Requirement for glucose in ligand-stimulated meiotic maturation of cumulus cell-enclosed mouse oocytes

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Summary. In this study, the effect of different energy sources used in Eagle's minimum essential medium on the meiotic maturation of mouse oocytes in culture was examined. The effects of glucose (5.5 mmol l\(^{-1}\)), pyruvate (0.23 mmol l\(^{-1}\)) and glutamine (2 mmol l\(^{-1}\)) in different combinations were tested on the maturation of denuded oocytes in the presence or absence of 300 μmol dibutyryl cAMP l\(^{-1}\) during 17–18 h of culture. In the absence of cyclic nucleotide, only oocytes from those groups containing pyruvate resumed maturation at a high frequency (99–100% germinal vesicle breakdown); all other combinations resulted in ≤54% germinal vesicle breakdown. When dibutyryl cAMP was introduced, all pyruvate-containing groups exhibited maturation frequencies of about 50%, whereas maturation in all other groups was negligible (≤10% GVB). Pyruvate was also important for the maintenance of viability in denuded oocytes (≥86% viability in pyruvate-containing medium; ≤35% viability in pyruvate-free groups). When cumulus cell-enclosed oocytes were cultured in medium without inhibitor, all combinations of energy substrates supported high frequencies of maturation (≥89% germinal vesicle breakdown) and viability (≥91%). The addition of dibutyryl cAMP resulted in inhibition of meiotic maturation (5–33% germinal vesicle breakdown) in all cultures except the pyruvate-alone group (97% germinal vesicle breakdown). Viability in cumulus cell-enclosed oocytes was greatest when two or more energy substrates were present in the medium. Follicle-stimulating hormone (FSH) produced a stimulation of meiotic maturation in all cultures of meiotically arrested cumulus cell-enclosed oocytes, but maximal induction of germinal vesicle breakdown was dependent upon D-glucose. Concanavalin A (ConA)-induced meiotic maturation was also dependent upon D-glucose. Uptake and metabolism of D-glucose by the cumulus cells is important in mediating the stimulatory effects of these ligands on oocyte maturation because (1) both FSH and ConA stimulated uptake of D-glucose and 2-deoxyglucose but not 3-O-methylglucose; (2) phloretin prevented the stimulatory action of FSH and ConA on germinal vesicle breakdown at a concentration that suppressed ligand-induced uptake of D-glucose; (3) 2-deoxyglucose, a hexose that suppresses glycolysis, prevented the induction of meiotic maturation by FSH and ConA and (4) D-mannose, a glycolysable sugar, was as effective as D-glucose in supporting the ligand effects. Thus, while pyruvate appeared to be most effective in supporting the spontaneous maturation of denuded oocytes, the presence of D-glucose is important in cumulus cell-enclosed oocytes for both the maintenance of meiotic arrest and ligand-induced meiotic maturation.

Keywords: glucose; oocyte maturation; mouse

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Introduction

The isolated oocyte–cumulus cell complex has been a popular model system for studying the control of meiotic maturation in mammalian oocytes. Removal of oocytes from Graafian follicles releases them from inhibitory constraints that originate in the somatic compartment of the follicle and leads to spontaneous germinal vesicle breakdown in culture. Supplementation of medium with agents that stimulate the production of cAMP prevents the resumption of maturation (Tsafiriri et al., 1982; Eppig & Downs, 1984). This inhibitory influence can, in turn, be abolished by the addition of stimulatory ligands such as follicle-stimulating hormone (FSH), epidermal growth factor (EGF) and mitogenic lectins that induce germinal vesicle breakdown despite the continued presence of inhibitory agents within the medium (Dekel & Beers, 1978; Downs et al., 1988; Fagbohun & Downs, 1990).

Previous studies have addressed the energy requirements of mammalian oocytes undergoing spontaneous meiotic maturation in culture. In mice, it was shown that denuded, cumulus cell-free oocytes do not exhibit spontaneous maturation when glucose is the sole energy source, but do undergo germinal vesicle breakdown in the presence of pyruvate or oxaloacetate (Biggers et al., 1967). Cumulus cell-enclosed oocytes, however, resume nuclear maturation in glucose-containing medium (Biggers et al., 1967), because cumulus cells metabolize glucose to pyruvate that is then made available to the oocyte (Donahue & Stern, 1968; Leese & Barton, 1985). It is also well established that cumulus cells mediate the stimulatory action of a number of ligands in promoting germinal vesicle breakdown in cultured oocytes (Downs et al., 1988; Fagbohun & Downs, 1990). It is therefore likely that modulation of both the type of energy source and its concentration within culture medium plays a vital role in the meiotic response of the oocyte in vitro.

Studies from this laboratory involving in vitro oocyte maturation have used Eagle’s minimum essential medium (MEM) containing D-glucose, pyruvate and glutamine as potential energy sources. The present study was carried out to determine the relative importance of each of these components in meiotic maturation of the mouse oocyte in culture, with particular emphasis on ligand-stimulated germinal vesicle breakdown. The data demonstrate that in the absence of cumulus cells, pyruvate is indeed the important energy source in supporting both meiotic maturation and oocyte viability. However, in cumulus cell-enclosed oocytes, glucose is the critical energy source, particularly for induction of meiotic maturation by FSH and Concanavalin A (ConA).

Materials and Methods

Experimental protocols and culture conditions

C57BL/6J × SJL/F1 female mice, 19–22 days old, were used in all experiments. Mice were primed with 5 IU pregnant mares’ serum gonadotrophin (Diosynth, Inc., Chicago, IL) and killed by cervical dislocation 48 h later. Ovaries were placed in medium containing 200 μmol 3-isobutyl-1-methylxanthine 1−1 (IBMX; Aldrich Chemical Co. Inc., Milwaukee, WI) to maintain meiotic arrest throughout the isolation procedure. Large antral follicles were punctured with sterile needles to release the cumulus cell-enclosed oocytes. Denuded oocytes were obtained by repeated pipetting of the cumulus cell-enclosed oocytes with sterile pasteur pipettes. Unless otherwise specified, cumulus cell-enclosed oocytes and denuded oocytes were washed in four changes of IBMX-free medium before transfer to 1 ml of test medium in stoppered borosilicate culture tubes, and cultured at 37°C for various periods. Tubes were gassed with a humidified mixture of 5% O2, 5% CO2 and 90% N2.

For the cumulus expansion experiment, cumulus cell-enclosed oocytes were isolated in inhibitor-free medium, washed and then transferred to 3 ml of appropriate test medium in 35 mm Petri dishes (Falcon no. 1008, Falcon Plastics, Los Angeles, CA). These were placed in modular incubator chambers (Billups-Rothenberg, Del Mar, CA), gassed and cultured in a water-jacketed incubator for 17–18 h at 37°C. The degree of expansion was assessed according to a subjective scale as described by Fagbohun & Downs (1990).

The culture medium used for all these studies was Eagle’s minimum essential medium (MEM) with Earle’s salts and antibiotics. The concentrations of energy substrates were 5.5 mmol D-glucose 1−1; 0.23 mmol pyruvate 1−1 and 2 mmol glutamine 1−1. For oocyte maturation experiments, the medium was supplemented with 3 mg crystallized lyophilized bovine serum albumin ml−1 (ICN ImmunoBiologicals, Lisle, IL) and cumulus expansion experiments were carried out in MEM containing 5% fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, Utah). For the maturation kinetics experiment denuded oocytes were cultured in the same energy supplement variations but this time
in 100 μl drops of medium under oil, and were examined after 3, 6, 12 and 21–22 h of culture. At each time point the number of dead oocytes was determined and the nuclear status in the remaining viable oocytes was assessed.

**Chemicals**

All hexoses, dibutyryl cAMP, phloretin, and ConA were purchased from Sigma Chemical Co. (St Louis, MO). Biological grade ovine FSH-16 (oFSH) was generously provided by the National Hormone and Pituitary Program of the NIDDK (Bethesda, MD). Stock solutions of ConA and FSH were prepared in phosphate-buffered saline containing 3 mg bovine serum albumin ml⁻¹ and stored at −20°C. ConA was used at concentrations of 10 μg ml⁻¹ and 25 μg ml⁻¹ for oocyte maturation and cumulus expansion experiments, respectively. FSH was used at a concentration of 0.1 μg ml⁻¹. These concentrations were previously determined to be efficient in producing the optimal stimulatory response of the oocyte-cumulus cell complex. All other agents were prepared fresh before each experiment.

**Uptake of radiolabelled sugars and ConA**

Uptake of tritiated D-glucose (66.6 Ci mmol⁻¹), 2-deoxyglucose (6.3 Ci mmol⁻¹) and 3-O-methylglucose (79.0 Ci mmol⁻¹) by oocyte-cumulus cell complexes was assayed during a 3 h culture period by the addition of 10 μCi of the respective radiolabelled sugar in 1 ml medium. All sugars were obtained from New England Nuclear (Boston, MA). For each treatment group, 20 complexes were washed through four changes of medium without radiolabel and transferred to a vial for scintillation counting. An equal volume of medium from the last wash dish served as a blank. Tissue was solubilized with NaOH, neutralized with HCl and assayed by scintillation spectroscopy. For measurement of ConA uptake, complexes were treated in identical fashion except that medium contained 2 μCi of the tritiated lectin (24.5 Ci mmol⁻¹; Amersham Corp., Arlington Heights, IL).

**Oocyte assessment and statistical analysis**

Each oocyte maturation experiment was performed three or more times with at least 40 oocytes per group per experiment and the data reported as mean percentage germinal vesicle breakdown ± SEM. Oocytes were assessed for maturation at the end of the culture period by scoring them for germinal vesicle breakdown, the first observable manifestation of maturation. All frequencies were subjected to arcsin transformation before statistical analysis. Differences between two groups were compared statistically by Student's t test. When more than two groups were involved, data were compared by analysis of variance (ANOVA) followed by Duncan's multiple range test. A P value <0.05 was considered to be significant.

At the end of culture, the gross morphological features of the oocytes were examined for assessment of viability. Most oocytes categorized as nonviable were characterized by a dense, shrunken, granulated remnant within the zona pellucida. A small number of oocytes were grossly abnormal and exhibited varying degrees of shrinkage, blebbing with or without fragmentation; these were also categorized as nonviable. Oocytes assessed as nonviable were not included in the maturation frequency data.

**Results**

**Effects of different MEM energy sources on denuded oocyte maturation**

The three potential energy sources in MEM – glucose, pyruvate and glutamine – were tested in different combinations, at concentrations normally found in the medium, for their effect on the maturation of denuded oocytes in the presence or absence of 300 μmol dibutyryl cAMP 1⁻¹ during a 17–18 h culture period. In the absence of nucleotide, only oocytes from those groups containing pyruvate resumed maturation at a high frequency (99–100% germinal vesicle breakdown, Fig. 1). All other combinations (no supplements, D-glucose or glutamine alone, or D-glucose plus glutamine) resulted in maturation frequencies no higher than 50%. When dibutyryl cAMP was included in the medium, oocytes resumed meiotic maturation in all pyruvate-containing groups at a frequency of about 50%, whereas maturation in all other groups was negligible (≤10% germinal vesicle breakdown). A significant decrease in oocyte viability was associated with the fall in meiotic maturation in pyruvate-free groups. Only 12–35% of the oocytes from these groups were still viable at the end of culture, compared to 86–90% viable oocytes in the pyruvate alone group and 95–98% in all other pyruvate-containing groups (Fig. 1).

Kim & Schuetz (1991a) have recently shown that when denuded mouse oocytes are cultured in pyruvate-free medium a nucleolus-like structure comprised of a small mass of condensed chromatin...
Fig. 1. Effect of different energy substrates on the meiotic maturation of denuded oocytes. Denuded oocytes were cultured for 17–18 h in Eagle's minimum essential medium containing D-glucose (Gluc, 5.5 mmol l⁻¹), pyruvate (Pyr, 0.23 mmol l⁻¹) or glutamine (Gln, 2 mmol l⁻¹) alone or in different combinations as designated under each bar, either in the presence (□) or absence (■) of 300 μmol dibutyryl cAMP l⁻¹. The percentages for germinal vesicle breakdown are given for those oocytes still viable at the end of culture. The percentage of viable oocytes is given below each bar. The — dibutryl cAMP and + dibutryl cAMP groups were analysed separately by ANOVA followed by Duncan's multiple range test. Groups with at least one identical letter at the top of the bar are not significantly different (P > 0·05).

is formed subsequent to germinal vesicle breakdown that might be interpreted under the stereomicroscope as a germinal vesicle. It was possible that oocytes cultured in the absence of pyruvate undergo germinal vesicle breakdown but then form these structures and are mistakenly categorized as immature. A maturation kinetics experiment was therefore included in the present study. Denuded oocytes were cultured in the same energy supplement variations and were examined after 3, 6, 12 and 21–22 h of culture. In this experiment the number of dead oocytes in the pyruvate-treatment groups was higher than in the preceding experiment (Table 1). The reason for this is not known. At all time points, an obvious suppression of germinal vesicle breakdown was observed in viable oocytes in all cultures lacking pyruvate. Nucleolus-like structures, similar to those described by Kim & Schuetz (1991a), were observed but not until at least 12 h of culture, and their frequency was limited. Fluorescent staining with Hoechst dye confirmed that they contained DNA. Nevertheless, identification of these structures required careful examination under the stereomicroscope; consequently, they would not be routinely categorized as germinal vesicles under normal nuclear assessment procedures.

The effects of FBS on denuded oocytes in two of the treatment groups, in MEM plus or minus pyruvate were compared with the effects of BSA, because Kim & Schuetz (1991a) used MEM supplemented with 10% FBS in their experiments. The use of FBS resulted in a significant increase in viability in the cultures containing glutamine and glucose but lacking pyruvate (only 25% dead oocytes compared with 99% in the corresponding BSA group after 21–22 h). An increase (as a percentage of the total number of oocytes cultured) in oocytes demonstrating nucleolus-like structures after 12 and 21–22 h in the pyruvate group supplemented with FBS was observed. However, it is impossible to determine whether the increase in the number of oocytes containing these structures was due to a component of FBS directly affecting their appearance or was an indirect result of the increased viability of the oocytes in FBS-supplemented medium.
Table 1. Effects of different energy substrates on the kinetics of the maturation of denuded oocytes

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<th>GV (% of viable)</th>
<th>GVB (% of viable oocytes)</th>
<th>Nucleolus-like structure (% of viable oocytes)</th>
<th>Number dead (% of total oocytes)</th>
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Denuded oocytes were cultured for 3–21 h in medium containing either 3 mg bovine serum albumin (BSA) ml⁻¹ or 10% fetal bovine serum (FBS) with various combinations of the following supplements: 5-5 mmol glucose 1⁻¹, 2 mmol glutamine 1⁻¹ and 0-23 mmol pyruvate 1⁻¹. At each time point, oocytes were assessed for viability, germinal vesicle breakdown (GVB), and for the presence of nucleolus-like structures. Maturation data do not include dead oocytes. GV, germinal vesicle-stage. Data are pooled from 3–4 experiments.
Effects of different MEM energy sources on cumulus cell-enclosed oocyte maturation

The same type of experiment as shown in Fig. 1 was carried out using cumulus cell-enclosed oocytes but with three different treatments. Oocytes were cultured for 17–18 h in control medium or in medium containing dibutyryl cAMP, the latter either with or without 0·1 µg FSH ml⁻¹ (Fig. 2). When control medium was tested, all of the combinations supported a high percentage of germinal vesicle breakdown (glutamine alone, 89%; all others, 99–100%; Fig. 2a). In addition, viability was greater than 90% in all groups. The data for the no supplement group are not presented because 96% of the oocytes were dead at the end of culture.

![Graph](image)

Fig. 2. Effect of different energy substrates on the meiotic maturation of cumulus cell-enclosed oocytes. Cumulus cell-enclosed oocytes were cultured for 17–18 h in Eagle’s minimum essential medium (MEM) containing D-glucose (Gluc, 5·5 mmol l⁻¹), pyruvate (Pyr, 0·23 mmol l⁻¹) or glutamine (Gln, 2 mmol l⁻¹) alone or in different combinations as designated under each bar. Three different media were tested: control, inhibitor-free MEM (a) and MEM containing 300 µmol dibutyryl cAMP l⁻¹ with (X) or without ( ) 0·1 µg FSH ml⁻¹ (b). The -dibutyryl cAMP and +dibutyryl cAMP groups were analyzed separately by ANOVA and Duncan’s multiple range test. Groups with at least one identical letter at the top of the bar are not significantly different (P > 0·05).
The addition of dibutyryl cAMP to the medium resulted in significant suppression of spontaneous meiotic maturation in all instances except the pyruvate-alone group (Fig. 2b). While the frequency of maturation in all other groups ranged from 5–33%, dibutyryl cAMP was unable to maintain meiotic arrest in medium containing pyruvate as the sole energy source (97% germinal vesicle breakdown). Apart from this pyruvate group, in which essentially all of the oocytes had already resumed meiotic maturation, FSH stimulated germinal vesicle breakdown in all cultures, and the greatest FSH effect was observed in media containing d-glucose (Fig. 2b). Maturation frequencies in glucose-containing groups ranged from 69 to 81%, whereas those in the other two responding groups (glutamine and glutamine plus pyruvate) were only 32–33%.

Viability of cumulus cell-enclosed oocytes was lowest in those groups containing only one energy source (85–87% viable in d-glucose, 87–91% in glutamine, 63–76% in pyruvate), but was highest in cultures containing more than one of these components (95–99% viability). The lowest viability was observed in the pyruvate group, which supported the highest viability in denuded oocytes (Fig. 1).

Effects of glucose and 2-deoxyglucose on ConA- and FSH-stimulated maturation of cumulus cell-enclosed oocytes

The previous experiment suggested that d-glucose was the MEM component that supported FSH stimulation of oocyte maturation in oocyte–cumulus cell complexes. We therefore compared the importance of this hexose in the action of FSH and ConA on oocyte–cumulus cell complexes. Like FSH, ConA has been shown to induce nuclear maturation in meiotically-arrested cumulus cell-enclosed oocytes in culture (Fagbohun & Downs, 1990). Cumulus cell-enclosed oocytes were cultured for 21–22 h in one of three media: complete MEM (all energy sources), MEM minus d-glucose, or MEM with d-glucose replaced with 2-deoxyglucose. All media contained 300 µmol dibutyryl cAMP l⁻¹ plus or minus FSH (0.1 µg ml⁻¹) or ConA (10 µg ml⁻¹). In the absence of d-glucose (but in the presence of pyruvate and glutamine), the induction of oocyte maturation by FSH or ConA was greatly reduced (from 74% to 18% germinal vesicle breakdown in FSH-containing cultures and from 79% to 33% in ConA-containing cultures; P < 0.05), and when d-glucose was replaced with 2-deoxyglucose, these responses were completely suppressed (only 7% of the oocytes underwent maturation in both groups) (Fig. 3).

**Fig. 3.** Effects of glucose and 2-deoxyglucose on Concanavalin A (ConA)- and follicle-stimulating hormone (FSH)-stimulated maturation of cumulus cell-enclosed oocytes. Cumulus cell-enclosed oocytes were cultured for 17–18 h in medium containing 300 µmol dibutyryl cAMP l⁻¹ alone (□) or supplemented with 0.1 µg FSH ml⁻¹ (˟) or 10 µg ConA ml⁻¹ (واء). Medium contained either no glucose (– Hexose), 5.5 mmol d-glucose l⁻¹ (+ Gluc) or 5.5 mmol 2-deoxyglucose l⁻¹ (+2deGluc). Different superscripts at the top of a bar denote a significant difference (P < 0.05).
Effects of ConA and FSH on uptake of D-glucose, 2-deoxyglucose and 3-O-methylglucose by oocyte–cumulus cell complexes

Sugar uptake was measured in oocyte–cumulus cell complexes cultured for 3 h in MEM containing one of three tritiated glucose compounds. While D-glucose and 2-deoxyglucose were readily taken up by complexes, only a negligible amount of 3-O-methylglucose was taken up (Fig. 4). The addition of ConA or FSH stimulated increased uptake of D-glucose and 2-deoxyglucose, but not that of 3-O-methylglucose.

![Graph](image)

**Fig. 4.** Effects of (a) Concanavalin A (ConA) and (b) follicle-stimulating hormone (FSH) on uptake of D-glucose (D-Gluc), 2-deoxyglucose (2-DeGluc) and 3-O-methylglucose (3-O-MeGluc). Oocyte–cumulus cell complexes were cultured for 3 h in medium containing 10 μCi radiolabelled sugar ml⁻¹ plus (❑) or minus (□) 10 μg ConA ml⁻¹ or 0.1 μg FSH ml⁻¹. An asterisk denotes a significant difference from the respective treatment group minus protein ligand (P < 0.05).

Effects of phloretin on D-glucose uptake and on stimulation of oocyte maturation and cumulus expansion by ConA and FSH

Experiments were carried out to determine the effect of phloretin, an inhibitor of glucose-facilitated diffusion (LeFevre & Marshall, 1959), on meiotic maturation and cumulus expansion in oocyte–cumulus cell complexes. The initial experiment established the dose dependency of phloretin action on [³H]D-glucose uptake by oocyte–cumulus cell complexes during a 3 h culture period. Uptake
was suppressed by 19%, 83% and 98% in the presence of 25, 100 and 250 μmol phloretin l⁻¹, respectively (Fig. 5a). The action of phloretin on glucose uptake was also examined under conditions used for ligand-stimulated oocyte maturation. Complexes were cultured in medium containing 300 μmol dibutyryl cAMP l⁻¹ plus either ConA or FSH. In the presence of 100 μmol phloretin l⁻¹, the uptake of [³H]d-glucose was suppressed in ConA- and FSH-supplemented cultures by 80% and 88%, respectively (Fig. 5b).

**Fig. 5.** Effects of phloretin on d-glucose uptake. (a) Dose response effect of phloretin on d-glucose uptake. Oocyte–cumulus cell complexes were cultured for 3 h in glucose-free Eagle’s minimum essential medium containing 10 μCi [³H]d-glucose ml⁻¹ and increasing concentrations of phloretin. Bars with different letters at the top are significantly different (P < 0.05). (b) Effect of phloretin on d-glucose uptake in ligand-stimulated complexes. Oocyte–cumulus cell complexes were cultured for 3 h in medium containing 300 μmol dibutyryl cAMP l⁻¹ plus 0.1 μg follicle-stimulating hormone (FSH) ml⁻¹ or 10 μg Concanavalin A (ConA) ml⁻¹, in the presence (■) or absence (□) of 100 μmol phloretin l⁻¹. An asterisk denotes a significant difference (P < 0.05) from the treatment group minus protein ligand.

The effects of 100 μmol phloretin l⁻¹ were next tested on germinal vesicle breakdown in cumulus cell-enclosed oocytes induced by ConA or FSH when meiotic arrest was maintained with 300 μmol dibutyryl cAMP l⁻¹. Phloretin had no effect on the meiotic arrest maintained by dibutyryl cAMP alone, but this compound eliminated the stimulation of maturation in both treatment groups (Fig. 6). In addition, this action was reversible. When, after 17–18 h of culture in both phloretin-containing treatment groups, oocytes that were still at the germinal vesicle stage were washed free of inhibitor and cultured for more than 6 h in control media, over 95% resumed meiotic maturation; and, if cultured long enough, many extruded a polar body.
ConA-stimulated
"See
ConA
Control
Treatment"
cumulus
dibutyryl
oocyte
glycolytically
pyruvate
ConA-stimulated
Effects
FSH
Control
FSH
Concanavalin A (ConA) ml⁻¹ or 0.1 µg follicle-stimulating hormone (FSH) ml⁻¹, in the presence (•) or absence (□) of 100 µmol phloretin l⁻¹. An asterisk denotes a significant difference (P < 0.05) from the respective treatment group without phloretin.

Phloretin had a similar suppressive effect on cumulus expansion. In the presence of 100 µmol phloretin l⁻¹, the cumulus expansion index in complexes from control medium was not affected, but in all cultures in which expansion was stimulated, phloretin prevented expansion of the cumulus oophorus (Table 2). Moreover, when the phloretin-treated complexes were then washed and cultured overnight (21–24 h) in medium containing 1 mmol 8-Br-cAMP l⁻¹, expansion did occur (Table 2). These results demonstrate that the viability of the cumulus cells was not compromised by phloretin treatment, and that the effects of the inhibitor were reversible.

**Table 2. Effects of phloretin on cumulus expansion**

<table>
<thead>
<tr>
<th>Treatment⁴</th>
<th>Number of oocytes</th>
<th>Degree of cumulus expansion</th>
<th>Phloretin-treated complexes plus 8-Br-cAMP</th>
<th>CEI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+1</td>
<td>+2</td>
<td>+3</td>
</tr>
<tr>
<td>Control</td>
<td>84</td>
<td>80</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control + phloretin</td>
<td>78</td>
<td>77</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ConA</td>
<td>82</td>
<td>4</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>ConA + phloretin</td>
<td>76</td>
<td>70</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>FSH</td>
<td>87</td>
<td>0</td>
<td>3</td>
<td>53</td>
</tr>
<tr>
<td>FSH + phloretin</td>
<td>90</td>
<td>82</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

⁴The concentrations of supplements were as follows: Concanavalin A (ConA) 2·5 mg ml⁻¹; follicle-stimulating hormone (FSH) 0.1 mg ml⁻¹; phloretin 100 mmol l⁻¹ and 8-Br-cAMP 1 mmol l⁻¹. CEI: cumulus expansion index.

Effects of glycolysable sugars and poorly metabolized glucose analogues on FSH- and ConA-stimulated maturation of the cumulus cell-enclosed oocytes

Since the removal of glucose from the culture medium produced a decrease in both FSH- and ConA-stimulated maturation of the cumulus cell-enclosed oocyte despite the availability of pyruvate and glutamine as energy sources, it was important to compare the effects of other glycolytically metabolizable sugars and poorly metabolized glucose analogues on ligand-stimulated oocyte maturation. Cumulus cell-enclosed oocytes were maintained in meiotic arrest with 300 µmol dibutyryl cAMP l⁻¹ and the medium was supplemented with one of the following sugars at a concentration of 5·5 mmol l⁻¹: D-glucose, D-mannose, D-fructose (which can all potentially be metabolized
via glycolysis), and L-glucose or 2-deoxyglucose, which cannot (Rogers & Yanagimach, 1975; Niwa & Iritani, 1978; Rogers et al., 1979; Morgan & Faik, 1981). In FSH-treated (Fig. 7a) and ConA-treated (Fig. 7b) cultures, D-glucose and D-mannose were most supportive of meiotic maturation. Neither L-glucose nor D-fructose supported ligand-induced germinal vesicle breakdown above the frequency observed in hexose-free medium. 2-Deoxyglucose was inhibitory to oocyte maturation.

**Fig. 7.** Effects of glycolysable sugars and poorly metabolized glucose analogues on Concanavalin A (ConA)-stimulated maturation of cumulus cell-enclosed oocytes. Cumulus cell-enclosed oocytes were cultured for 21–22 h in medium containing 300 µmol dibutyryl cAMP l⁻¹ with [] or without (□) (a) 0·1 µg follicle-stimulating hormone (FSH) ml⁻¹ or (b) 10 µg ConA ml⁻¹. The medium was supplemented with 5·5 mmol l⁻¹ of the different hexoses (Control: no hexose; D-Gluc: D-glucose; L-Gluc: L-glucose; D-Mann: D-mannose; D-Fruc: D-fructose; 2-DeGluc: 2-deoxyglucose). Different letters at the top of a bar denote a significant difference (P < 0·05).

**Effect of phloretin on FSH-induced meiotic maturation in MEM containing D-mannose**

Because D-mannose supported a high frequency of germinal vesicle breakdown in meiotically arrested cumulus cell-enclosed oocytes treated with FSH or ConA, phloretin was added to these cultures to test its specificity of action on D-glucose-mediated meiotic maturation. Cumulus cell-enclosed oocytes were maintained in meiotic arrest for 17–18 h with 300 µmol dibutyryl cAMP l⁻¹ in medium containing D-mannose instead of D-glucose (5·5 mmol l⁻¹). The addition of FSH
produced, as expected, a stimulation of maturation (from 12% to 76% germinal vesicle breakdown), but the further addition of 100 µmol phloretin l\(^{-1}\) to FSH-containing cultures did not significantly influence the frequency of maturation (57% germinal vesicle breakdown; Fig. 8). Interestingly, when phloretin was added to cultures containing dibutyryl cAMP and ConA, the oocytes died during overnight culture. The reason for this decrease in viability is puzzling, since phloretin had no apparent detrimental effect on oocyte health in D-glucose-containing medium. This result establishes a specificity for phloretin action in that D-glucose-supplemented cultures are more sensitive to suppression of FSH-induced maturation by phloretin than D-mannose-supplemented cultures.

![Diagram](https://via.placeholder.com/150)

**Fig. 8.** Effect of phloretin on follicle-stimulating hormone (FSH)-induced meiotic maturation in D-mannose-supplemented medium. Cumulus cell-enclosed oocytes were cultured for 17–18 h in Eagle's minimum essential medium containing 300 µmol dibutyryl cAMP l\(^{-1}\) and 5-5 mmol D-mannose l\(^{-1}\), in the presence or absence of 0-1 µg FSH ml\(^{-1}\). Phloretin (Z) was added to cultures at a concentration of 100 µmol l\(^{-1}\). All four groups were analysed by ANOVA and Duncan's multiple range test and different letters at the top of the bars represent a significant difference (P < 0.05).

**Discussion**

This study has demonstrated that the energy substrate requirements for spontaneous meiotic maturation, maintenance of meiotic arrest and ligand-induced resumption of meiotic maturation differ significantly in mouse oocytes cultured in vitro. In addition, removal of the cumulus cells results in dramatic changes in the response of oocytes to culture conditions. Although pyruvate appears to be most effective in supporting the spontaneous maturation of denuded oocytes, the presence of D-glucose was important in cumulus cell-enclosed oocytes for the maintenance of meiotic arrest and for ligand-induced meiotic maturation. Uptake and metabolism of glucose, possibly via glycolysis, appear to mediate the facilitative effect of this hexose on FSH- and ConA-induced oocyte maturation.

It has previously been shown that pyruvate alone supports spontaneous meiotic maturation of denuded mouse oocytes in vitro (Biggers et al., 1967). The data reported here are consistent with this finding. In addition, pyruvate was important for maintaining oocyte viability. Neither D-glucose nor glutamine, either alone or in combination, could substitute for pyruvate, which indicates a role for pyruvate in supporting both the health of the oocyte and in promoting meiotic maturation. The maturation kinetics experiment demonstrated that the lower frequency of germinal vesicle breakdown observed in pyruvate-deficient medium was due to an actual suppression of oocyte maturation and not to the reformation of germinal vesicle-like structures, as has been shown to occur in spontaneously maturing (Kim and Schuetz, 1991a) and metaphase II-arrested (Kim &
Schuetz, 1991b) mouse oocytes in pyruvate-free medium. An increase (as a percentage of the total number of oocytes cultured) in oocytes demonstrating nucleolus-like structures after 12 and 21-22 h in the pyruvate group supplemented with FBS was observed. However, it is impossible to determine whether the increase in the number of oocytes containing these structures was due to a component of FBS directly affecting their appearance or was an indirect result of the increased viability of the oocytes in FBS-supplemented medium. A requirement for oxygen for the beneficial effect of pyruvate on oocyte maturation has been demonstrated (Haidri et al., 1971; Zeilmaker et al., 1972). The gas phase in the present experiments included 5% O₂, so it is possible that oxidative metabolism of pyruvate provides conditions conducive to oocyte maturation that are not present in the absence of this energy source. However, this may not be true for oocytes of other mammalian species. For example, pyruvate cannot serve as the sole energy source for ova from rats (Zeilmaker & Verhamme, 1974) or rabbits (Bae & Foote, 1975; Kane, 1972).

The presence of cumulus cells profoundly influences the meiotic response of mouse oocytes in culture. MEM containing D-glucose, pyruvate or glutamine alone supported high percentages of viability and meiotic maturation in cumulus cell-enclosed oocytes in inhibitor-free medium. Cumulus cells metabolize glucose to pyruvate (Donahue & Stern, 1968; Leese & Barton, 1985) that the oocyte can use as an energy source (Biggers et al., 1967; Eppig, 1976). Thus, glucose alone can indirectly supply the cumulus cell-enclosed oocyte with a suitable energy source. Since denuded oocytes do not survive well in glutamine alone, the survival of cumulus cell-enclosed oocytes indicates that the cumulus cells must take up this amino acid and either transfer it to the oocyte via the gap junctional coupling pathway or provide metabolic products for use by the oocyte. Glutamine is an important energy source for a variety of mammalian cells (Zielke et al., 1984) and can support the spontaneous maturation of rabbit oocytes in the absence of carbohydrate (Bae & Foote, 1975).

In the presence of dibutyryl cAMP, all but one of the energy substrate combinations, that of pyruvate alone, supported significant suppression of meiotic maturation (5-33% germinal vesicle breakdown). In pyruvate alone, 97% of the cumulus cell-enclosed oocytes underwent germinal vesicle breakdown, which is interesting in light of the finding that 48% of denuded oocytes were maintained in meiotic arrest under identical conditions. Apparently, processing of pyruvate by cumulus cells produces conditions that negate the meiosis-arresting action of dibutyryl cAMP on oocyte maturation. Perhaps increased respiration due to transit of pyruvate through the TCA cycle is responsible for this effect. Other energy substrates added to the medium might suppress the respiratory activity and permit the inhibitory action of dibutyryl cAMP to be manifested. For example, the addition of D-glucose might produce a Crabtree effect (Koobs, 1972) by promoting glycolytic activity at the expense of respiration. Confirmation of such a mechanism must await further experimentation.

When meiotic arrest was maintained with dibutyryl cAMP, stimulatory ligands promoted the resumption of oocyte maturation, and this effect was maximal in the presence of D-glucose. Removal of D-glucose from MEM produced a significant drop in the maturation frequency in the presence of FSH (32 and 33% germinal vesicle breakdown in the glutamine and glutamine plus pyruvate groups, respectively, compared with 69-81% in D-glucose-containing groups). The importance of D-glucose in ligand-stimulated maturation was further demonstrated by the finding that a comparable loss of meiotic induction was observed for FSH- and ConA-treated cultures when D-glucose was absent from the medium. The decrease in germinal vesicle breakdown in glucose-free medium cannot be attributed to an absence of energy substrates for the oocyte because the medium contained pyruvate and glutamine, a combination that supports meiotic maturation. Obviously, there are significant differences in the energy requirements for ligand-stimulated and spontaneous maturation.

A number of observations underscore the importance of uptake and metabolism of glucose by the cumulus cells in mediating the stimulatory action of FSH and ConA on oocyte maturation: (1) both ligands stimulated increased uptake of 2-deoxyglucose and D-glucose, but not 3-O-methylglucose,
by oocyte–cumulus cell complexes. Since 3-O-methylglucose cannot be metabolized but the other sugars can be at least partially metabolized (see below), the effects of these agents are unlikely to result from a direct action on the membrane glucose transporter but, rather, to a mass action effect through increased glucose use; (2) 2-deoxyglucose completely suppressed ligand-stimulated oocyte maturation. This glucose analogue is phosphorylated by hexokinase with the resultant accumulation of nonmetabolizable 6-phospho-2-deoxyglucose; this, in turn, irreversibly blocks further glycolytic activity (Wick et al., 1957). Thus, glycolysis is suppressed and phosphate energy is diverted to the glucose analogue. In the oocyte–cumulus cell complex, a glycolytic block and a drop in FSH concentrations in the presence of 2-deoxyglucose could prevent the stimulatory action of FSH and ConA. The ability of oocytes to respond, albeit in limited fashion, to ligand stimulation in the absence of exogenous d-glucose is probably due to endogenous levels of hexose present at the time of isolation. (3) Phloretin prevented the uptake of d-glucose by oocyte–cumulus cell complexes and this was coincident with suppression of ligand-stimulated oocyte maturation. Since this drug prevents facilitated glucose diffusion across plasma membranes (LeFevre & Marshall, 1959), these data suggest that d-glucose needs to be internalized to produce its effect. Evidence that phloretin was not acting by a toxic effect was provided by the observations that its actions on oocyte maturation were reversible; it did not prevent the stimulatory action of FSH when medium was supplemented with d-mannose, and cumulus cells could still undergo expansion after overnight culture in the presence of the drug. (4) Glycolysable sugars can substitute for d-glucose in supporting ligand-stimulated oocyte maturation. The finding that d-glucose and d-mannose augment the action of FSH and ConA on oocyte maturation to a greater extent than d-fructose, L-glucose or 2-deoxyglucose is consistent with results in other culture systems showing the importance of glycolysable sugars in supporting cell growth (Harris & Kutsky, 1953; Eagle et al., 1958; Melnykovych & Bishop, 1972; Burns et al., 1976; Faik & Morgan, 1976; Scannell & Morgan, 1980). Similarly, previous studies have demonstrated that glucose and mannose are more effective than fructose in facilitating sperm capacitation and penetration of eggs in vitro (Niwa & Iritani, 1978; Rogers & Perrault, 1990). The influence of hexose on ConA action in the present system is not likely to be due to binding to the lectin and alteration of its membrane interactions, because binding/uptake of [3H]ConA by the complex was not affected by the presence or absence of d-glucose in MEM (data not presented).

The data herein suggest that ligand-stimulated meiotic maturation in isolated cumulus cell-enclosed oocytes requires increased metabolism of glucose via glycolysis. However, increased production of pyruvate leading to augmented respiration as the mechanism for stimulation of oocyte maturation is not supported by earlier studies. High concentrations of pyruvate added to culture medium do not stimulate germinal vesicle breakdown in isolated Graafian follicles (Lindner et al., 1974), even though it stimulates respiration in isolated oocyte–cumulus cell complexes (Hillensjo et al., 1975). In addition, glycolytic activity in rat ovarian follicles can be dissociated from meiotic maturation. Treatment of isolated follicles with gonadotrophin results in increased glucose uptake and glycolysis (Ahren et al., 1969; Nilsson, 1974; Hillensjo, 1976; Tsafiriri et al., 1976), and isolated granulosa cells exhibit increased lactate production in vitro in response to gonadotrophin stimulation (Hillier et al., 1985; Harlow et al., 1987). At a low concentration of the glycolytic inhibitor iodoacetate, gonadotrophin stimulation of lactate production in cultured follicles was prevented but resumption of meiotic maturation was unaffected; at a higher concentration both activities were suppressed (Tsafiriri et al., 1976). These data demonstrate that suppression of follicular glycolytic activity did not necessarily affect oocyte maturation. However, the response of the entire follicle may not reflect the microenvironment of the oocyte–cumulus cell complex within the follicle; for example, the small contribution of the complex to the total follicular response may be masked by the greater amount of membrana granulosa tissue in the follicle. This idea is supported by the observation that, while oxygen consumption by isolated membrana granulosa cells increases in response to gonadotrophin stimulation (Hamberger, 1968), oocyte–cumulus cell complexes exhibit a reduced respiratory rate (Hillensjo et al., 1975; Dekel et al., 1976;
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Billig & Magnusson, 1985). This change in metabolism by cumulus cells may reflect increased glycolysis which would provide suitable substrate for oocyte respiration (Dekel et al., 1976). Indeed, cumulus cells exhibit increased lactate production in response to gonadotrophin treatment in vitro (Billig et al., 1983; Billig & Magnusson, 1985). Although the reduction in respiration by complexes is associated with increased respiration by the oocyte, these changes occur only after resolution of meiotic maturation (Magnusson et al., 1977; Magnusson, 1980). Hence, increased oocyte respiration is not a prerequisite for germinal vesicle breakdown. It is possible that subtle differences in oocyte respiration, not readily detectable by assay methods, play a causal role in the resolution of meiosis. On the other hand, it is possible that specific cumulus cell products of glycolysis or other glucose-metabolizing pathways positively influence the meiotic status of the oocyte through a mechanism not dependent upon respiration.

It should be pointed out that these potential energy sources could exert effects on oocyte maturation through mechanisms unrelated to energy generation. Of particular interest is the possible interaction of glutamine and glucose with purine metabolism. Purines have been implicated as important modifiers of mammalian oocyte maturation (Downs, 1990). Glutamine is required for the de novo synthesis of purines and may influence oocyte development through modulation of de novo pathway activity. Glucose may have varied effects on purine metabolism through the generation of ribose-5-phosphate via the pentose phosphate pathway. The pyrophosphate of ATP is added to this compound to form phosphoribosyl pyrophosphate (PRPP). PRPP serves as an early substrate for de novo purine synthesis and is also a cosubstrate with hypoxanthine or guanine for the salvage enzyme hypoxanthine-guanine phosphoribosyltransferase. Pyruvate has been shown to have protective effects on oxygen radicals, and the possibility has recently been raised that the positive actions of pyruvate on development may be related to this capability (Leese, 1990).

The present study has shown that altering the types of energy substrates in culture medium can dramatically influence both spontaneous and ligand-induced meiotic maturation in isolated mouse oocytes. It will be important to determine in future experiments how different energy sources and their relative concentrations affect glycolysis as well as the TCA cycle and respiratory activity in the oocyte and oocyte–cumulus cell complex, and how this affects meiotic maturation. It is important to emphasize that spontaneous and ligand-induced meiotic maturation are under separate metabolic control insofar as energy substrate requirements are concerned. It is also clear that manipulation of energy substrates in culture medium can determine whether a particular medium is inhibitory to meiotic maturation or promotes germinal vesicle breakdown. These are important considerations when analysing the effects of exogenous factors on oocyte maturation and may prove critical in devising optimal media for the cytoplasmic maturation of oocytes in vitro.

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