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Energy substrates and the completion of spontaneous meiotic maturation

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Summary

This study was carried out to examine how different combinations of pyruvate and glucose affect spontaneous meiotic maturation of cumulus-cell-enclosed mouse oocytes (CEO) to metaphase II (MII). Most experiments used an open system in which oocytes were cultured in 1 ml medium in plastic tubes. Initial experiments examined the dose response effects of pyruvate or glucose alone in the presence or absence of 2 mM glutamine. When medium lacked both pyruvate and glucose, more than 91% of the oocytes died in glutamine-free medium during 15 h of culture; viability was restored with the addition of glutamine, but only 11% of the CEO reached MII. In the absence of glutamine, 62–68% of oocytes completed maturation in 0.23–2.3 mM pyruvate, while 44–60% MII was observed in 0.55–27.8 mM glucose. The addition of glutamine to these cultures had a general suppressive effect on the completion of maturation. When glucose was added to pyruvate-containing cultures, the combination of 1 mM pyruvate/5.5 mM glucose was most effective in supporting maturation (about 90% MII), with little effect of glutamine. No further increase in maturation was observed when glucose was increased five-fold (to 27.8 mM). The positive effect of glucose was in part attributed to stimulation of glycolysis and increased production of pyruvate, since a reduced culture volume (8 µl), which allows the accumulation of secreted pyruvate, improved maturation in glucose-containing, but not pyruvate-containing, medium, and FSH, which stimulates glycolysis, increased progression to MII in glucose-containing, but not pyruvate-containing, medium. Yet these results also suggest that glucose has a beneficial effect on maturation apart from simple provision of pyruvate. The pyruvate effect was directly on the oocyte, because denuded oocytes responded more effectively than CEO to this energy substrate. The highest percentage of MII oocytes (96–97%) occurred in microdrop cultures containing glucose but lacking glutamine. These results indicate that glutamine supports oocyte viability but is not an adequate energy source for the completion of spontaneous meiotic maturation and may be detrimental. In addition, while pyruvate and glucose alone can each support meiotic progression of CEO to MII, optimal maturation requires the provision of both substrates to the culture medium when a large volume (1 ml) is used. It is concluded that careful attention to specific energy substrate supplementation and culture volume is important to optimise spontaneous meiotic maturation *in vitro*.

Keywords: Glutamine, Glucose, Metaphase II, Pyruvate

Introduction

The completion of meiotic maturation is an important prerequisite for fertilisation and subsequent embryo development, since oocytes that fail to progress beyond MI are not readily activated by sperm under normal conditions (Iwamatsu & Chang, 1972; Niwa & Chang, 1975). Although nuclear and cytoplasmic maturation usually occur at about the same time, these two processes are not dependent upon one another, and cytoplasmic maturation can proceed even in the absence of complete nuclear maturation (Eppig, 1996). For example, activation of rodent oocytes by calcium ionophore (Ducibella *et al*., 1990; Eppig *et al*., 1994; McConnell *et al*., 1995), IP_3 (Fujiwara *et al*., 1993) or spermatozoa (Eppig *et al*., 1994; Mehlmann & Kline,

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1994) can occur between germinal vesicle breakdown and metaphase II (MII), undoubtedly due, at least in part, to the increase in oocyte calcium stores that occurs during meiotic maturation (Jones *et al*., 1995). In addition, oocytes from mouse strains such as LT/Sv and I/LnJ that exhibit restricted maturation beyond metaphase I (MI) will either spontaneously activate (Stevens & Varnum, 1974; Eppig, 1978; Kaufman & Howlett, 1986; Eppig *et al*., 1996) or undergo successful fertilisation (Eppig & Wigglesworth, 1994), and each can subsequently develop to the blastocyst stage. However, mouse oocytes fertilised at MI are predominantly triploid (Eppig *et al*., 1994), and Kaufman & Speirs (1987) have reported that LT/Sv oocytes fertilised at the MI stage fail to develop to term. Thus, postimplantation development depends on the completion of meiotic maturation before fertilisation.

Removal of mammalian oocytes from preovulatory antral follicles and transfer to a suitable culture medium leads to spontaneous meiotic maturation *in vitro*. Since this important observation in the mid-1930s (Pincus & Enzmann, 1935), the isolated oocyte culture system has been used extensively to study numerous parameters of oocyte physiology and is an important means of producing mature, fertilisable ova. The culture medium for these various studies has usually included both pyruvate and glucose as potential energy sources. It has been shown that pyruvate, but not glucose, is readily metabolised by denuded rodent oocytes (DO) and can serve as the sole energy source (Biggers *et al*., 1967). Glucose alone will support oocyte maturation, but only in the presence of follicle cells, which supply pyruvate to the oocyte through glycolytic metabolism of glucose (Biggers *et al*., 1967; Donahue & Stern, 1968; Leese & Barton, 1985). The beneficial effect of pyruvate requires oxygen, presumably to generate ATP through pyruvate-fuelled oxidative phosphorylation (Haidri *et al*., 1971; Zeilmaker & Verhamme, 1974). Experiments by Kim & Schuetz (1991*a*) indicate that pyruvate is required by the oocyte for both germinal vesicle breakdown and completion of meiotic maturation and is involved in regulating protein synthesis.

Another potential energy source for oocytes included in many culture media is the amino acid glutamine, which can generate ATP after conversion to α-ketoglutarate and transit through the tricarboxylic acid (TCA) cycle. Glutamine serves as an important energy source for rabbit (Bae & Foote, 1975) and hamster (Gwatkin & Haidri, 1973) oocyte maturation, but fails in this capacity in denuded mouse oocytes; it does, however, support viability and germinal vesicle breakdown in cumulus-cell-enclosed mouse oocytes (CEO) (Fagbohun & Downs, 1992).

If glucose can provide the energy required for successful oocyte maturation, then the necessity for pyruvate in the culture medium for follicle- or cumulus-cell-enclosed oocytes may be questioned. Indeed, some culture media such as Eagle's minimum essential medium or Waymouth's MB 752/1 medium contain no pyruvate in their formulations. Most general cell culture media originally included pyruvate because it supported somatic cell survival under conditions of low plating density, and this effect has been attributed to an antioxidant function (O'Donnell *et al*., 1987). But the oocyte is unusual in its requirement for pyruvate, and the many effects of this substrate are probably not due simply to a non-specific extracellular neutralisation of reactive oxygen species. For example, we have demonstrated that in the presence of millimolar concentrations of pyruvate, meiotic inhibitors such as hypoxanthine and dibutyryl cyclic AMP maintained meiotic arrest very poorly, but the inclusion of a small amount of glucose dramatically restored this capacity (Downs & Mastropolo, 1994). On the other hand, the addition of pyruvate to Waymouth's MB 752/1 medium, which contains 27.8 mM glucose, reduced the frequency of maturation in inhibitor-treated CEO (Downs & Mastropolo, 1997). Thus, striking a balance in energy substrate supplementation is important in maintaining normal meiotic control, as each of the two substrates is capable of modifying consumption of the other (Downs *et al*., 1997).

In a previous study, we reported that polar body formation by CEO in several different culture media was compromised in the absence of pyruvate, despite a high (5.5 mM) glucose concentration (Downs & Mastropolo, 1997). This indicates that glucose alone, while supporting oocyte viability and meiotic resumption, may not suffice as an energy source for the completion of meiotic maturation in cultured CEO. The present study was therefore undertaken to determine the role of pyruvate and glucose in driving spontaneous meiotic maturation *in vitro*. We have examined the effects of differing combinations of pyruvate and glucose alone and in combination, in the presence or absence of glutamine, and have also tested the influence of follicle-stimulating hormone (FSH) and culture volume on the completion of the first meiotic division in culture.

Materials and methods

Oocyte isolation and culture conditions

Immature (C57BL/6J × SJL/J) F_1 mice, 20–23 days old, were used for all experiments. Mice were primed with 5 IU pregnant mare serum gonadotropin and killed 48 h later by cervical dislocation. Ovaries were placed in culture medium and large antral follicles were punctured with sterile needles. Oocyte–cumulus cell complexes were collected, washed free of extraneous

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ovarian tissue, and transferred to the appropriate treatment. Some of the oocytes were denuded of their cumulus cells by repeated pipetting with a Pasteur pipette. The culture medium for all oocyte maturation experiments was Eagle's minimum essential medium containing Earle's salts, antibiotics and 3 mg/ml lyophilised crystallised BSA (ICN ImmunoBiologicals, Lisle, IL). In all experiments utilising glutamine, this amino acid was added at a concentration of 2 mM. Oocytes were cultured in either 1 ml medium in plastic tubes or in 8 µl microdrops in Petri dishes under oil. All oocytes were gassed with a humidified mixture of 5% CO_2, 5% O_2 and 90% N_2 and cultured at 37 °C for the appropriate time period.

Chemicals

All media components were obtained from Sigma Chemical Co. (St Louis, MO) except for MEM amino acids, which were purchased from GIBCO BRL (Rockville, MD). Highly purified ovine FSH-20 (4453 IU/mg) was provided by the National Hormone and Pituitary Program of NIDDK (Bethesda, MD).

DNA staining of oocytes

Cumulus-cell-free oocytes were fixed for 30–45 min in 3% formaldehyde in phosphate-buffered saline, transferred in a small volume to a microscope slide and dried on a warming tray. A 10–20 µl drop of glycerol:PBS (1:1) containing 1 µg/ml Hoechst 33342 (Polysciences, Warrington, PA) was applied over the oocytes and a cover slip was then added and sealed with nail polish. Oocytes were observed under a Leitz Ortholux II fluorescence microscope.

Statistical analysis

Unless stated otherwise, maturation experiments were carried out a minimum of three times with at least 30 oocytes per group per experiment, and data from each group are presented as the mean percentage of MII oocytes ± SEM. Kinetics data are presented as the mean of two experiments. In one experiment, germinal vesicle breakdown (GVB) was used as the endpoint. Viable MII oocytes were scored for maturation after fixation and staining, while GVB was immediately assessed at the conclusion of culture after removal of the cumulus cells and observation under a stereomicroscope. Normal viability, characterised by healthy-appearing oocytes as assessed by simple morphological examination, is considered to be ≥93% (Downs & Mastropolo, 1994), and exceptions are noted in the text. Maturation frequencies were subjected to arcsin transformation and groups of three or more were analysed by ANOVA followed by Duncan's multiple range test. Paired comparisons were carried out using Student's *t*-test. A p value less than 0.05 was considered significant.

Results

Effects of energy substrates and FSH on oocyte maturation in CEO during 15 h cultures

In this study, apart from the GVB kinetics experiment discussed below, more than 95% of the oocytes in each treatment group had resumed meiotic maturation at all time points examined. Thus, the emphasis in data presentation is on the relative numbers of oocytes that completed the first meiotic division, manifested by polar body extrusion and arrest at MII.

CEO were cultured for 15 h in stoppered tubes in 1 ml MEM/BSA supplemented with varying concentrations of pyruvate (0.23–2.3 mM) or glucose (5.5–27.5 mM) alone, in the presence or absence of 2 mM glutamine, and then assessed for meiotic status after fixation and Hoechst staining. In the absence of substrate, less than 9% of the oocytes survived, but viability was restored by either pyruvate or glucose. Meiotic maturation to MII was 62–68% in pyruvate-containing cultures and 44–60% in glucose-containing cultures (Fig. 1, left). Increasing pyruvate from 1 to 2.3 mM or glucose from 5.5 to 27.8 mM failed to augment further the number of oocytes completing maturation.

Viability was maintained when medium was supplemented with glutamine alone, but only 11% of these oocytes progressed to MII (Fig. 1, right). The addition of pyruvate or glucose to glutamine-containing medium again increased the number of oocytes completing maturation, but percentages were generally lower than in glutamine-free medium (44–64% in pyruvate-containing and 33–50% in glucose-containing medium). Maturation rates again peaked at 1 mM pyruvate and 5.5 mM glucose, with higher concentrations of either substrate failing to increase maturation; in fact, increasing pyruvate to 2.3 mM proved detrimental to maturation. We did not observe the reformation of germinal-vesicle-like structures reported by Kim & Schuetz (1991*a*, *b*) following culture of maturing and MII-arrested mouse oocytes in pyruvate-free medium, probably due to the presence of cumulus cells or to the lack of fetal bovine serum that was used in the earlier studies (cf. Fagbohun & Downs, 1992). These results demonstrate that, under the above conditions, pyruvate or glucose alone are limited in their ability to support completion of maturation.

The next set of experiments tested the effectiveness of combinations of pyruvate and glucose on meiotic maturation. CEO were cultured in medium containing 0.23 or 1 mM pyruvate, plus 0, 5.5 or 27.8 mM glucose.

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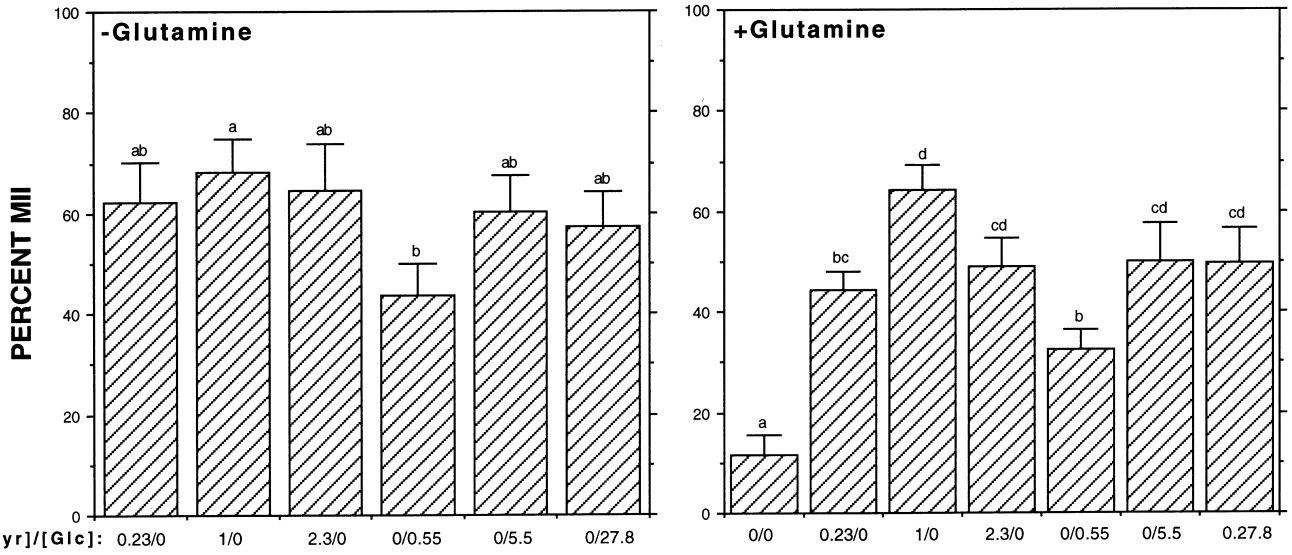

Figure 1 Effects of pyruvate and glucose alone on oocyte maturation. CEO were cultured for 15 h in 1 ml medium lacking (left panel) or containing (right panel) 2 mM glutamine plus increasing concentrations of either pyruvate (0.23–2.3 mM) or glucose (0.55–27.8 mM). The number of MII oocytes in each group was then determined by Hoechst staining. The –glutamine experiment was conducted five times and the +glutamine experiment three times. Groups that share a common letter at the top of the bar are not significantly different.

Similar results were obtained in either the presence or absence of glutamine. In all instances, the addition of glucose to pyruvate-containing medium significantly increased the number of oocytes reaching MII after 15 h of culture (Fig. 2). The optimal combination proved to be 1 mM pyruvate plus 5.5 mM glucose, which resulted in 90–91% MII whether or not glutamine was present. Increasing the concentration of glucose to 27.8 mM had no beneficial effect on maturation, and, in fact, reduced the percentage of oocytes reaching MII in medium containing glutamine plus 1 mM pyruvate (Fig. 2, right). These results therefore demonstrate that the presence of both pyruvate and glucose supports the highest levels of meiotic maturation.

To determine the effect of FSH on oocyte maturation, CEO were cultured in tubes in various energy substrate combinations in the presence or absence of 0.01 μg/ml FSH in medium containing 2 mM glutamine. We reasoned that in medium containing only glucose, FSH stimulation of glycolysis might increase pyruvate availability to the oocyte and augment oocyte maturation. The data support this idea. As shown in Fig. 3, maturation frequencies in 5.5 and 27.8 mM glucose alone were 33–34%, but were increased to 50–52% upon addition of FSH. Gonadotropin treatment had no beneficial effect in pyruvate-containing cultures; moreover, in medium supplemented with 1 mM pyruvate, a significant decrease in maturation (27–35%) was observed, regardless of the presence or absence of glucose. Thus, FSH was beneficial to maturation only when pyruvate was absent from the medium.

Our data suggest that glutamine suppresses the completion of meiotic maturation, but up to this point its effects had not been directly compared in the same experiment. The influence of glutamine on maturation of CEO was therefore tested in medium containing 5.5 mM glucose in the presence or absence of FSH. In glutamine-free medium, 59% of the oocytes developed to MII, and this number was increased to 75% upon addition of FSH (Fig. 4). Including 2 mM glutamine in the medium decreased the incidence of MII in both the absence (35%) and presence (55%) of FSH. Hence, glutamine blunted the effect of glucose on maturation, but did not prevent the increase in the MII percentage induced by FSH.

Effects of medium volume on oocyte maturation

To compare the effects of small versus large medium volumes on meiotic progression, CEO were cultured 15 h in glutamine-supplemented medium containing 0.23 mM pyruvate or 5.5 mM glucose, plus or minus FSH, in 1 ml medium in culture tubes or in 8 μl microdrops overlaid with oil. In each experiment, 40 CEO were cultured per tube, while 10 CEO were included in each of four microdrops. These data are presented in Fig. 5. When cultured in tubes, 59% and 43% of CEO reached MII in pyruvate- and glucose-supplemented medium, respectively (Fig. 5*A*). Consistent with previous results, the addition of FSH decreased slightly the maturation percentage in pyruvate-containing medium but increased this percentage in the presence of glucose. When cultured in microdrops, the number

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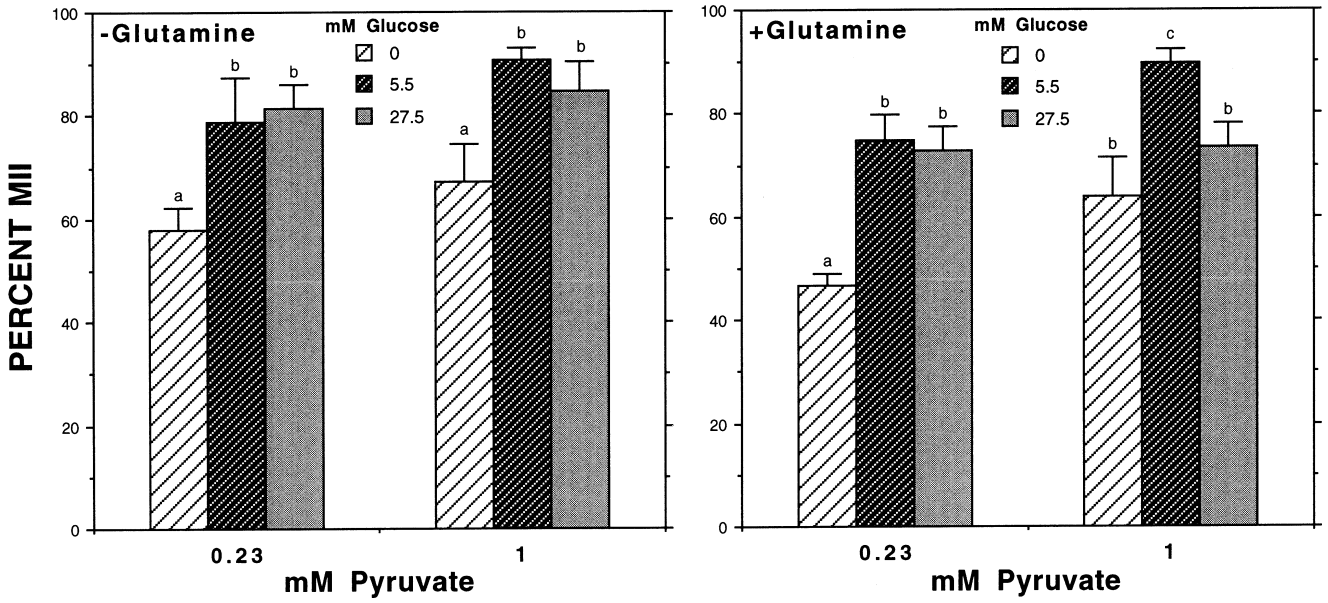

Figure 2 Effects of substrate combinations on oocyte maturation. CEO were cultured for 15 h in 1 ml medium lacking (left panel) or containing (right panel) 2 mM glutamine plus different combinations of pyruvate and glucose. The number of MII oocytes in each group was then determined by Hoechst staining. The –glutamine experiment was conducted three times and the +glutamine experiment five times. A different letter at the top of the bar denotes a significant difference.

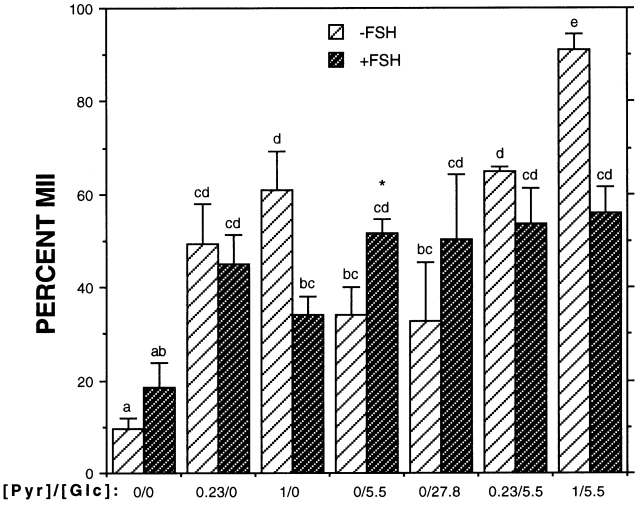

Figure 3 Effects of FSH on oocyte maturation. CEO were cultured for 15 h in 1 ml medium containing 2 mM glutamine plus millimolar energy substrate supplementation as shown. The number of MII oocytes in each group was then determined by Hoechst staining. This experiment was conducted three times. Groups that share a common letter at the top of the bar are not significantly different. *, significantly different from corresponding –FSH group, by Student's *t*-test.

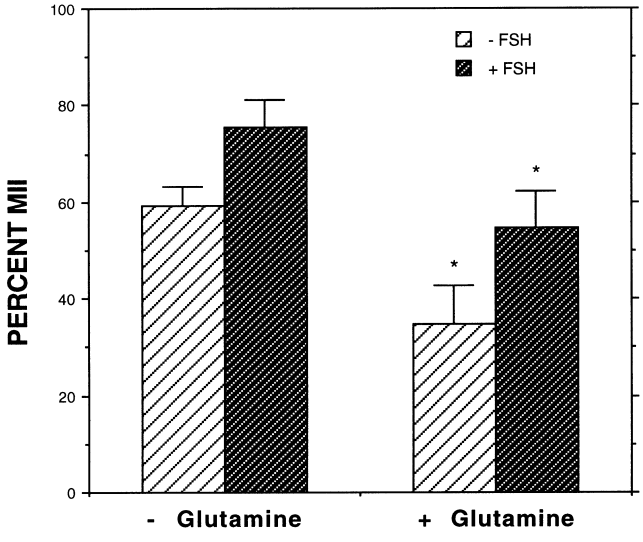

Figure 4 Effects of glutamine on oocyte maturation. CEO were cultured for 15 h in 1 ml medium containing 5.5 mM glucose, plus or minus FSH, in the presence or absence of 2 mM glutamine. The number of MII oocytes in each group was then determined by Hoechst staining. This experiment was conducted three times. As determined by Student's *t*-test, (1), FSH increased the number of MII oocytes in both the presence and absence of glutamine, and (2) glutamine decreased the number of MII oocytes in both the presence and absence of FSH. *, significantly different from the corresponding –glutamine group.

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of pyruvate-treated CEO completing maturation was decreased by 19% to 40%, while that for glucose-treated CEO was increased by 16% to 59%. FSH had a small, but insignificant, stimulatory effect on glucose-treated CEO but was without effect in pyruvate-treated CEO.

To examine further the effect of small volumes on oocyte maturation, CEO were cultured in microdrops in the presence or absence of 2 mM glutamine, in medium containing 0.23 mM pyruvate, 5.5 mM glucose or the two substrates together. When glutamine was absent from the medium, only 59% of the CEO cultured in pyruvate alone progressed to MII, but this number reached 96% in glucose alone (Fig. 5*B*). The addition of pyruvate to glucose-containing medium had no effect on maturation (97% MII). As was the case in tubes, addition of 2 mM glutamine to microdrop cultures suppressed the completion of meiosis. MII levels were reduced in pyruvate-containing medium by 16% to 41% and in glucose-containing medium by 27% to 67% (Fig. 5*B*). This decrease was much less pronounced in medium containing both pyruvate and glucose (from 97% to 91%). It is important to note that the maturation percentages in the –glutamine groups containing glucose were the highest obtained in this study.

An additional consideration is the viability of oocytes under these conditions. Although glutamine has a suppressive effect on the completion of maturation, it has a protective effect in terms of viability. In the absence of glutamine, 81% and and 84% of pyruvate-treated and glucose-treated CEO were viable after 15 h as determined by morphological assessment; the addition of this amino acid increased those percentages to 100% and 95%, respectively (Fig. 5*B*), but coincidentally reduced the rate of polar body formation. In the absence of glutamine, the addition of pyruvate to glucose-containing medium restored viability as well as supporting a high meiotic completion rate.

Substrate effects in denuded oocytes

To examine effects of different pyruvate concentrations (from 0.23 to 2.3 mM) on meiotic maturation, DO or CEO were cultured for 15 h in 1 ml glutamine-supplemented medium in tubes. In CEO, comparable levels of MII were reached in the three concentrations of pyruvate (62–64%; Fig. 6*A*). At 0.23 and 1 mM, these percentages were shifted upward in DO by 29% and 17%, respectively, to 90% and 83% MII. A slight drop to 81% MII was observed in DO at the highest dose of pyruvate (2.3 mM). It is therefore apparent that cumulus cells do not mediate the action of pyruvate on the oocyte, but may in these conditions impede maturation. When the same pyruvate doses were tested in the absence of glutamine, no differences in MII percentages were observed (Fig. 6*A*).

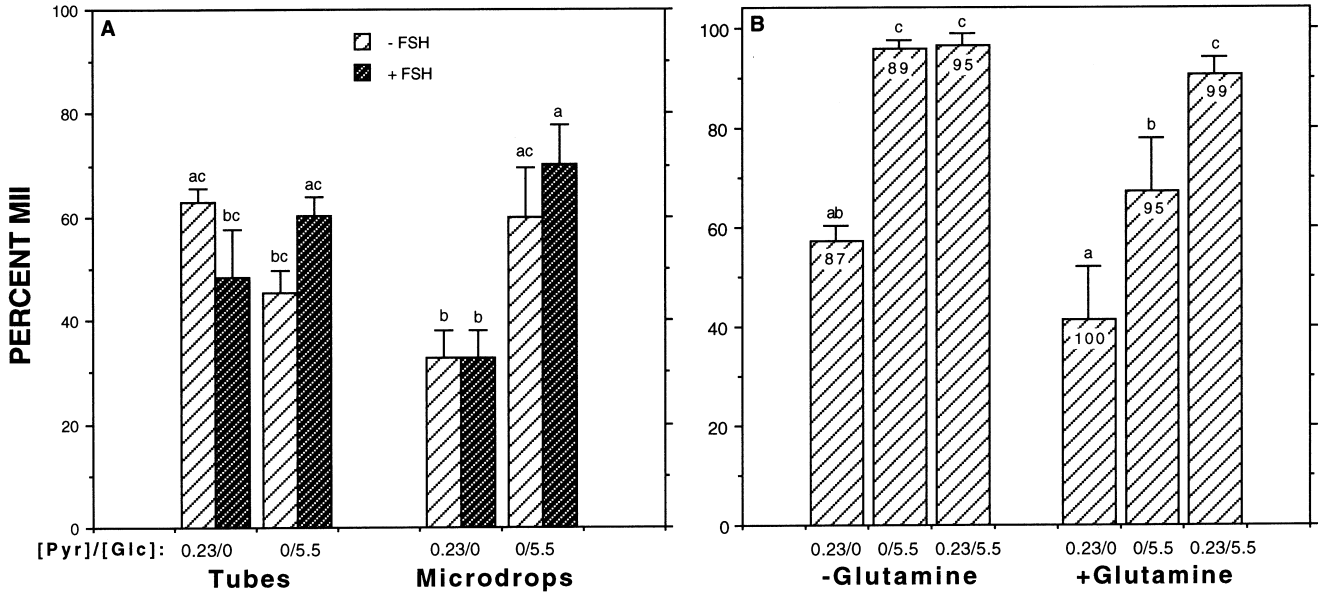

Figure 5 Effects of medium volume on oocyte maturation. (*A*) CEO were cultured for 15 h in either 1 ml medium in tubes (40 CEO per tube) or 8 µl microdrops under oil (10 CEO per microdrop), containing 2 mM glutamine plus either pyruvate or glucose alone, in the presence or absence of FSH. (*B*) CEO were cultured 15 h in microdrops containing different combinations of pyruvate and glucose, in the presence or absence of 2 mM glutamine. The number of MII oocytes in each group was determined by Hoechst staining. The experiment in (*A*) was conducted four times, that in (*B*) three times. The numbers within the bars in (*B*) denote the percentage of viable oocytes. Groups that share a common letter at the top of the bar are not significantly different.

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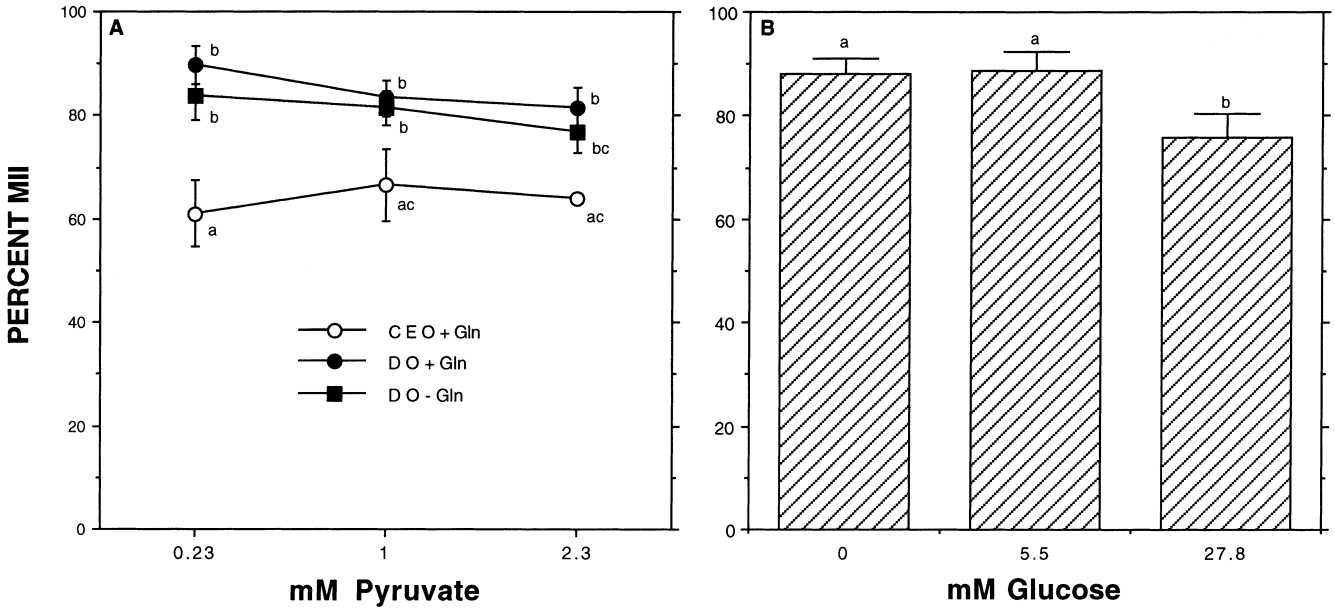

Figure 6 Effects of energy substrates on maturation of denuded oocytes. (*A*) CEO or DO were cultured for 15 h in 1 ml medium containing 2 mM glutamine plus increasing concentations of pyruvate from 0.23 to 2.3 mM. An additional DO group was exposed to the same pyruvate concentrations in medium lacking glutamine. (*B*) DO were cultured for 15 h in 1 ml medium containing 2 mM glutamine and 0.23 mM pyruvate, plus 0, 5.5 or 27.8 mM glucose. The number of MII oocytes in each group was determined by Hoechst staining. Each experiment was conducted five times. Groups that share a common letter are not significantly different.

To assess the effect of glucose on maturation of DO, cultures were maintained for 15 h in medium containing 2 mM glutamine and 0.23 mM pyruvate, plus 0, 5.5 or 27.8 mM glucose. In the absence of glucose, 86% of DO progressed to MII (Fig. 6*B*). Glucose had no effect at 5.5 mM but at 27.8 mM reduced the number of MII oocytes by 12% to 72%. Thus, glucose had no additional beneficial effect on the meiotic maturation of DO.

Effects of energy substrates on kinetics of oocyte maturation

Kinetics experiments were performed wherein CEO were cultured in 1 ml medium in tubes for 8, 12, and 16 h to assess maturation to MII in glutamine-supplemented medium in the presence or absence of FSH under the following four energy substrate conditions: (1) no additional substrate; (2) 1 mM pyruvate; (3) 5.5 mM glucose; and (4) pyruvate plus glucose. These data are presented in Fig. 7.

In all groups, in the presence or absence of FSH, no MII oocytes were observed after 8 h. When medium contained glutamine alone, only 10% of the oocytes had reached MII by 12 h, and no further maturation was observed 4 h later. When FSH was added, similar kinetics were observed in this treatment group, with only a marginal increase in MII oocytes.

Supplementing the medium with 5.5 mM glucose increased the number of MII oocytes to 38% at 12 h, but no further maturation was observed after this time point (40% MII at 16 h). The addition of FSH to glucose-containing medium reduced the number of MII oocytes at 12 h to 30% but increased this number at 16 h to 55%.

Pyruvate at 1 mM produced a maturation rate at 12 h comparable to that observed in 5.5 mM glucose (43%), but a higher percentage was observed at 16 h (63%; Fig. 7, left). FSH reduced these percentages at both time points by 20–31%.

The combination of pyruvate and glucose produced the greatest percentage of MII oocytes at all time points, in either the presence or absence of FSH. Because FSH is known to produce a transient arrest of spontaneous maturation (Vivarelli *et al*., 1983; Downs *et al*., 1985), and because we earlier showed that FSH reduces the meiotic completion rate in this treatment group by 35% (see Fig. 3), the kinetics were extended to 20 h. This was to determine whether the FSH effect was simply to delay maturation or to suppress it on a more permanent basis. As shown in Fig. 7, the maturation rate peaked by 16 h of culture (91% MII) in the absence of FSH, with only a marginal increase after 20 h. The inclusion of FSH reduced the frequency of MII oocytes at 16 h by 21% to 70%, with little change during the next 4 h. These results therefore indicate that FSH

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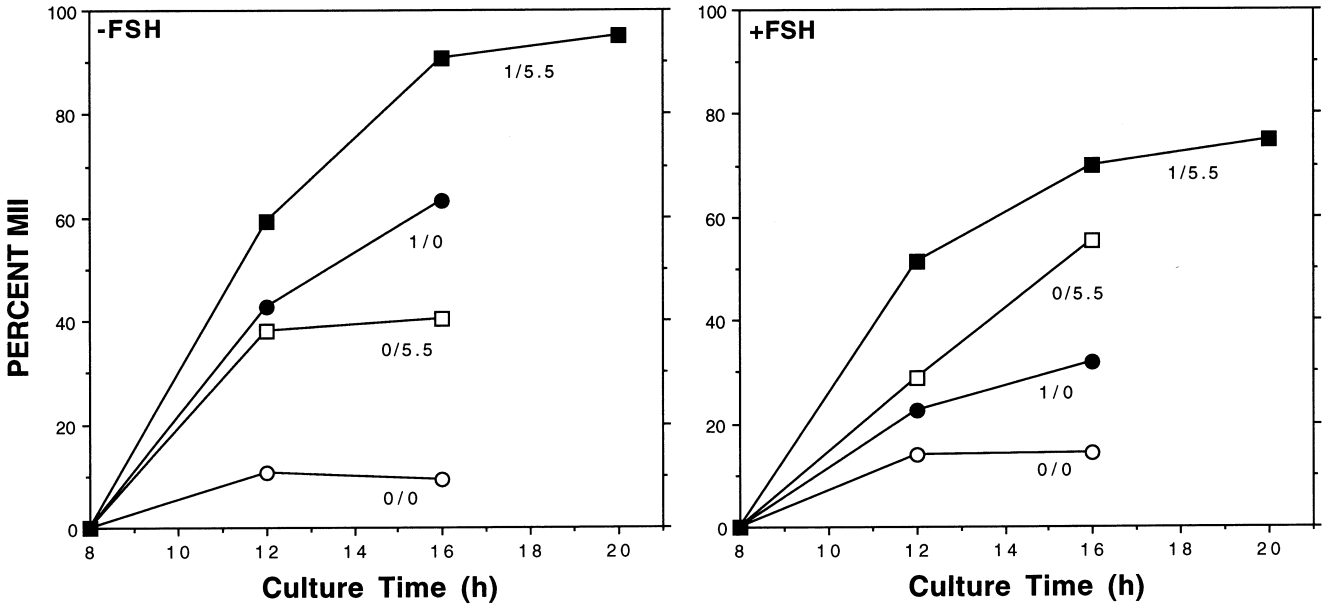

Figure 7 Kinetics of polar body formation. CEO were cultured for varying periods from 8 to 20 h in medium containing 2 mM glutamine (0/0) or glutamine plus 1 mM pyruvate (1/0), 5.5 mM glucose (0/5.5) or pyruvate plus glucose (1/5.5), in either the absence (left panel) or presence (right panel) of FSH. At the appropriate time point, the number of MII oocytes in each was determined by Hoechst staining. Each data point represents the mean of two determinations.

exerts a long-term suppression of maturation that is not related strictly to its transient inhibition of meiotic resumption (see below).

To relate the kinetics of meiotic completion to earlier effects of culture conditions on meiotic resumption, the same treatment groups were analysed for their effects on the kinetics of GVB in the presence or absence of FSH. Cultures were carried out for up to 2.25 h in the absence of FSH and for up to 3.75 h in the presence of FSH. As shown in Fig. 8, in the absence of FSH meiotic resumption occurred with the fastest kinetics in medium containing pyruvate, with 51% GVB after only 0.75 h and 99% by 1.25 h. The maturation rate was delayed slightly with the addition of glucose to pyruvate-containing medium, and even slower kinetics were observed in 5.5 mM glucose. Oocytes exhibited the slowest maturation in the absence of both pyruvate and glucose. Under these latter conditions 21% of the oocytes were still in the germinal vesicle stage after 1.75 h, but 95% GVB was observed by 2.25 h.

The addition of FSH delayed maturation in all four groups, and the relative kinetics were similar to those observed in the –FSH groups. Pyruvate-containing groups, with or without glucose, exhibited about 50% maturation after 2.25 h, but complete maturation was manifested by 3 h (Fig. 8). Meiotic resumption was slower in the absence of pyruvate and was not complete until 3.75 h of culture.

Discussion

Results of this study confirm that energy substrates are an important consideration in the optimisation of culture conditions for oocyte maturation *in vitro*. In the mouse, not only do the relative concentrations of glutamine, pyruvate and glucose determine the extent of spontaneous maturation, but they also influence the meiotic response to stimulatory ligands such as FSH. Thus, even relatively subtle changes in medium composition can have a significant impact on meiotic regulation. In addition, the data provide further evidence that cumulus cells mediate both positive and negative signals for oocyte maturation; the balance between the two is influenced by energy supplementation and determines the extent of maturation of CEO in culture.

While 2 mM glutamine alone can supply enough energy to fuel GVB and maintain the viability of CEO during 15 h of culture, it failed to effectively support the completion of meiotic maturation and, in fact, proved detrimental when added to medium containing pyruvate or glucose. It is therefore a poor choice as sole energy substrate for mouse oocyte maturation. On the other hand, this amino acid supports the maturation of rabbit (Bae & Foote, 1975) and hamster (Gwatkin & Haidri, 1973) oocytes. The reason for its suppressive action on mouse oocyte maturation cannot be deduced from these results, but may be related to its

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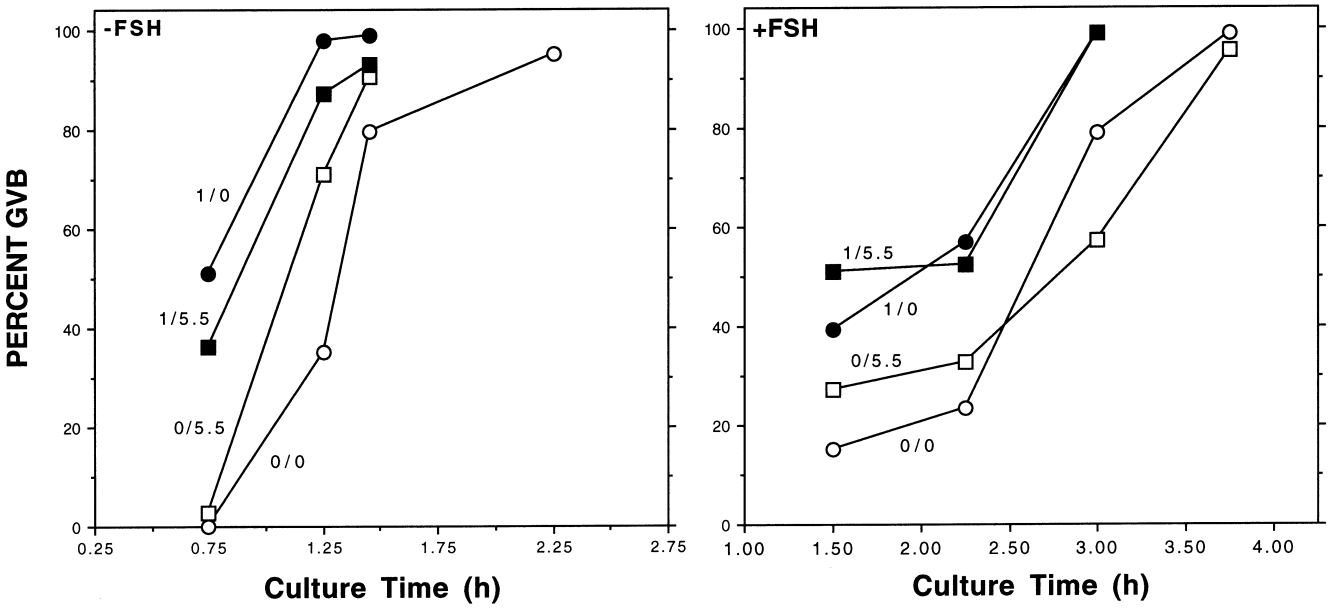

Figure 8 Kinetics of germinal vesicle breakdown. CEO were cultured for varying periods in medium containing 2 mM glutamine (0/0) or glutamine plus 1 mM pyruvate (1/0), 5.5 mM glucose (0/5.5) or pyruvate plus glucose (1/5.5), in either the absence (left panel) or presence (right panel) of FSH. At the appropriate time point, cumulus cells were removed and unstained oocytes were assessed for GVB under a stereomicroscope. Each data point represents the mean of two determinations.

participation in *de novo* purine synthesis. Glutamine has been shown to augment both *de novo* purine synthesis and the meiosis-suppressing action of hypoxanthine and dbcAMP in CEO (Downs, 1999).

When cultured in 1 ml medium in tubes, CEO were able to progress meiotically to the MII stage in the presence of glucose alone, but the success rate was usually below 50%. The amount of glucose was not limiting, because increasing the concentration five-fold from 5.5 to 27.8 mM had no further positive effect on maturation and, in some circumstances, was inhibitory. Under these conditions, pyruvate was generally a more effective substrate for supporting the completion of maturation, but maximal maturation levels still only reached 64–68%. Hence, simply providing high levels of glucose or pyruvate alone in the medium will not suffice to optimise maturation of CEO, and may even prove detrimental.

Although 1 mM was the most effective concentration of pyruvate for oocyte maturation, addition of 5.5 mM glucose to pyruvate-containing medium increased the MII percentage by 24–26%. There was no further enhancement with higher levels of glucose, but in the presence of glutamine a supraphysiological level of glucose (27.8 mM) led to a 16% decline in MII oocytes. These results demonstrate that when a large volume of medium (1 ml) is used for the culture system both pyruvate and glucose are required to achieve the highest number of MII oocytes and that the proper ratio of the two substrates can be critical to achieve this end. Kim & Schuetz (1991*a*) reported that pyruvate deficiency compromises polar body formation, and we have previously shown that adding pyruvate to glucose-containing medium can increase the percentage of polar body formation in spontaneously maturing CEO (Downs & Mastropolo, 1997). On the other hand, the beneficial effect of glucose when added to pyruvate-containing medium is not exclusively the result of increased pyruvate availability, because raising pyruvate from 1.0 to 2.3 mM in the absence of glucose failed to enhance the MII frequency. Consequently, glucose must provide an additional, meiosis-promoting effect unrelated to changes in pyruvate level, be it through glycolytic ATP, products of the pentose phosphate/purine metabolic pathways, or by some other means. It is important to caution that some culture media such as Waymouth's MB 752/1 contain the higher concentration of glucose (27.8 mM), and under certain conditions this could prove suboptimal for oocyte maturation. However, simply increasing pyruvate concentrations in such medium may not completely resolve this condition, since no difference in MII percentage was observed in 0.23 versus 1 mM pyruvate when CEO were cultured in high glucose (see Fig. 2).

Hormone supplementation is often used to stimulate meiotic maturation and/or promote developmental competence in oocytes matured *in vitro*. For example, FSH induces meiotic resumption in isolated CEO maintained in meiotic arrest by a variety of inhibitory

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compounds (Downs *et al*., 1988). Furthermore, including FSH during oocyte maturation has been shown to increase the 2-cell to blastocyst transition after *in vitro* fertilisation (Downs *et al*., 1986; Schroeder *et al*., 1988; Eppig *et al*., 1992). It was therefore of interest to test the effects of this gonadotropin on spontaneous maturation under a variety of energy substrate conditions. Interestingly, a positive action of FSH on completion of the first meiotic division was observed only in medium containing glucose but no pyruvate. We propose that the positive action of FSH is due, at least in part, to its stimulation of glycolysis and the provision of pyruvate to the oocyte. The stimulatory action of FSH on glycolysis in oocyte–cumulus cell complexes is well documented (Billig *et al*., 1983; Zuelke & Brackett, 1992; Downs *et al*., 1996), as is the release of pyruvate by cumulus cells (Donahue & Stern, 1968; Leese & Barton, 1985; Downs & Mastropolo, 1994). The released pyruvate could act in a paracrine fashion, perhaps in concert with non-pyruvate-related actions of glucose, to promote maturation. Other glucose metabolites or glucose-stimulated paracrine factors could also serve in this capacity.

The inhibitory effect of FSH in medium containing 1 mM pyruvate was unexpected and raises the possiblity that gonadotropin stimulation diverts pyruvate from a meiosis-promoting metabolic route to one that is without effect or perhaps even inhibitory. The mechanism whereby this occurs is unclear, but the TCA cycle is unlikely to be involved, since FSH has no effect on the oxidation of radiolabelled pyruvate by oocyte–cumulus cell complexes (Downs, 2000). Nor does the presence or absence of glucose seem to matter, since FSH produced the same effect under either condition. These results demonstrate that culture conditions, specifically energy substrate supplementation, can determine how hormones influence nuclear maturation.

A paracrine effect of cumulus cells is supported by the results of the microdrop experiments, in which the CEO/µl ratio was increased from 40/1000 (0.04) in tubes to 10/8 (1.2) in microdrops, a 30-fold change. Under microdrop conditions, MII percentages were reduced in medium containing only pyruvate but increased in glucose-containing medium when compared with maturation in tubes. The result in pyruvate-containing medium may be due to consumption of substrate and an inability of the oocyte–cumulus cell complex to adequately replenish pyruvate in the absence of glucose. The higher percentage of maturation in glucose-containing medium is consistent with an extracellular accumulation of glucose-derived, meiosis-promoting paracrine factor(s). One potential candidate is pyruvate, since microdrops of conditioned glucose-supplemented medium after 18 h of CEO culture showed a pyruvate level of 0.16 mM compared with only 0.01 mM in the conditioned medium from tubes (Downs, Humpherson and Leese, unpublished results). Other possibilities include a meiosis-activating sterol, present in follicular fluid (Byskov *et al*., 1995) and purportedly released by cumulus cells in culture (Guoliang *et al*., 1994; Byskov *et al*., 1997), and activin, a protein released by granulosa cells (Sidis *et al*., 1998; Silva & Knight, 1998) that promotes maturation in rat (Itoh *et al*., 1990; Sadatsuki *et al*., 1993), primate (Alak *et al*., 1996) and bovine (Silva & Knight, 1998) oocytes.

Glutamine had the greatest impact on oocyte maturation in microdrop cultures. Of particular significance was the finding that 96% of the CEO cultured in microdrops containing 5.5 mM glucose progressed to MII in the absence of glutamine compared with only 67% in its presence. Such an increase in maturation levels cannot be explained simply by the loss of glutamine, since the percentage of MII in oocytes cultured in identical medium in tubes was only 44–59% (Figs. 1, 4). It is proposed that the smaller culture volume facilitates accumulation of paracrine factors released by the complex that promote completion of meiotic maturation, especially when the inhibitory influence of glutamine has been eliminated. These results demonstrate that essentially all oocytes derived from eCG-primed immature mice are competent to complete nuclear maturation, but the ability to do so *in vitro* is dictated principally by culture conditions.

Evidence linking increased tissue/culture volume ratios with putative paracrine or autocrine factors has previously been demonstrated using embryos (Wiley *et al*., 1986; Kane, 1987; Paria & Dey, 1990; Lane & Gardner, 1992; Gardner *et al*., 1994; McKiernan & Bavister, 1994; Sherbahn *et al*., 1996) and oocytes (Downs & Mastropolo, 1994; Guoliang *et al*., 1994; Byskov *et al*., 1997; Liu *et al*., 1997). It is also important to note that studies by Biggers *et al*. (1967), Donahue & Stern (1968) and Kim & Schuetz (1991*a*) demonstrating high levels of MII after culture of mouse oocytes with cumulus cells in the presence of glucose were carried out using microdrops. The above studies support the premise that release of positive factors such as energy substrates or growth factors within the microdrop promotes development of embryos or oocytes. However, often ignored in this type of study is the fact that culture in microdrops could also facilitate the depletion of potentially toxic or inhibitory substances present in the medium by partitioning into the oil or through consumption by the cultured tissue. Consistent with this idea is the finding that removal of glutamine from the medium improved the percentage of oocytes reaching MII. Thus, although the benefits of a reduced culture volume have been demonstrated, further work will be required before definitive conclusions can be made concerning the mechanism involved.

Cumulus cells impose an inhibitory constraint on spontaneous oocyte maturation. When oocytes were

cultured in glutamine- and pyruvate-supplemented medium in tubes, 16–29% more DO than CEO developed to MII. In addition, glutamine did not have the same effect on DO as it did on CEO, since dose response curves for DO in the presence or absence of this amino acid were essentially superimposable. A similar inhibitory effect of cumulus cells on the spontaneous maturation of both I/LnJ and LT/Sv oocytes has been reported (Eppig & Wigglesworth, 1994). It is possible that cumulus cells compete with the oocyte for pyruvate and other beneficial medium components and impede their availability to the germ cell. Alternatively, cumulus cells may transmit inhibitory molecules to the oocyte, perhaps through the gap junctional pathway, and this effect may be accentuated by the presence of glutamine. On the other hand, cumulus cells also mediate a positive action of glucose on oocyte maturation, presumably in large measure through their high glycolytic activity and release of pyruvate. Therefore, cumulus cells can provide both positive and negative signals for maturation, and the relative influence of each of these signals may determine the extent, as well as the kinetics (see below), of nuclear maturation in CEO. These characteristics no doubt extend to the membrana granulosa cells within the follicle and are a major influence in the acquisition of developmental competence by the oocyte (Moor *et al*., 1998).

The kinetics experiments revealed that the rate of meiotic resumption is influenced by the energy substrate composition. GVB kinetics were fastest when medium contained pyruvate, but were attenuated with the inclusion of glucose and were even slower when both pyruvate and glucose were eliminated. These results demonstrate opposing stimulatory and inhibitory functions for pyruvate and glucose, respectively, on spontaneous meiotic resumption and are in agreement with the actions of these substrates on meiotically arrested oocytes in culture (Downs & Mastropolo, 1994). The slow rate of maturation in glutamine alone may indicate that glutamine provides just enough energy for this response; alternatively, the contribution by glutamine may be nominal, with residual energy present at the time of isolation mediating this function. FSH suppressed meiotic resumption in all groups, but less effectively in pyruvate-containing medium. The addition of pyruvate to glucose-containing medium shifted the maturation curve upward by 22–42%, an action probably due to a suppression of glucose utilisation (Downs *et al*., 1997), and reduced glycolytic production of ATP that is thought to be inhibitory to spontaneous maturation (Downs, 1995).

The kinetics of polar body formation were also fastest in pyruvate-containing medium. In addition, while the percentage of MII oocytes continued to rise throughout the culture period in the presence of pyruvate, it peaked by 12 h in medium without pyruvate. This indicates utilisation of pyruvate for the duration of maturation, from GVB through polar body extrusion, in agreement with Kim & Shuetz (1991*a*). Although FSH reduced the extent of polar body formation, this effect cannot be attributed solely to a transient arrest of meiotic resumption, since the latter suppression was of such short duration: GVB in FSH-treated oocytes cultured in pyruvate plus glucose was delayed by only 1.25 h, yet the number of MII oocytes in this group reached its maximum by 16 h, with no significant increase after 4 more hours of culture. Therefore, FSH must have an additional inhibitory action on meiotic progression separate from, and subsequent to, GVB.

In the present study we have not considered cytoplasmic maturation, the process whereby the developing oocyte acquires competence for fertilisation and subsequent embryo development. While competence for both nuclear and cytoplasmic maturation develops near the end of the oocyte's growth phase, these two processes are not necessarily coordinated, as the completion of nuclear maturation does not always guarantee successful fertilisation, let alone pre- or postimplantation development (Eppig, 1996). Yet a reduction division must precede fertilisation or problems with ploidy will compromise the viability of the resulting embryo. While some aspects of cytoplasmic maturation occur in the absence of nuclear maturation, others are implemented following GVB (Eppig, 1996). Thus, optimising conditions for the completion of nuclear maturation should help promote these aspects of cytoplasmic maturation as well. The choice of culture medium for oocyte maturation has been shown to affect the developmental capacity of mouse (van de Sandt *et al*., 1990) and bovine (Rose & Bavister, 1992) oocytes, but in these studies it is not clear which component(s) of the preferred medium conferred an advantage to the oocyte. Recent efforts have been made to define the nutrient conditions that benefit developmental competence in oocytes during meiotic maturation *in vitro* (e.g. Rose-Hellekant *et al*., 1998). A better understanding of how energy substrates and other medium components affect meiotic maturation will enable further refinements that not only increase the percentage of maturing oocytes but also augment their developmental capacity.

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