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Observations on Rat Oocyte Maturation *in Vitro*: Morphology and Energy Requirements

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Denuded ovarian oocytes, obtained from prepuberal rats, were incubated under oil and their development was studied by time-lapse cinemicrography. Polar bodies were formed about 8 h after autopsy and showed active movements immediately following abstriction. The oocyte membrane did not show contraction wrinkles during polar body formation as had been observed in mouse oocytes. Unfertilized tubal oocytes formed a second polar body *in vitro* about 45 min after isolation.

Rat ovarian oocytes were able to utilize pyruvate, lactate, and possibly even an endogenous energy source for maturation. The absence of oxygen prevented maturation. It is suggested that oxygen (oxidation–reduction potential changes) may trigger oocyte maturation *in vivo*.

The formation of the second polar body *in vitro* is not inhibited by KCN, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone, or the absence of oxygen, which indicates that the initiation of the second maturation division, unlike that of the first meiotic division, does not require generation of ATP by oxidative metabolism. Tubal oocytes lose the capacity to form a second polar body in the evening on the day of estrus.

It is likely that *in vitro* maturation of mouse oocytes depends on the availability of ATP, generated by oxidative phosphorylation (Biggers *et al.*, 1967; Zeilmaker *et al.*, 1972). Lactate can be utilized only when exogenous NAD is added to the medium (Zeilmaker *et al.*, 1972; Sorensen, 1972). Experiments with human and mouse oocytes have led to the conclusion that pyruvate is a key nutrient for maturation *in vitro* (Kennedy and Donahue, 1969; Biggers *et al.*, 1967). The failure to utilize glucose, glycogen, and lactate is probably due to a cytoplasmic failure such as shortage of NAD. The question arises whether the abovementioned unique metabolic requirements prevent maturation *in vivo* since, in a metabolically active follicle, lactate rather than pyruvate may be expected to be present, due to the probably limited amount of oxygen available in the central part of the follicle.

In the present study, the requirements for rat oocyte maturation *in vitro* were analyzed in order to gain information on the apparent energy requirements for this process. As signs of rat oocyte maturation *in vitro* have been mentioned only briefly by Edwards (1962), a short description of the formation of the first polar body *in vitro* is included. Data on the formation of the second polar body *in vitro* in isolated tubal oocytes are presented as well.

MATERIALS AND METHODS

Ovarian oocytes were obtained by puncturing ovaries from 30- to 40-day-old (R × U)F_1 hybrid rats in a balanced salt solution (Biggers *et al.*, 1967) of which part of the sodium chloride was replaced by the substrate being investigated (0.05 mM pyruvate, 10 mM lactate) to maintain osmolarity. Surrounding cumulus cells were removed by forcing the cell masses containing the oocytes through a tapered pipet. This treatment was necessary in order to make the germinal vesicle visi-

ble. Hyaluronidase treatment did not dissociate the cumulus of freshly isolated oocytes. Lactate stock solutions were prepared twice weekly. These solutions contained less than 0.05% pyruvate.

Tubal oocytes were isolated from virgin adult $(R \times U)F_1$ rats killed by cervical dislocation on the day of estrus (or 1 day later) by flushing the oviduct with balanced salt solution. In some experiments the cumulus oophorus was removed by exposure of the eggs to hyaluronidase (300 IU/ml in Hanks' solution containing 1 mg polyvinylpyrrolidone/ml).

Oocyte maturation was studied after incubation in 100-μliter drops of medium under paraffin oil at 37°C. One experiment was performed using anaerobic conditions (5% CO_2 in nitrogen) by incubating oocytes in depression slides which were placed in a modified Roux bottle (Wensinck and Ruseler van Embden, 1971). The incubation medium had been equilibrated in the bottle before the oocytes were added. In these particular experiments the interval between isolation and incubation was minimized (about 5 min).

In two experiments, cinemicrographs were taken in a temperature-controlled room through an inverted microscope in order to study the time sequence of maturation. Pictures were taken every 5 or 30 sec using electronic flash.

Tubal eggs were sectioned after transfer to ant cocoons (Mintz, 1971) and embedding in paraffin.

RESULTS

Experiment 1

Formation of the First Polar Body in Vitro

Analysis of five time-lapse cinemicrographs showed that the germinal vesicle of 40 inspected oocytes disappeared 75–130 min after isolation and incubation in pyruvate-containing medium under 5% CO_2 in air. The abstriction of the first polar body in 34 oocytes was completed 8 h and 20 min after isolation (5 cultures, SE: 20 min). This process was preceded by a change in the properties of the oocyte membrane at the site of future polar body formation. A circular indentation increased in diameter; at the same time this indentation contracted progressively so that in 24–36 min a polar body was abstricted. Even before the abstriction was completed the membrane of the future polar body showed undulating movements. Immediately after its formation the polar body made ameboid movements, which lasted about 3 h. Thereafter, the polar body became more quiet and often degenerated (Fig. 1). Even when the culture period was prolonged for another 12 h the formation of the second polar body was not observed.

With some experience, oocytes with degenerated polar bodies could easily be distinguished from oocytes which had not formed a polar body by turning the oocytes with the pipet so that the remnants of the first polar body can be seen between the vitelline membrane and the zona pellucida.

Experiment 2

Effects of Oxygen and Energy Substrate on Rat Oocyte Maturation in Vitro

In this experiment the effect of using lactate as an energy source was tested. Unexpectedly, oocytes incubated in medium without pyruvate or lactate also showed maturation. For this reason oocytes obtained from one rat were allotted to three groups for incubation in pyruvate, lactate, or salt solution. Care was taken to use clean pipets for each incubation and to remove the granulosa cells from the oocytes.

The results, presented in Table 1, indicate that polar bodies were formed even

TABLE 1

SUBSTRATE AND RAT OOCYTE MATURATION

Energy source	Number of incubations	Number of polar bodies[a]/number of oocytes studied
None	18	60/192[b] (31%)
0.50 mM pyruvate	20	74/187 (39%)
10 mM lactate	15	61/129 (47%)

[a] Including degenerated polar bodies.

[b] 40 Oocytes had degenerated after pb. formation.

Legend: Oocytes obtained from prepuberal rats were incubated for 18 h under 5% CO_2 in air in oil-drop cultures.

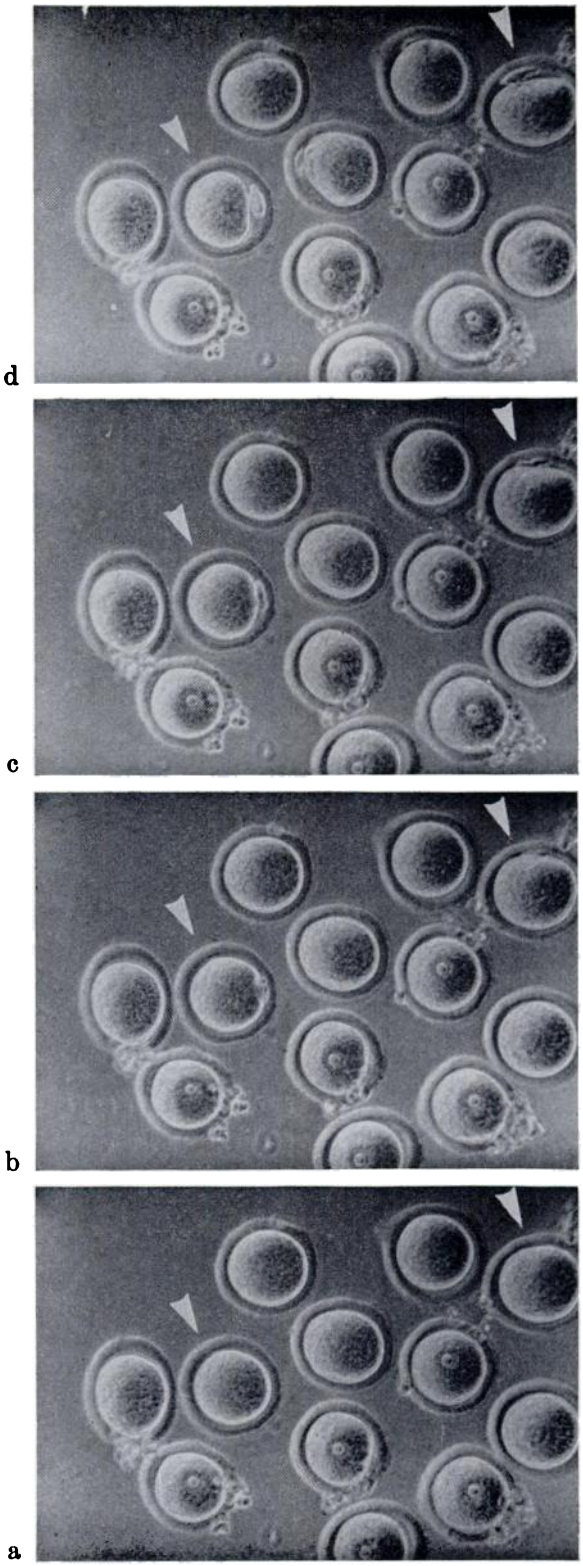


FIG. 1. Ovarian oocytes, isolated from prepuberal rats, cumulus cells removed by pipetting. Pictures taken at 8 h (a); 8 h 10′ (b); 8 h 15′ (c) and 8 h 30′ (d) after incubation. Abstriction of polar body visible in third oocyte from top and in oocyte at bottom right.

TABLE 2

EFFECT OF OXYGEN ON GERMINAL VESICLE DISAPPEARANCE IN MEDIUM WITH 10 mM LACTATE

	No. of oocytes undergoing GV breakdown/total	No. with polar body/total after standard culture conditions
After 4 h 0% O_2	2/57	23/51 (45)%
After 4 h 95% O_2	13/30	13/30 (43%)

Legend: Oocytes were incubated with lactate for 4 h under 0% O_2 or 95% O_2. The number of oocytes undergoing germinal vesicle breakdown was recorded after 4 h and the oocytes were then placed under 5% CO_2 in air for a further period of 14 h and the number of polar bodies obtained is recorded.

when no energy substrate was added; however, degeneration occurred more frequently under these circumstances. (Figure 1 shows granulosa cells which were not removed for the morphological study of Exp I but which were carefully removed for the metabolic studies.)

Since in a model study (Zeilmaker *et al.*, 1972) evidence was obtained that mouse oocyte maturation could be initiated by the addition of oxygen to a system containing pyruvate, a similar experiment was performed with rat oocytes. Oocytes were incubated for 4 h in an atmosphere containing 0% or 95% O_2. The high oxygen content of the gas phase was chosen with the expectation that the contrast with the anaerobic condition would be optimal. Later (unpublished) observations have shown that essentially the same results are obtained when 5% CO_2 in air is used. After this period of 4 h the oocytes were incubated under oil and air with 5% CO_2 for 14 h more. Lactate (10 mM) was used as an energy source. The results, presented in Table 2, show that oxygen was required for the initiation of oocyte maturation since the germinal vesicle did not disappear under anaerobic conditions but only when oxygen was available. The subsequent formation of polar bodies shows that the oocytes which showed delayed maturation were viable.

TABLE 3

In Vitro MATURATION OF RAT OOCYTES AND OXYGEN CONCENTRATION IN GAS PHASE

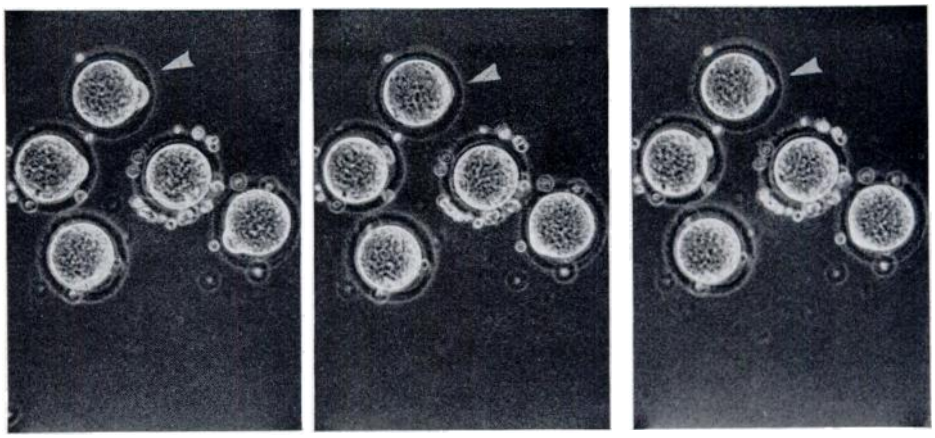
% O_2 in gas phase	No. of oocytes with 1st polar body/total
0	0/18 (0%)
5	57/207 (28%)[a]
20	22/96 (23%)[a]

[a] Not significantly different $\chi^2 = 0.51$; $df = 1$; $p > 0.3$

Legend: Oocytes were incubated in medium with 0.50 mM pyruvate in modified Roux bottles for 17–24 h. The lower incidence of maturation in this experiment, as compared with the data of Tables 1 and 2, is probably due to the fact that the oocytes were cultured in depression slides placed in Roux bottles for the duration of the experiment.

The effect of different oxygen tensions in the gas phase on maturation *in vitro* was also tested. Denuded oocytes from 19 rats were isolated in medium containing 0.50 mM pyruvate and incubated under 0, 5, or 20% oxygen, with 5% CO_2 and nitrogen in modified Roux bottles. After 17–24 h of incubation, the cultures were inspected for signs of maturation. The results are presented in Table 3. From these data it may be concluded that rat oocytes mature equally well with 5 or 20% O_2 in the presence of pyruvate.

Finally, 53 oocytes obtained from 4 prepuberal rats were incubated under 5% O_2, 5% CO_2 and nitrogen in medium without energy source, but which contained 1 mM KCN, in order to investigate whether activity of the cytochrome oxidase system is required for oocyte maturation. After 20 h in 39 of these oocytes a germinal vesicle could be seen; the remainder of the cells had degenerated. This indicates that for the induction of germinal vesicle breakdown oxidative processes are required.



a b c

FIG. 2. Tubal oocytes, isolated at estrus and cumulus cells removed by hyaluronidase treatment. Pictures taken at 25 (a); 45 (b) and 55 (c) min after incubation. Maximal bulging (a), disappearance of protrusion (b) and second polar body (c) in oocyte at the top. In the rat the first polar body has disappeared before ovulation.

Experiment 3

Observations on the Formation of the Second Polar Body in Vitro

Fifteen oocytes obtained from the oviducts of three rats were denuded with hyaluronidase. At this stage all oocytes had formed a protruding bulge which disappeared 27–35 min after isolation and incubation in pyruvate medium. A polar body was abstricted in 8–16 min at a place adjacent to this area. The second polar body membrane undulated slightly and less than that of the first polar body after its formation (Fig. 2).

Groups of 18 tubal oocytes were collected and transferred to ant cocoons immediately after isolation from the oviduct and after 1, 2, and 4 h of incubation in pyruvate medium. Following histological processing it could be seen that all oocytes fixed after isolation from the oviduct were in the metaphase II stage. The first polar body could not be detected. After 1 h of incubation the second polar body was abstricted in most cases. After 4 h of incubation the oocyte chromosomes were slightly scattered; a pronucleus was never observed.

In order to study the effect of the oxygen concentration in the gas phase on second polar body formation, tubal oocytes isolated on the morning of estrus were incubated with or without the cumulus in medium without energy source. Five hours later the eggs were inspected. The results are presented in Table 4. It may be seen that even in the absence of oxygen a second polar body was formed in 55% of the cases. This polar body was sometimes smaller (diam 7 μm) than a normal second polar body (diam 18 μm). The presence of the

TABLE 4

OXYGEN CONCENTRATION AND SECOND MATURATION DIVISION *in Vitro*

	0% oxygen	5% oxygen	20% oxygen
With cumulus	10/18 (55%)	33/33	10/10
Without cumulus	7/29 (24%)	22/22	23/23

Legend: Tubal oocytes isolated at estrus were incubated in medium without energy source under 5% CO_2 in various oxygen concentrations for 5 h. The number of oocytes with a second polar body is recorded.

cumulus had no effect on second polar body formation.

Fifty-five tubal oocytes with cumulus were incubated in medium without energy source in the presence of 1 mM KCN or 5 or 10 μM carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) an uncoupler of oxydative phosphorylation, in order to test whether formation of ATP by oxydative phosphorylation is required for the induction of the second maturation division. As none of these treatments suppressed the formation of the second polar body as inspected after 4 h (Table 5), it seems that the above mentioned process is not essential for the completion of meiosis.

It was subsequently determined how late on the day of estrus oocytes lose the capacity to complete the second meiotic division *in vitro*. Groups of tubal oocytes with cumulus cells were incubated in pyruvate medium under 20% oxygen at 9, 17, and 23 h of the day of estrus and at 9 h of the day of metestrus. All oocytes formed a polar body following incubation at 9 or 17 h of estrus. At 23 h (5 rats, 41 oocytes) all oocytes obtained from a given rat behaved in the same way: either all matured or none matured. Incubation at 9 h on metestrus never led to polar body formation (31 oocytes). It seems, therefore, that late in the evening of the day of estrus oocytes lose the capacity to form a second polar body.

TABLE 5

EFFECTS OF 1 mM KCN OR 5–10 μM FCCP[a] ON THE FORMATION OF THE SECOND POLAR BODY *in Vitro*

Addition to medium	No. of oocytes with second polar body/total
1 mM KCN	28/33 (85%)
5 μM FCCP	15/15 (100%)
10 μM FCCP	6/7 (85%)

[a] Carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone.

Legend: Tubal oocytes were incubated in medium without energy source under 5% CO_2 in air and inspected for the presence of the second polar body 4 h later.

DISCUSSION

The present study shows that isolated rat oocytes form the first polar body *in vitro*. Both the way in which the polar body is formed and the metabolic requirements for its formation are different in the rat and mouse. It appears that the interval between isolation and complete maturation *in vitro* is about 4 h shorter in the rat than in the mouse (see Van Vliet and Zeilmaker, 1972). This may also be deduced from studies *in vivo*. In mice, most polar bodies appear 11 h after HCG treatment (Edwards and Gates, 1959), whereas, in rats, polar bodies begin to appear 8 h after the LH surge (Tsafriri and Kraicer, 1972).

The circular indentation of the cell surface of the mouse oocyte before polar body extrusion, progressively contracts leading to the formation of an initially small polar body, which is later filled with cytoplasm. This process is the result of active contractions of the oocyte membrane, since contraction wrinkles are visible at the end of the filling phase. In the rat oocyte, this circular indentation seems to move excentrically from a central area over the cell surface; moreover, contractions of the oocyte membrane are not observed. Behind the indentation, even before the polar body is abstricted, the membrane shows active undulating movements. Immediately following its abstriction, the polar body of the rat oocyte shows active ameboid movements. Mouse polar bodies start some ameboid movements only 30–50 min after abstriction (Kuhl, 1941; van Vliet and Zeilmaker, 1972). The circular indentation, leading to polar body abstriction in the mouse, does not move over the cell surface but starts to constrict where it is first visible. This may explain the fact that, in our mouse oocyte cultures, polar bodies were occasionally observed which were nearly as large as the remaining oocyte (cf. Donahue, 1970). In the rat only small polar bodies were formed.

Not only does the mode of polar body

formation differ in rats and mice, but also the energy requirements for maturation are different. The most interesting observation in the present experiments was that polar body formation occurred in defined media in which albumin was the only potential energy source present. Although oocytes in this medium frequently showed signs of degeneration, polar bodies formed in 30% of the cases. Our finding of a requirement for oxygen for germinal vesicle disappearance and the observation that 1 mM KCN prevents germinal vesicle disappearance in rat oocytes, as has been observed in mice (Zeilmaker *et al.*, 1972), suggest that ATP is required for initiation of maturation in both rat and mouse oocytes.

In contrast to the situation in mouse oocytes (Biggers *et al.*, 1967), it appears that rat oocytes can utilize lactate and an endogenous energy source to a certain extent. When lactate is added to the medium the oocytes have a healthy appearance as compared with oocytes which have matured without an exogenous energy source. These metabolic requirements make it unlikely that the availability of specific nutrients (Pincus and Enzmann, 1935; Donahue and Stern, 1968) is the limiting factor in the timing of oocyte maturation. Instead, it seems likely that oxygen is a limiting factor, preventing oocyte maturation in mice, rats and possibly other species. As early as 1955, Chang discussed a possible role of oxygen in triggering oocyte maturation. Exposure of the follicle to an ovulating surge of LH causes profound changes in follicular cell metabolism and properties of the follicular wall (Lipner and Smith, 1971) which may lead to a change in oxidation–reduction potentials in the follicular fluid. Other studies on the role of oxygen in egg development have emphasized that a high oxygen concentration in the medium is detrimental to development of zygotes and oocytes (Whitten 1969; Haidri *et al.*, 1971).

Our experiments also show that rat tubal oocytes resume meiosis following explantation *in vitro*. The application of a cold shock *in vitro* (Thibault, 1949) is not required. It is well-known that in the rat the abstriction of the second polar body *in vivo* is normally a consequence of sperm penetration. Exposure of the female rat to ether anesthesia or local cooling (Thibault, 1949; Austin and Braden, 1954) also leads to the formation *in vivo* of the second polar body in tubal rat oocytes. In a study with rabbit eggs, explantation *in vitro* sometimes led to the formation of the second polar body (Pincus, 1939).

Similar treatment of tubal mouse oocytes did not result in the formation of a second polar body (Zeilmaker, unpublished).

The absence of an effect of addition of KCN or FCCP on the formation of the second polar body in the rat indicates that formation of ATP by oxydative phosphorylation is not required for the abstriction of the second polar body. The stimulus for the initiation of the second maturation division therefore is probably of a different nature than that leading to the disappearance of the germinal vesicle.

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