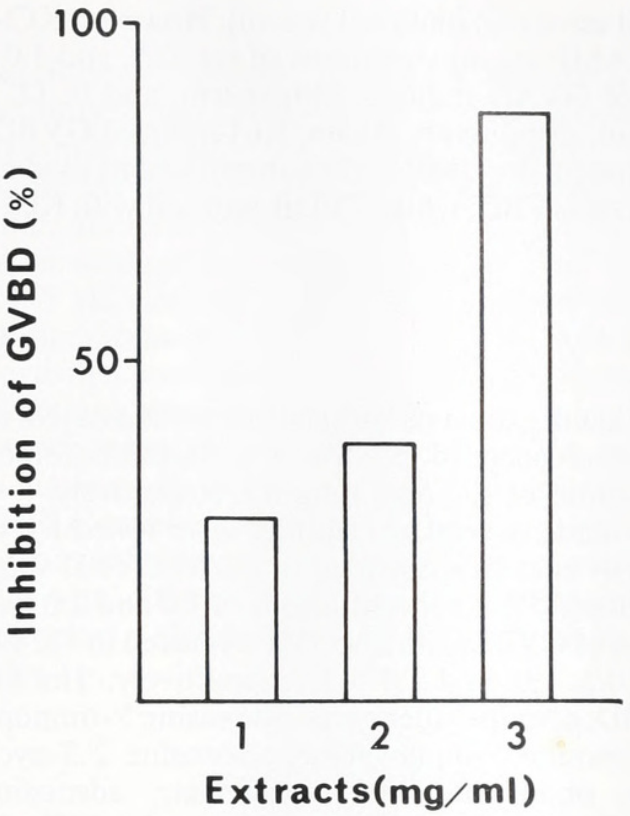

FIGURE 1. Effect of *Spisula* factor on the maturation of *Spisula* oocytes incubated with serotonin (5 μM). A lyophilized 70% ethanol extract of *Spisula* tissues was used.

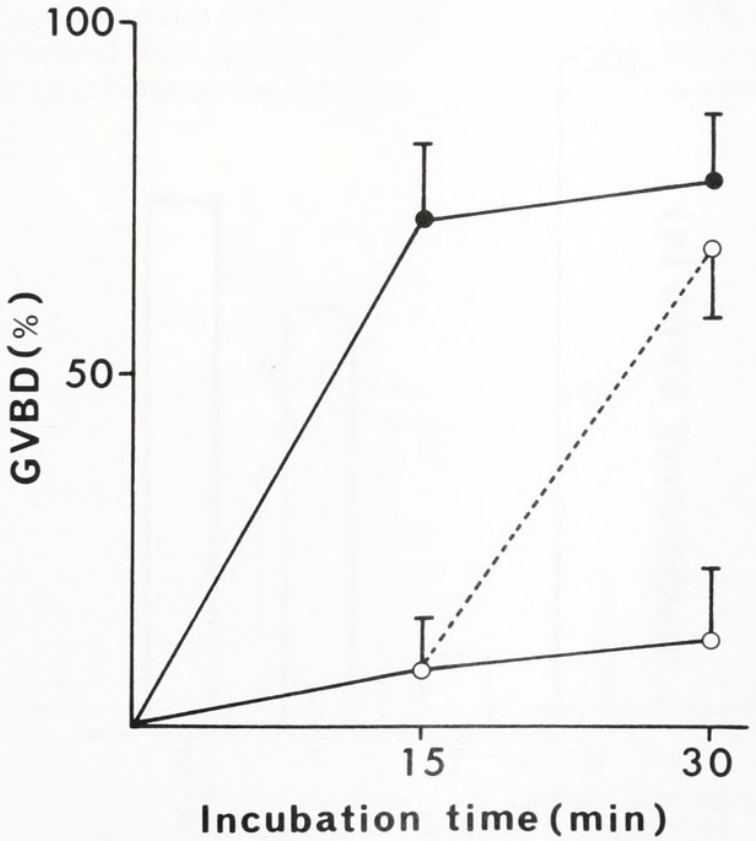

FIGURE 2. Effect of *Spisula* extract on the time course of GVBD of *Spisula* oocyte induced with serotonin (5 μM). ● —— ●, oocytes incubated in control medium, ○ —— ○, incubated with *Spisula* extract, ○ ----- ○, after 15 min of treatment, the oocytes were washed in ASW three times, and transferred to control medium.

induced by sperm and serotonin (data not shown). However, KCl-induced maturation was not affected. dbcAMP at concentrations of 0.1, 0.5, and 1.0 m*M*, exerted 28, 68 and 70% inhibition of GVBD induced with sperm, and 0, 11, and 24% inhibition induced with serotonin, respectively. Again, KCl-induced GVBD was not affected by dbcAMP (data not shown). dbcGMP at the concentrations used had a slight inhibitory effect on sperm-induced GVBD, while GVDB-induced with KCl or serotonin was not affected.

Chaetopterus *oocytes*

When the crude ethanol extract of *Spisula* tissues was assayed, it blocked maturation of *Chaetopterus* oocytes suspended in NSW (Fig. 3). Inhibition of GVBD was 13, 68, and 79% at concentrations of 1, 2 and 3 mg/ml, respectively.

As control compounds, several nucleotides were tested for their ability to block GVBD in *Chaetopterus* oocytes suspended in NSW. GVBD was inhibited in 18 and 46% of oocytes with dbcAMP at concentrations of 1.0 and 2.0 m*M*, respectively (data not shown). Inhibition of GVBD with dbcGMP occurred in 46, 84, and 96% of oocytes at concentrations of 0.5, 1.0, and 2.0 m*M*, respectively. The following compounds had no effect on GVBD: adenine, adenosine, adenosine 5′-monophosphate, adenosine 2′,5′-diphosphate, adenosine 5′-diphosphate, adenosine 2′,3′-cyclic monophosphate, adenosine 2′,3′-cyclic phosphate 5′-monophosphate, adenosine 3′,5′-diphosphate, 8-bromoadenosine 3′-5′-cyclic monophosphate, guanosine 3′,5′-cyclic monophosphate, and guanosine 5′-triphosphate.

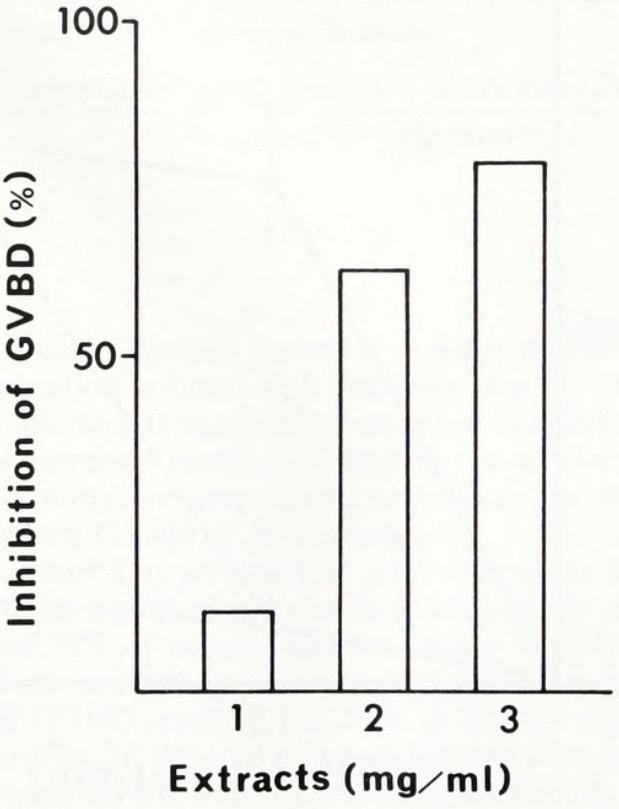

FIGURE 3. Effect of the *Spisula* factor on the maturation of *Chaetopterus* oocytes in NSW. Oocytes were examined for GV after 30 min suspension in NSW. A lyophilized 70% ethanol extract of *Spisula* tissues was used.

Mouse oocytes

Spisula extract was further purified by chromatography on a Dowex 1-×8 column. It was assayed for its ability to prevent GVBD in isolated cumulus-free mouse oocytes (Fig. 4). The GVBD inhibiting activity was found in the 0.1 *N* HCl fraction eluted from the Dowex column. At the third hour of incubation, 78% of the control oocytes had undergone GVBD. dbcAMP (50 μM) and the 0.1 *N* HCl fraction (2.5 mg/ml) added individually to the medium had a slight inhibitory effect. However, when varying concentrations of 0.1 *N* HCl Dowex fraction of the *Spisula* substance were coupled with 50 μM dbcAMP significant inhibition of GVBD occurred. GVBD was prevented in 47 and 59% of oocytes at concentrations of 1.0 and 2.5 mg/ml of *Spisula* substance (0.1 *N* HCl Dowex fraction), respectively.

The *Spisula* substance separated on the Dowex column was further purified by HPLC (Fig. 5). Two major peaks were eluted. The second peak possessed the GVBD inhibiting activity. HPLC-purified *Spisula* factor tested alone at concentrations of 5 and 10 μg/ml did not influence the spontaneous maturation of mouse oocytes (Fig. 4). When added with dbcAMP (50 μM) the frequency of GVBD of isolated mouse oocytes was blocked. Inhibition was 68 and 85% at concentrations of 5 and 10 μg/ml, respectively.

Discussion

Compounds that increase the intracellular cAMP level or derivatized cAMP such as cholera toxin, forskolin, dbcAMP, theophylline, and IBMX inhibit maturation of oocytes, suggesting that the arrest of meiosis at the dictyate state may be regulated by intracellular cyclic nucleotides (Dekel and Beers, 1978; Sato and Koide, 1984a). The present results obtained with *Spisula* and *Chaetopterus* oocytes support this thesis. One distinct difference between the oocytes of *Spisula* and *Chaetopterus* has been the

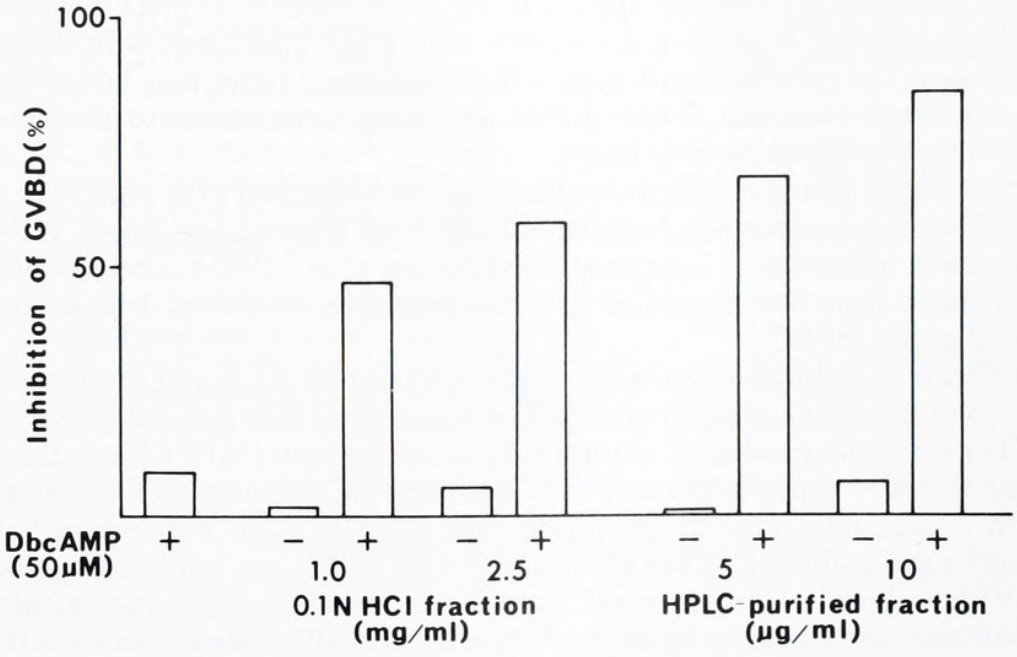

FIGURE 4. *In vitro* effect of purified *Spisula* factor on GVBD of isolated mouse oocytes. Spisula factor was purified by chromatography on a Dowex 1-×8 column and by high performance liquid chromatography on Waters Radial Pak column (Fig. 5).

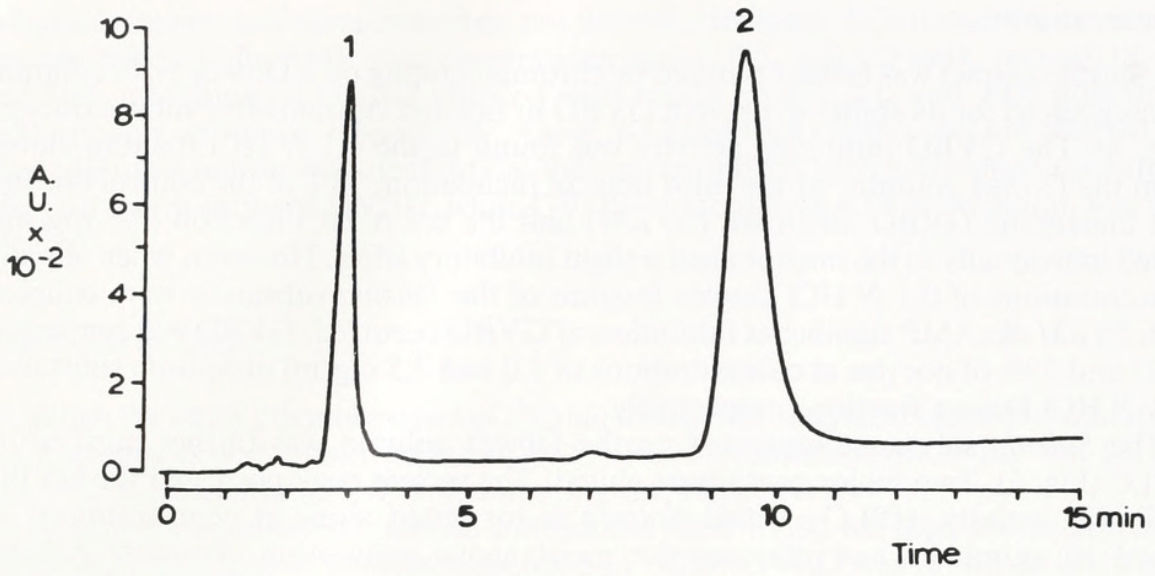

FIGURE 5. Elution pattern of *Spisula* factor purified by high performance liquid chromatography. Conentrated sample of the 0.1 *N* HCl fraction obtained from Dowex 1-8× column was applied to a Water Radial Pak column, eluted with 0.05 *M* $NH_4H_2PO_4$ in 10% aqueous ethanol, pH 5.0, at a flow rate of 2 ml/min.

effectiveness of cAMP *versus* cGMP. In *Spisula* oocytes dbcAMP was a very potent inhibitor of GVBD when induced with sperm or serotonin. In *Chaetopterus,* dbcGMP was the more potent suppressor of GVBD. It was reported that both dbcAMP and dbcGMP can arrest mammalian oocytes at the dictyate stage except that the suppressive activity of dbcAMP was more pronounced than dbcGMP (Hubbard and Terranova, 1982). dbcGMP may exert its inhibitory effects through the cumulus cells, while dbcAMP appears to act directly on the oocytes (Hubbard and Terranova, 1982). The observed variation in sensitivity of oocytes of different species to dbcAMP and dbcGMP deserves further attention.

It was demonstrated that the *Spisula* factor alone blocked GVBD in *Spisula* and *Chaetopterus* oocytes. Inhibition is induced only when combined with dbcAMP in mouse oocytes. The present results suggest that the *Spisula* factor may be an important meiotic arresting substance. Experiments are being undertaken to determine the chemical structure of the *Spisula* factor.

An inhibitor of mouse oocyte maturation was demonstrated to be present in porcine follicular fluid which acts synergistically with dbcAMP (Downs and Eppig, 1984). This compound was identified as hypoxanthine (Downs *et al.,* 1985). The *Spisula* factor can be separated from hypoxanthine by chromatography on Dowex 1-8× column and by reversed phase HPLC.

GVBD can be induced in *Spisula* oocytes with sperm, KCl, and serotonin (Allen, 1953; Hirai *et al.,* 1984). Hirai *et al.* (1984) demonstrated that *Spisula* oocytes treated with KCl or serotonin produced maturation promoting fator (MPF). Since the *Spisula* factor blocked serotonin-induced GVBD and not KCl-induced GVBD, the site of inhibition is probably prior to the production of MPF. This contention is further supported by the findings that GVBD induced with KCl is not inhibited by dbcAMP, while GVBD induced with sperm and serotonin are blocked. Thus, the *Spisula* factor and cAMP analogs appear to block the formation of MPF rather than its action.

The *Spisula* factor also suppressed the forward motility of *Spisula* sperm. Within 2 or 3 min after incubation at concentrations of 1 and 2 mg/ml, sperm became immobile. The *Spisula* factor is not cAMP since this nucleotide stimulates sperm motility (Hoskins *et al.,* 1974, 1975; Acott and Hoskins, 1978; Acott *et al.,* 1979).

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