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Hypoxanthine and Adenosine in Murine Ovarian Follicular Fluid: Concentrations and Activity in Maintaining Oocyte Meiotic Arrest

JOHN J. EPPIG,[2] PATRICIA F. WARD-BAILEY, and DOUGLAS L. COLEMAN

The Jackson Laboratory
Bar Harbor, Maine 04609

ABSTRACT

The concentrations of hypoxanthine and adenosine in ovarian follicular fluid were estimated, using high-performance liquid chromatography, for three groups of mice: 1) pregnant mare's serum gonadotropin (PMSG)-primed mice; 2) PMSG-primed mice 2 h after injection with human chorionic gonadotropin (hCG); and 3) PMSG-primed mice 5 h after injection with hCG. The concentration of hypoxanthine in follicular fluid of Group 1 mice was 2–4 mM and of adenosine was 0.35–0.70 mM. There was no difference in the concentrations of these purines in the follicular fluid of Group 2 mice, in which maturation had been induced with hCG but the samples were taken just before germinal vesicle breakdown (GVBD). Therefore, a decrease in the concentrations of these purines does not appear to induce GVBD. A significant decrease in the concentrations of hypoxanthine and adenosine was observed in the follicular fluid of Group 3 mice in which GVBD had already occurred. This decrease was probably a result of an increase in follicular fluid volume.

Adenosine had a significant, but transient, effect in maintaining both cumulus cell-enclosed and denuded oocytes in meiotic arrest; all oocytes had undergone GVBD by 100 min incubation in 1 mM adenosine. When GVBD was assessed after 3 h culture, concentrations up to 5 mM adenosine failed to maintain meiotic arrest. In contrast, hypoxanthine (2–5 mM) had a dose-dependent effect in maintaining both cumulus cell-enclosed and denuded oocytes in meiotic arrest that was sustained up to 24 h. Cumulus cell-enclosed oocytes were always more sensitive to hypoxanthine than were denuded oocytes. There was a strong synergistic effect of adenosine and hypoxanthine in maintaining meiotic arrest; 4 mM hypoxanthine and 0.75 mM adenosine maintained more than 95% of the oocytes in meiotic arrest for culture periods up to 24 h. This action was completely reversible by withdrawal of the purines. It is hypothesized that the synergistic effect of these purines may result both by promoting cyclic adenosine monophosphate synthesis (adenosine), and by preventing its hydrolysis (hypoxanthine).

INTRODUCTION

The mammalian ovarian follicle maintains the oocyte in meiotic arrest until the preovulatory surge of gonadotropins or follicular degeneration. Controversy has surrounded the proposed existence of a low molecular weight substance in ovarian follicular fluid that maintains the oocyte in meiotic arrest. Although such substances have been reported to be present in pig, hamster, sheep, cow, and human follicular fluid (Chang, 1955; Tsafriri and Channing, 1975; Gwatkin and Andersen, 1976; Jagiello et al., 1977; Tsafriri et al., 1977; Chari et al., 1983), the existence of these substances has been controversial, since some investigators have failed to obtain an inhibitory effect of follicular fluid on maturation (Leibfried and First, 1980; Racowsky and McGaughey, 1982; Fleming et al., 1983). We have shown that porcine follicular fluid (PFF) contains a low molecular weight, protease-insensitive, ether-insoluble substance that maintains meiotic arrest in murine oocytes and that the activity of this substance is greatly augmented by cyclic adenosine monophosphate (cAMP) (Downs and Eppig, 1984). We have shown further that the principal inhibitory component of this low molecular weight PFF fraction is hypoxanthine (Downs et al., 1985a). In addition to hypoxanthine (1.4 mM), the preparation of PFF analyzed contained high concentrations of other purines and pyrimidines including adenine (0.06 mM), uracil (0.44 mM), and 7-methylinosine (0.19 mM). However, these other purines and pyrimidines did not appear to contribute significantly to the maturation-arresting activity of the PFF fraction (Downs et al., 1985a). Nevertheless, other purines, not detected in the analyzed prepara-

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tion of PFF, may participate in the maintenance of meiotic arrest and other aspects of follicular function.

Adenosine is of particular interest because it has been shown to elevate granulosa cell cAMP levels (Polan et al., 1983), and the elevation of cumulus cell cAMP levels has been positively correlated with the maintenance of meiotic arrest (Eppig et al., 1983; Schultz et al., 1983a; Ekholm et al., 1984; Racowsky, 1984). Moreover, adenosine is a metabolic precursor of hypoxanthine. The aims of the present study were to determine whether hypoxanthine and/or adenosine is present in mouse follicular fluid, to estimate their concentrations, and to assess the effect of these concentrations of hypoxanthine and adenosine on maintaining meiotic arrest in mouse oocytes in vitro. The results show that murine follicular fluid contains 2–4 mM hypoxanthine and 0.35–0.70 mM adenosine, and that the concentrations of these purines do not appear to decrease immediately before gonadotropin-induced germinal vesicle breakdown (GVBD). In addition, these two purines synergistically and reversibly maintain meiotic arrest up to 24 h in culture.

MATERIALS AND METHOFS

The mice used in this study were (C57BL/6J × LT/Sv)F_1 females, 21–22 days of age. The mice were either injected with 5 IU of pregnant mares' serum gonadotropin (PMSG; Diosynth, Inc.) 48 h prior to use (Group 1), or injected with 5 IU of PMSG followed 48 h later by an injection of 5 IU human chorionic gonadotropin (hCG; Sigma Chemical Co., St. Louis, MO) and used 2 h (Group 2) or 5 h (Group 3) later. About 90% of the oocytes in large Graafian follicles had undergone germinal vesicle breakdown (GVBD) by 5 h after the hCG injection. Therefore, follicular fluid was obtained from follicles containing immature oocytes prior to the induction of maturation in vivo (Group 1), oocytes just before gonadotropin-induced GVBD (Group 2), and oocytes having undergone GVBD (Group 3).

Mouse follicular fluid was obtained for purine analysis by immersing the ovaries in Dulbecco's phosphate-buffered saline (without added protein), but containing 10 μM Pentostatin (deoxycoformycin), an adenosine deaminase inhibitor generously donated by the Warner-Lambert Company (Ann Arbor, MI). The ovaries of 5 mice were collected in 1 ml of saline and as many as possible of the large Graafian follicles were burst with a fine needle to release the follicular fluid into the saline. The debris was removed by centrifugation and 100% (w/v) trichloroacetic acid (TCA) was added to a final concentration of 5%. After clearing the samples by 5 min centrifugation with an Eppendorf Microfuge, the TCA was extracted three times with 10 vol of ether. When ^{3}H-hypoxanthine was equilibrated with similar preparations prior to addition of TCA and ether extraction, the recovery of hypoxanthine in the aqueous phase was found to be 100%.

Purines were separated and measured using high-performance liquid chromatography (HPLC). The samples were analyzed using an Altex ultrasphere ODS reversed-phase column with 100 mM phosphate buffer (pH 2.9) as the eluting agent and an elution rate of 1 ml/min as described by Durre and Andreesen (1982), and as used in our previous study (Downs et al., 1985a). The retention time of peaks in the samples was compared to known standards, and the peaks detected at 260 nm were quantified by peak height. The estimation of the concentration of purines in mouse follicular fluid is based on the assumption that the concentration of protein in the follicular fluid is between 50% and 100% of the concentration of protein in the serum, and that this concentration does not change with follicular maturation (Caravaglios and Cilotti, 1957; McGaughey, 1975; Andersen et al., 1976; McNatty, 1978). In this case, the concentration of protein in the serum of these mice was 40 mg/ml. A sample of the saline containing mouse follicular fluid was taken for protein estimation after centrifugation to remove debris and before TCA addition. Protein concentration was estimated using the Bio-Rad (Richmond, CA) kit using bovine serum albumin as the standard. The concentrations of hypoxanthine or adenosine in the various groups were compared using one-way analysis of variance followed by the Student-Newman-Kuels Multiple Range Test.

To assess the effect of hypoxanthine and/or adenosine on the maintenance of meiotic arrest, oocyte-cumulus cell complexes were isolated from mice 48 h after priming with PMSG by puncturing the large Graafian follicles while the ovaries were immersed in minimum essential medium containing 5% fetal bovine serum (MEM/FBS), or MEM/FBS containing the substance(s) to be tested. The complexes were washed by serial passage through four dishes containing 2.5 ml of medium with the aid of a micropipet. Cumulus cells were removed from some groups of oocytes by drawing the complexes in and out of a Pasteur pipet held at about a 45° angle to the bottom of a petri dish. Complexes or cumulus cell-denuded oocytes were incubated in 1.5 ml of medium in glass culture tubes for 3 or 24 h at 37°C, with an atmosphere of 5% O_2/5% CO_2/90% N_2. The complexes were then stripped of cumulus cells and the oocytes were assessed for GVBD. The frequency of GVBD in various groups was compared by chi-square analysis.

RESULTS

Estimates of the concentration of hypoxanthine in murine follicular fluid are shown in Table 1. Henceforth, when referring to these concentrations, we report low and high estimates. These estimates reflect the variation reported in the relative concentration of protein in follicular fluid compared to serum in various species (see McNatty, 1978); low estimates are based on 50% and high estimates are based on 100% of the concentration of protein in serum. It is also assumed that the concentration of protein in follicular fluid does not change

significantly during follicular maturation (Caravaglios and Cilotti, 1957; McGaughey, 1975; Andersen et al., 1976). Accordingly, the concentration of hypoxanthine in the follicular fluid of mice 48 h after priming with PMSG is 2–4 mM. In addition, adenosine was found in these preparations at a concentration of 0.35–0.70 mM.

Germinal vesicle breakdown begins in mouse follicles about 2 h after injecting PMSG-primed mice with an ovulatory dose of hCG (Edwards and Gates, 1959). As shown in Table 1, the concentrations of hypoxanthine and adenosine in mouse follicular fluid obtained at this time are the same as those prior to hCG injection. Therefore, it appears that a decrease in hypoxanthine or adenosine concentration in the follicular fluid does not immediately precede GVBD.

There was a significant decrease in the concentrations of hypoxanthine and adenosine measured in the follicular fluid of mice 5 h after injection of hCG (Table 1). The oocytes in more than 90% of these follicles had undergone GVBD and mucification had been initiated. However, more importantly, there had been a 46% increase in ovarian weight during the 5-h period since hCG injection (ovaries from PMSG-primed mice weigh 6.55 ± 0.18 mg, and 5 h after injecting the PMSG-primed mice with hCG they each weigh 9.54 ± 0.28 mg; n=10 for each group). Most of this increase is probably the result of an increase in fluid volume rather than cell proliferation. Therefore, the decrease observed in hypoxanthine and adenosine concentrations is probably the result of increased follicular fluid volume.

The effect of adenosine and hypoxanthine on oocyte maturation was assessed by incubating oocyte-cumulus cell complexes or denuded oocytes in concentrations of these purines up to 5 mM for 3 h. The effect on the maintenance of meiotic arrest is shown in Fig. 1. (Note that the data are presented in Figs. 1–5 as "Percent GVBD." We refer to the oocytes with an intact GV as being maintained in meiotic arrest.) Hypoxanthine had a dose-dependent effect in both cumulus cell-enclosed and denuded oocytes. However, the effect on cumulus cell-enclosed oocytes was always greater than on the denuded oocytes. More than 85% of the cumulus cell-enclosed oocytes were maintained in meiotic arrest by 5 mM hypoxanthine. In contrast, adenosine, up to 5 mM, was totally ineffective in maintaining meiotic arrest when assessed at this time.

Since hypoxanthine and adenosine are both present in murine follicular fluid, the effect of these two purines together, at approximately the proportion found in murine follicular fluid, was assessed after culture periods of 3 and 24 h. These data are shown in Fig. 2. Two and 4 mM hypoxanthine alone maintained, respectively, 20–30% and 55–65% of the oocytes in meiotic arrest for 24 h in culture. In contrast, adenosine alone, 0.375 or 0.75 mM, had no effect when assessed at these times. The effect of combining hypoxanthine and adenosine, at both the high and low concentrations, was a significant (P<0.01) synergistic activity in maintaining meiotic arrest. For example, the high-estimate concentrations of hypoxanthine and adenosine combined maintained 97% of the oocytes in meiotic arrest for 24 h. More than

TABLE 1. Estimated concentrations (mM) of hypoxanthine and adenosine in murine follicular fluid.[a]

Group	Hypoxanthine	Adenosine
PMSG (n=10)	2.1–4.3 ± 3%	0.34–0.68 ± 8%
2 h post-hCG (n=9)	2.2–4.4 ± 4%	0.39–0.78 ± 6%
5 h post-hCG (n=10)	1.8–3.6 ± 8%*	0.26–0.53 ± 9%**

[a]Follicular fluid was obtained as described in *Materials and Methods* from mice 1) 48 h after PMSG injection 2) 2 h after injecting the PMSG-primed mice with hCG, and 3) 5 h after injecting the PMSG-primed mice with hCG. The low estimate for each group is based on the assumption that the concentration of protein in murine follicular fluid is 50% of the concentration in serum, while the high estimate is based on the assumption that the protein concentration is 100% of that in serum. The standard errors are expressed as percentage of the mean.

*Significant difference from the other groups with P<0.05 using one-way analysis of variance followed by the Student-Newman-Kuels Multiple Range Test.

**Significantly different with P<0.01 using one-way analysis of variance followed by the Student-Newman-Kuels Multiple Range Test.

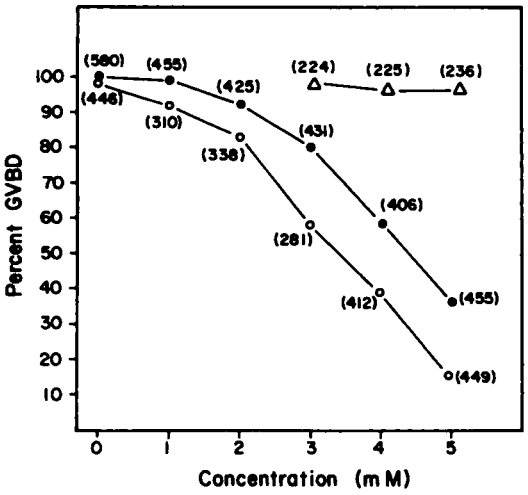

FIG. 1. The effect of hypoxanthine and adenosine on GVBD in murine oocytes. *Open circles* indicate cumulus cell-enclosed oocytes and *closed circles* indicate denuded oocytes incubated in medium containing various concentrations of hypoxanthine. The *triangles* indicate cumulus cell-enclosed oocytes incubated in medium containing various concentrations of adenosine. All groups were incubated for 3 h and then assessed for GVBD. The number of oocytes included in each point is indicated within the parentheses.

95% of these arrested oocytes underwent GVBD when removed from the purine-containing media and recultured in control medium (data not shown).

The relationship of hypoxanthine and adenosine in maintaining meiotic arrest was further assessed by determining the effect of various concentrations of adenosine with constant hypoxanthine concentrations. Although adenosine alone, at concentrations up to 5 mM (Fig. 1), had no maturation-arresting activity when assessed after 3 h culture, it had dose-dependent activity when added with hypoxanthine (Fig. 3). Accordingly, the frequency of meiotic arrest was dependent on the concentrations of both adenosine and hypoxanthine.

Other purines, adenine, inosine, and xanthosine, were assessed for synergistic activity with hypoxanthine by incubating cumulus cell-enclosed or denuded oocytes in medium containing 2 or 3 mM hypoxanthine, or 2 mM hypoxanthine plus a 1 mM concentration of an alternative purine. We have shown previously (Downs et al., 1985a) that 1 mM concentrations of these purines alone had no significant maturation-arresting activity when assessed at 3 h.

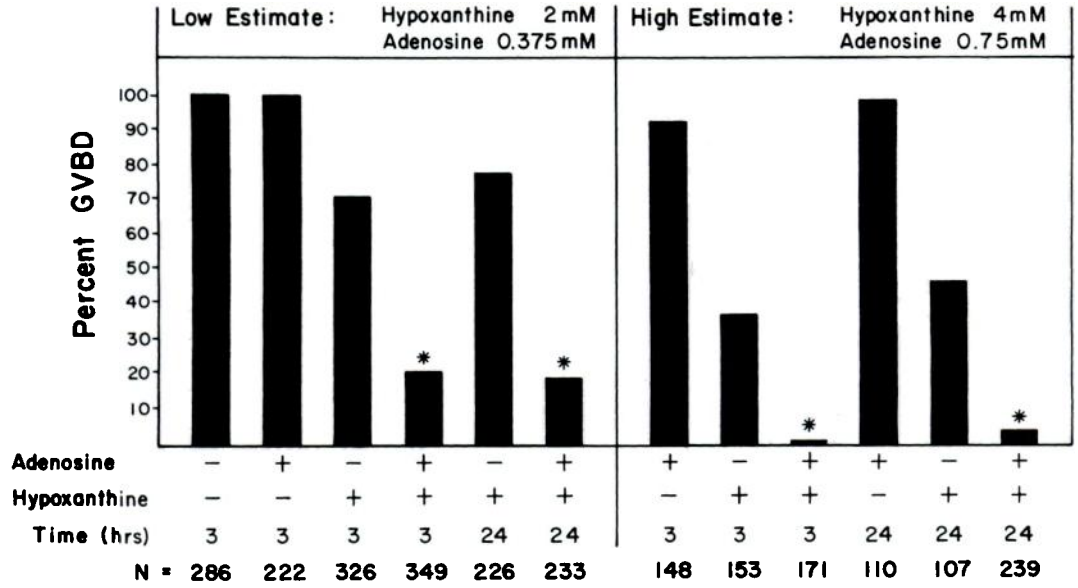

FIG. 2. The effect of hypoxanthine and adenosine together on GVBD in murine oocytes. Cumulus cell-enclosed oocytes were incubated in media containing no added purines, adenosine or hypoxanthine alone, or adenosine plus hypoxanthine for 3 or 24 h as indicated, then assessed for GVBD. A * on top of the bar indicates a significant difference ($P<0.01$) from the group incubated in hypoxanthine alone using chi-square analysis.

As shown in Fig. 4, only adenosine and inosine had a synergistic action with hypoxanthine on cumulus cell-enclosed oocytes, and only adenosine acted synergistically with hypoxanthine on denuded oocytes.

Although adenosine alone had no apparent maturation-arresting action when assessed after 3 h culture, it did have a transient effect on both cumulus cell-enclosed and denuded oocytes. Both groups of oocytes were cultured in control medium (no added purines), or medium containing 1 mM adenosine, and oocytes were assessed for GVBD at 20-min intervals beginning at 40 min. The results are shown in Fig. 5. After 40, 60, and 80 min of culture, there were significantly ($P<0.01$) more oocytes maintained in meiotic arrest in medium containing adenosine than in the control medium for both the cumulus cell-enclosed and denuded groups. In addition, denuded oocytes in control and adenosine-treated groups underwent GVBD earlier than their corresponding cumulus cell-enclosed oocyte groups. Nevertheless, almost all the oocytes in all groups had undergone GVBD by 100 min of culture.

DISCUSSION

The estimated concentrations of hypoxanthine and adenosine in murine follicular fluid are 2–4 and 0.35–0.70 mM, respectively. These concentrations did not appear to decrease immediately before hCG-induced GVBD. Therefore, it is unlikely that a decrease in the concentration of these purines in follicular fluid initiates oocyte maturation. Accordingly, if hypoxanthine, adenosine, and/or their derivatives are physiologically important for maintaining meiotic arrest, their concentrations within the oocyte may be more important than their concentrations in the extracellular follicular fluid. Germinal vesicle breakdown during the preovulatory period may result from 1) metabolic conversion of the purines to inactive derivatives, 2) transport of hypoxanthine and/or adenosine (or their metabolites) out of the oocyte, and/or 3) a gonadotropin-induced stimulus that bypasses the inhibitory influence of these purines.

Adenosine was not detected in the preparations of porcine follicular fluid used in our previous study (Downs et al., 1985a). However, these preparations were probably not collected

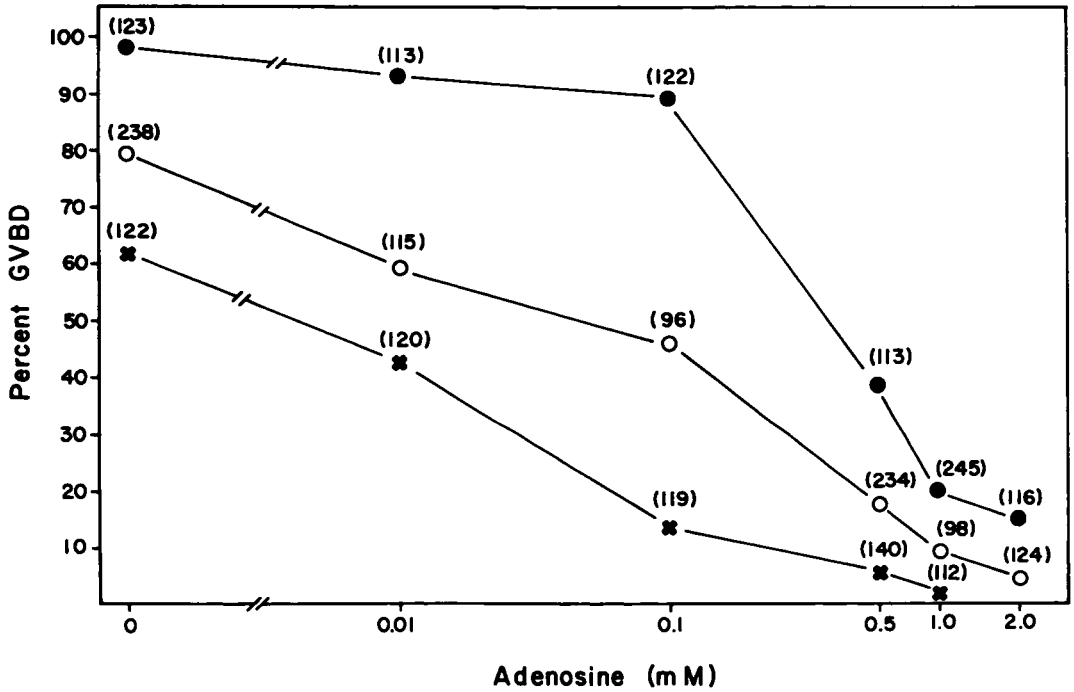

FIG. 3. The effect of various concentrations of adenosine in the presence of 1 (●), 2 (○), or 3 (X) mM hypoxanthine on GVBD in cumulus cell-enclosed oocytes. Groups were incubated for 3 h before the oocytes were assessed for GVBD. The number of oocytes assessed for each point is indicated within the parentheses.

under conditions that could inhibit the metabolism of adenosine to inosine and hypoxanthine. In the present study, the murine follicles were punctured immediately after collection in a solution containing 10 μM pentostatin, a potent adenosine deaminase inhibitor. Adenosine was found only in trace amounts when the inhibitor was not present during follicular puncture. We cannot exclude the possibility, however, that some adenosine metabolism occurred between the time of animal killing and follicular puncture. Nevertheless, injection of animals with 0.1 ml of 1 mM pentostatin 1 h before cervical dislocation had no effect on the measured levels of hypoxanthine or adenosine (data not shown).

Both cumulus cell-enclosed and denuded oocytes were maintained in meiotic arrest by hypoxanthine alone, at concentrations estimated to be present in murine follicular fluid. Arrest was sustained for 24 h in culture. The frequency of meiotic arrest was always significantly higher in the cumulus cell-enclosed than in the denuded oocytes. Therefore, while it appears that hypoxanthine enters denuded oocytes, it is possible that additional hypoxanthine, or its derivatives, enters oocytes via the gap junctions that metabolically couple the oocyte with cumulus cells (Heller and Schultz, 1980; Moor et al., 1980; Salustri and Siracusa, 1983; Racowsky and Satterlie, 1985).

Data presented here suggest that hypoxanthine and adenosine could act in concert to maintain the oocyte in the germinal vesicle stage. Adenosine alone had a significant, but transient, activity in maintaining meiotic arrest in both cumulus cell-enclosed and denuded oocytes. Concentrations of adenosine up to 5 mM were found to be unable to maintain meiotic arrest when GVBD was assessed after 3 h culture. In contrast, adenosine synergized dramatically with hypoxanthine to maintain meiotic arrest. Incubation of cumulus cell-enclosed oocytes in 2 mM hypoxanthine for 3 h results in the maintenance of meiotic arrest in about 35% of the oocytes (Fig. 4.). This percentage is increased to about 50% by 3 mM hypoxanthine. In contrast, 1 mM adenosine added to 2 mM hypoxanthine resulted in almost complete (95%) meiotic arrest, a true synergism. This combined action of hypoxanthine and adenosine is not likely due to toxicity for the following reasons. First, the meiosis-arresting synergism maintained for 24 h in culture was completely reversible. Second, when maturation was induced by withdrawal of the purines after 24 h maintenance of meiotic arrest in vitro, 47% of the ova were competent for fertilization and 30% of these developed to the expanded blastocyst stage (Downs et al., 1985b).

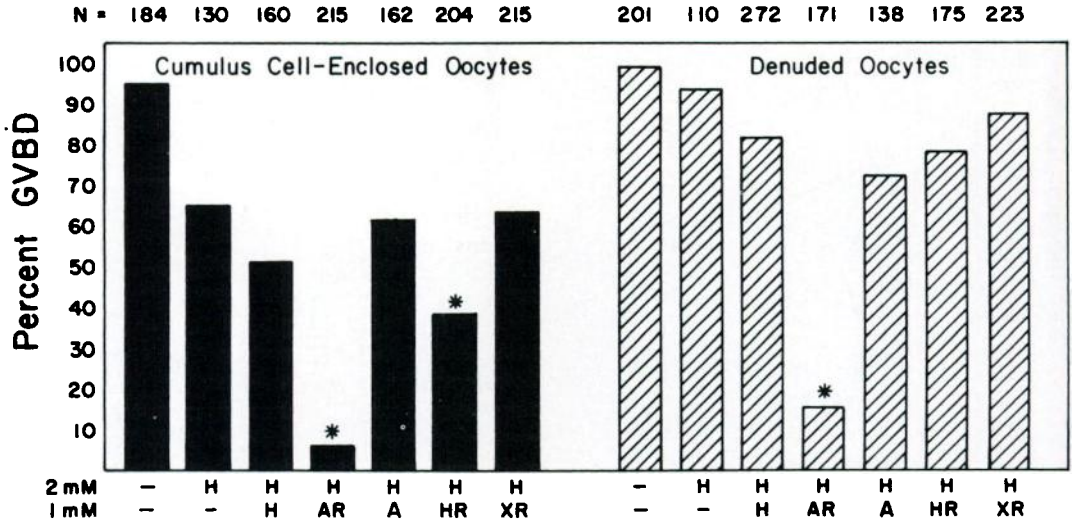

FIG. 4. An effect of adenosine (*AR*, adenine ribonucleoside), adenine (*A*), inosine (*HR*, hypoxanthine ribonucleoside), or xanthosine (*XR*, xanthine ribonucleoside) on the inhibition of GVBD by hypoxanthine (*H*). A * indicates a significant difference ($P<0.01$) from the group incubated in 3 mM hypoxanthine (2 mM H plus 1 mM H). Cumulus cell-enclosed (*solid bars*) or denuded (*striped bars*) oocytes were incubated for 3 h in the media indicated before assessing for GVBD.

Various substances have oocyte maturation-inhibiting activity when tested on isolated oocytes from a number of different species. The most consistent finding is that elevating or maintaining oocyte cAMP levels with membrane-permeable analogs of cAMP or with phosphodiesterase inhibitors maintains oocyte meiotic arrest (Cho et al., 1974; Wassarman et al., 1976; Magnusson and Hillensjo, 1977; Dekel and Beers, 1978; Schultz et al., 1983b). Moreover, a decline in mouse oocyte cAMP levels is coincident with a commitment to undergo GVBD (Schultz et al., 1983b). This decrease in oocyte cAMP could be the result of secretion of cAMP and/or the action of phosphodiesterase. Recent studies have demonstrated that mouse oocytes have abundant phosphodiesterase (Bornslaeger et al., 1984). In addition, several investigators have demonstrated that forskolin, which activates the catalytic subunit of adenylate cyclase (Seamon et al., 1981), both raises the level of cAMP in cumulus cell-free (denuded) mouse oocytes and inhibits GVBD (Eppig et al., 1983; Urner et al., 1983; Sato and Koide, 1984; Bornslaeger and Schultz, 1985). Thus, the identification of molecules with the potential for regulating oocyte cAMP levels may be key for understanding the physiologic regulation of oocyte maturation. Hypoxanthine, like other xanthines, has been shown to be a phosphodiesterase inhibitor in other cell systems (Chasin and Harris, 1976; Oleshansky, 1980). Moreover, adenosine has been shown to increase cAMP in granulosa and luteal cells (Hall et al., 1981; Polan et al., 1983). Perhaps these purines act synergistically to maintain oocyte meiotic arrest by this combined action of stimulation of cAMP generation by adenosine and inhibition of cAMP hydrolysis by hypoxanthine.

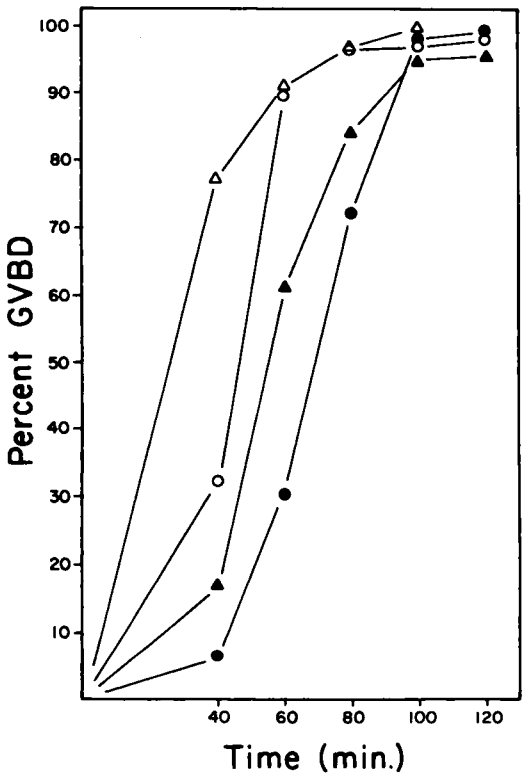

FIG. 5. A kinetics analysis of the effect of adenosine on the maturation of murine oocytes. Cumulus cell-enclosed (*circles*) or denuded (*triangles*) oocytes were incubated in control medium (*open symbols*) or in medium containing 1 mM adenosine (*closed symbols*) for the times indicated and then assessed for GVBD. *N* = (●) 186, 214, 184, 181, 58; (○) 195, 192, 180, 188, 194; (▲) 136, 174, 280, 157, 197; (△) 172, 174, 169, 134, 213.

Additionally, or alternatively, derivatives of adenosine and hypoxanthine may participate in the maintenance of meiotic arrest. Metabolism of adenosine to adenosine triphosphate could provide the substrate for adenyl cyclase. Hypoxanthine could be "salvaged" by the hypoxanthine phosphoribosyltransferase pathway to produce inosinic acid and thus potentiate conversion to other purines such as guanosine, which is exceptionally active in the maintenance of meiotic arrest (Downs et al., 1985a).

It is important to emphasize that the evidence presented here that hypoxanthine and adenosine may participate in the maintenance of meiotic arrest does not preclude a role for other molecular species in this process. Such molecules may function by regulating purine metabolism or the intracellular responses to purines or by means unrelated to the action of purines.

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