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Meiotic competence *in vitro* of pig oocytes isolated from early antral follicles*

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Summary. Pig oocytes were isolated from early antral follicles of different sizes and their abilities to resume and complete meiotic maturation *in vitro* were compared. After 24 h of culture, more than 80% of the oocytes from follicles 0·3–0·7 mm in diameter remained at the germinal vesicle stage, while 66, 94·3 and 100% oocytes from follicles 0·8–1·6, 1·7–2·2 and 3–5 mm in diameter, respectively, completed germinal vesicle breakdown. After 48 h of culture, 35% of the oocytes in the smallest follicle class progressed to prometaphase and only 4% to metaphase I. Of the oocytes from follicles 0·8–1·6 mm in diameter, 23% reached metaphase I and 17·3% metaphase II. About 50 and 76% of the oocytes from follicles 1·8–2·2 mm and 3–5 mm in diameter, respectively, extruded the first polar body.

The ability to resume meiosis (i.e. to undergo germinal vesicle breakdown) is reached by porcine oocytes when they approach their full size in antral follicles >0·8 mm in diameter and before they are capable of completing it (i.e. reaching metaphase II). The ability to complete meiotic maturation acquired in antral follicles of about 2 mm in diameter coincided with a significant decrease in the nucleolar transcriptional activity of the oocytes.

Introduction

When mammalian oocytes are released from preovulatory follicles, meiosis spontaneously resumes. However, conflicting results concerning the ability of pig oocytes from small antral follicles to resume maturation *in vitro* have been reported. While McGaughey, Montgomery & Richter (1979) claimed that about 80% of oocytes from follicles of 1–2 mm in diameter are capable of undergoing germinal vesicle breakdown, Tsafriri & Channing (1975) and Anderson & Hillensjö (1982) indicated that the incidence of maturation of oocytes from the same class is only 15–25%. Crozet, Motlik & Szöllösi (1981) showed that important morphological and functional changes occur *in vivo* in the nucleolus of pig oocytes during the early stages of antrum formation.

In the present study, oocytes were isolated from different classes of early antral follicles and their abilities to resume and complete meiotic maturation were compared. Oocyte competence has been related to oocyte size as well as to nucleolar structure and function.

Materials and Methods

Cyclic gilts of miniature pig crosses of the Minnesota and Göttingen strains were slaughtered on Days 2–4 and 8–10 of the cycle. The excised ovaries were immediately placed in warm phosphate-

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buffered saline (pH 7·4, 285 mosmol) and then cut into small pieces and transferred into the culture medium (see below) which was continuously bubbled with 5% CO_2 in air. Antral follicles were isolated under a dissection microscope, measured with a micrometer (× 25 magnification) and divided into four classes according to diameter: Class A: 0·3–0·7 mm; Class B: 0·8–1·6 mm; Class C: 1·7–2·2 mm; Class D: 3–5 mm.

The oocytes were isolated by rupture of the follicular wall, and only those surrounded by cumulus cells were cultured in 0·1 ml culture medium as drops under paraffin oil at 38°C under 5% CO_2 in air. The culture medium contained 72 ml isotonic TC 199 medium (Usol, Prague); 18 ml 1·45% $NaHCO_3$ + 0·002% phenol red; 10 ml 5·5% (w/v) glucose solution; 0·004 g sodium pyruvate; 10 mg freeze-dried calf serum growth protein (Usol)/ml; 50 i.u. penicillin and 5 mg streptomycin/ml. The supplements were dissolved in 3 times distilled water. After 24 or 48 h of culture, the cumulus cells were removed mechanically and the oocytes were mounted on slides, fixed in acetic alcohol (1 : 3 v/v) for 24 h, stained with 1% orcein and examined by phase-contrast microscopy. The diameter of at least 40 oocytes from each follicular class was measured with an ocular micrometer (× 200 magnification) immediately after isolation and removal of the cumulus cells. These oocytes were fixed and stained (see above) and served as control for germinal vesicle configuration before culture. Mean diameter and standard error of the mean were evaluated for each follicular class. Differences between the classes were tested by one-way analysis of variance.

For electron microscopy, oocytes were washed immediately after culture in cold (+4°C) PBS, fixed for 60 min in 2·5% (v/v) glutaraldehyde and 0·75% paraformaldehyde in 0·075 M-cacodylate buffer containing 0·1–1·5% potassium ferricyanide. They were then post-fixed for 60 min in 2% osmium tetroxide, washed in distilled water and stained overnight in a 0·5% aqueous uranyl acetate solution at 4°C. The oocytes were embedded in Durcupan (Fluka). Thin sections were stained with uranyl acetate for 30 min and with lead citrate for 10 min.

Results

Morphology

Nucleoli with small and large vacuoles were observed in the germinal vesicle of all Class A oocytes (Pl. 1, Figs 1 & 2). More than 50% of Class B oocytes showed the formation of a compact nucleolus, while large nucleolar vacuoles still prevailed in the other oocytes. About 60% of the oocytes isolated from Class C follicles had a compact nucleolus (Pl. 1, Fig. 3), sometimes

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PLATE 1

Figs 1–4. Pig oocytes before culture mounted on slides, fixed in acetic alcohol (1 : 3 v/v) and stained with 1% orcein (Loba-Chemic, Vienna) were photographed with phase-contrast microscopy. × 1600.

Fig. 1. Two nucleoli of an oocyte from a follicle 0·4 mm in diameter. The arrow indicates the position of the smaller nucleolus (see inset). Numerous nucleolar vacuoles are visible. Heterochromatic knobs in the nucleoplasm are indicated by arrowheads.

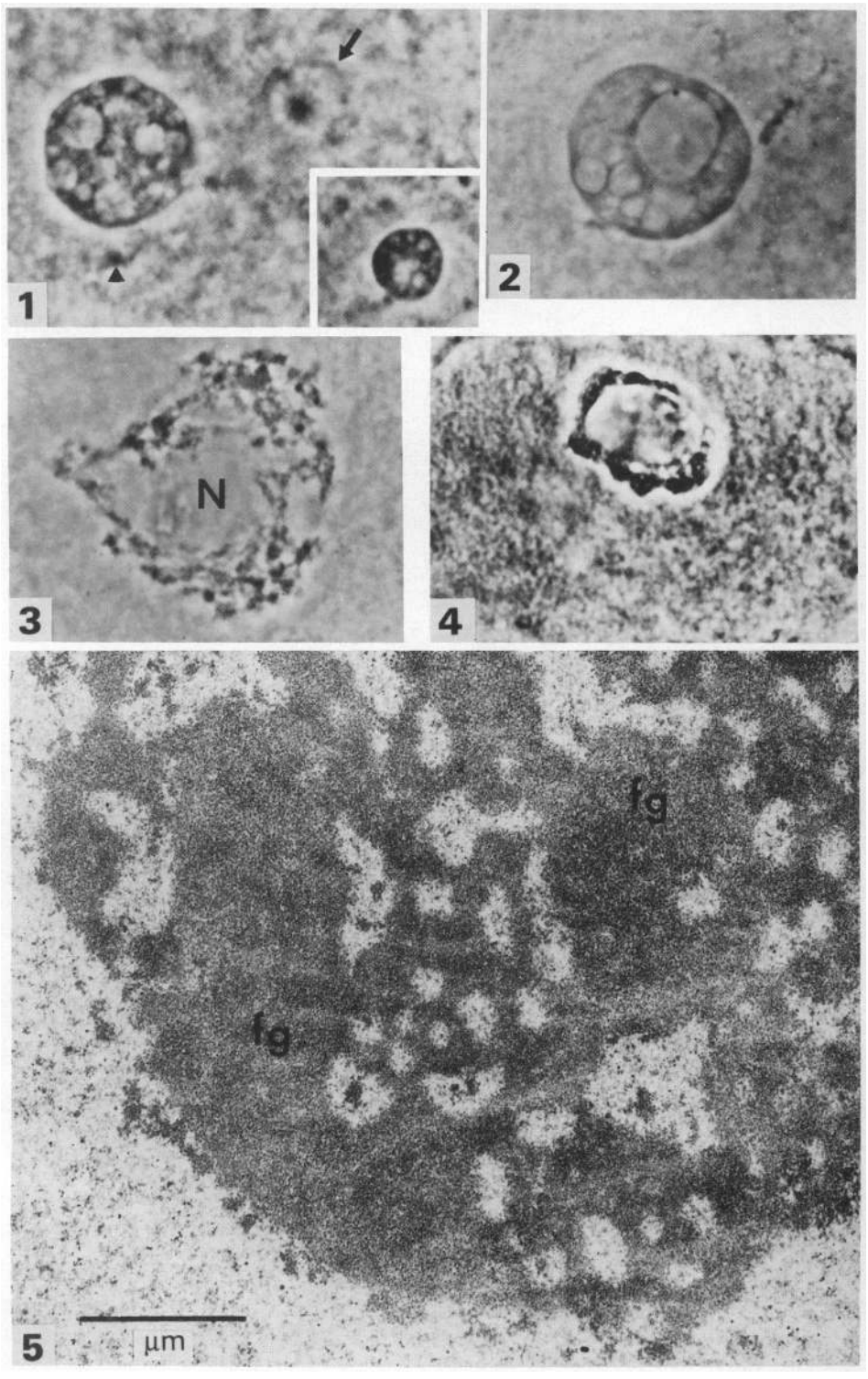
Fig. 2. Nucleolus with small and large vacuoles of an oocyte from a follicle 0·6 mm in diameter.

Fig. 3. Oocyte isolated from a follicle 1·6 mm in diameter; fine filamentous bivalents surround the nucleolar area (N). The nucleolus is out of focus.

Fig. 4. Germinal vesicle of an oocyte from a follicle 2·2 mm in diameter with a compact nucleolus. Well-stained condensed chromatin is localized around the nucleolus. No chromatin condensation is apparent in the finely stained granulated nucleoplasm.

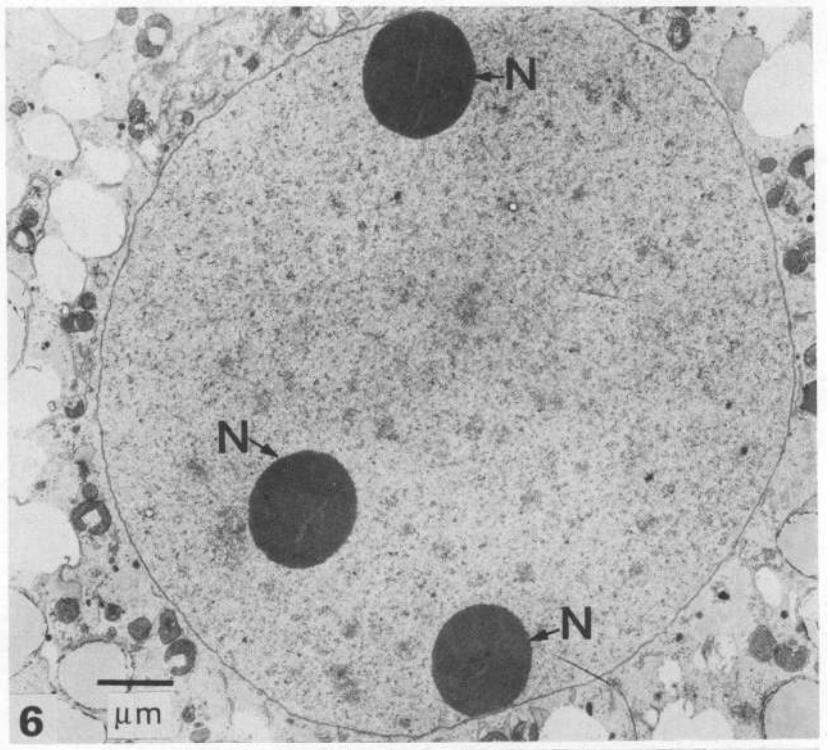
Fig. 5. Electron micrograph of a vacuolated nucleolus composed of fibrillo-granular elements (fg) of an oocyte from a follicle 0·3–0·7 mm in diameter cultured for 24 h. × 24 500.

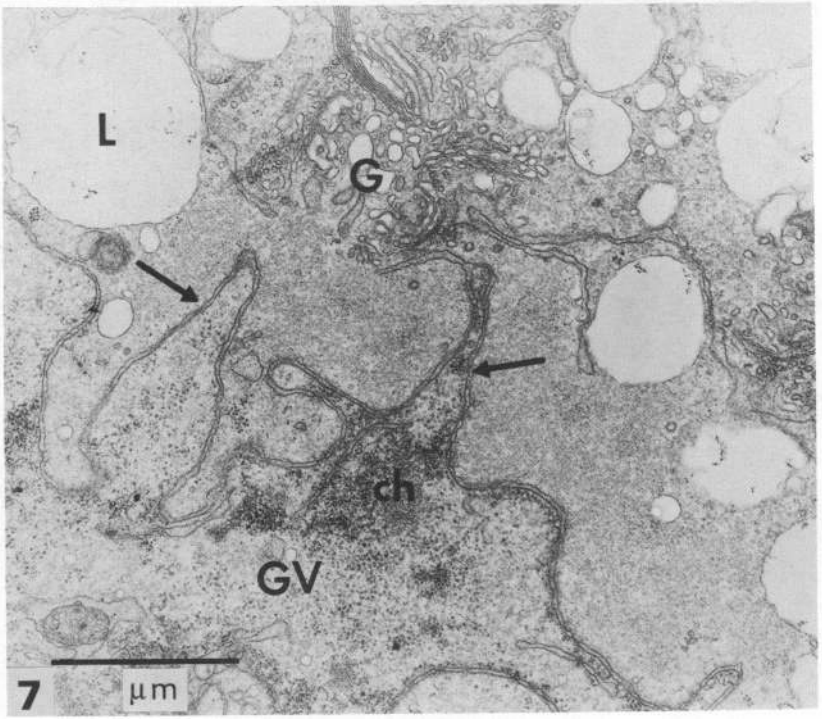
PLATE 1



(*Facing p.* 324)

PLATE 2





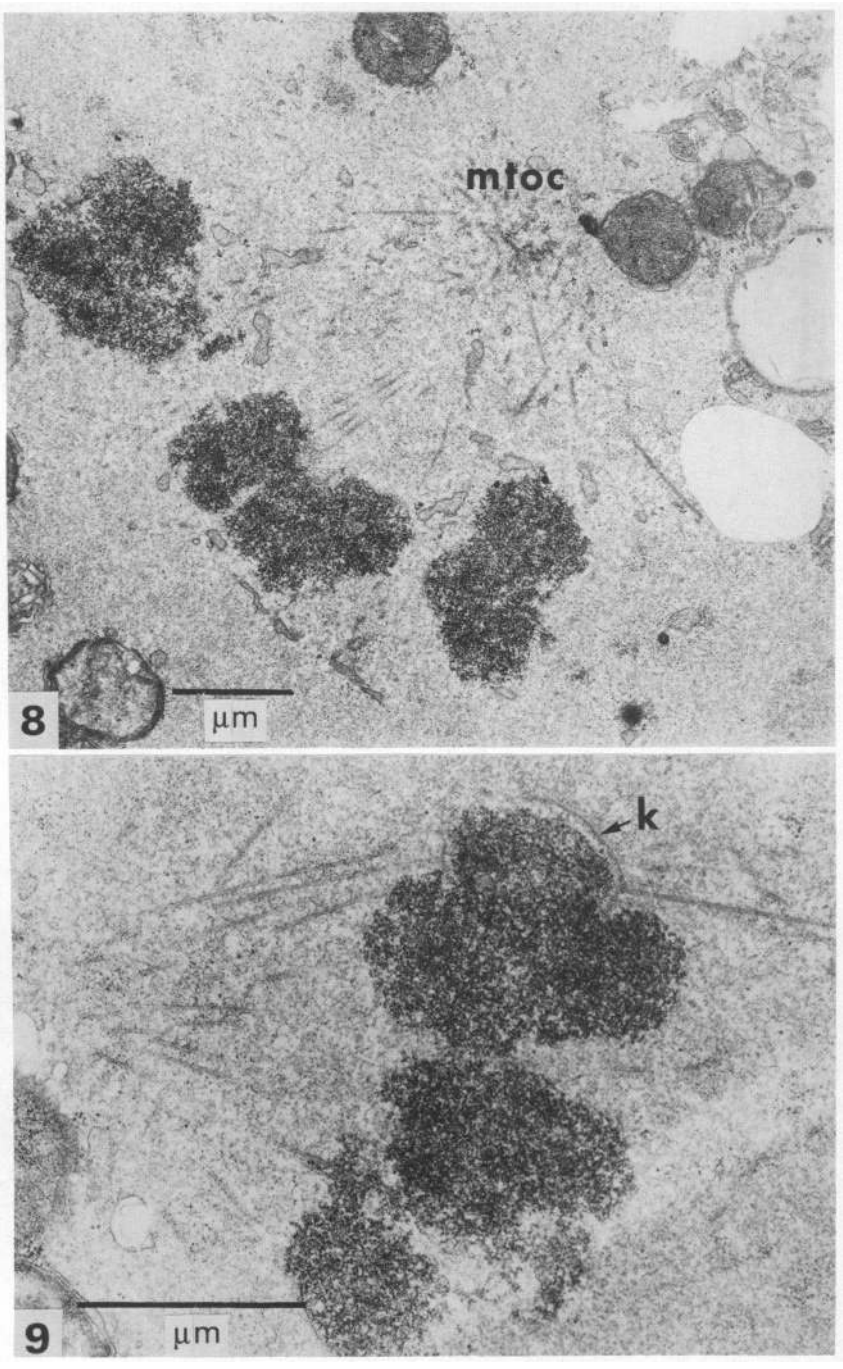


Fig. 8. Metaphase I of an oocyte from a follicle 0·8–1·6 mm in diameter cultured for 48 h; mtoc, microtubule organizing centre. × 16 000.

Fig. 9. Metaphase II spindle of an oocyte from a 1·8–2·2 mm follicle cultured for 48 h; k, kinetochore. × 30 000.

surrounded by filamentous chromatin. However, condensed heterochromatin was present around the compact nucleolus of all Class D oocytes isolated from the ovaries at Days 8–10 of the cycle (Pl. 1, Fig. 4).

The mean ± s.e.m. diameter (excluding the zona pellucida) of oocytes from the smallest follicles (Class A) was about 100 μm (101·85 ± 3·68 μm). Further oocyte growth was observed in Classes B and C in which oocyte diameter reached 109·59 ± 2·65 and 115·09 ± 2·17 μm respectively. Mean oocyte diameter in Class D was 119·87 ± 3·76 μm. The differences between the 4 classes were significant ($P < 0·01$). These data demonstrate that the pig oocytes from early antral follicles of up to 2·2 mm diameter had not completed growth.

Culture for 24 h

Oocytes from Class A follicles showed a very limited ability to initiate nuclear maturation after 24 h of culture *in vitro* (Table 1); more than 80% remained at the germinal vesicle stage. Under the electron microscope, the nucleoli of 6 oocytes analysed showed a vacuolated and fibrillo-granular structure (Pl. 1, Fig. 5) like that of the oocytes before culture (not shown). Only 12% of these Class A oocytes continued to prometaphase. In contrast 66, 94·3 and 100% of oocytes from follicles from Classes B, C, and D, respectively, progressed beyond the germinal vesicle stage.

Table 1. Maturation of pig oocytes isolated from antral follicles after culture

Culture period	Follicular diam. (mm)	Total no. of oocytes	No. of exps	Percentage of oocytes in: Germinal vesicle	Pro-metaphase	Metaphase I	Metaphase II	Clumps of condensed chromatin*
24 h	0·3–0·7 (Class A)	177	5	85 ± 1·5	12 ± 1·33	0	0	3 ± 0·6
	0·8–1·6 (Class B)	146	5	28 ± 1·2	53 ± 5	13 ± 1·9	0	6 ± 2·1
	1·7–2·2 (Class C)	140	5	6 ± 3·9	46 ± 0·4	49 ± 4·3	0	0
	3–5 (Class D)	138	5	0	43 ± 1·3	48 ± 0·6	8 ± 4·2	0
48 h	0·3–0·7 (Class A)	155	5	41 ± 12·1	35 ± 11·4	4 ± 0·4	0	20 ± 2·5
	0·8–1·6 (Class B)	121	5	17 ± 1·2	18 ± 1·1	23 ± 2·5	17 ± 0·3	25 ± 2·1
	1·7–2·2 (Class C)	122	5	3	0	31 ± 0·2	49 ± 4·2	17 ± 3·3
	3–5 (Class D)	116	5	0	0	24 ± 1·6	76 ± 2·5	0

Values are mean ± s.e.m.
* Oocytes with pycnotic chromatin indicating no further competence to mature.

PLATE 2

Fig. 6. Germinal vesicle of an oocyte from a 0·5–0·7 mm follicle cultured for 48 h; 3 small compact nucleoli (N) are present in the round nucleus. × 10 000.

Fig. 7. Part of the germinal vesicle (GV) of an oocyte from a follicle 0·5–0·7 mm in diameter cultured for 48 h. Condensed chromatin (ch) is close to the convoluted nuclear envelope (arrows); large Golgi structures (G) are proximal to the GV. L, lipid droplets. × 25 000.

Culture for 48 h

After 48 h of culture (Table 1), 41% of Class A oocytes still remained at the germinal vesicle stage, whereas 35% had progressed to prometaphase and only 4% to metaphase I. In Class B, 23% of the oocytes had progressed to metaphase I, while 17·3% reached metaphase II. The percentage of oocytes that completed the first meiotic division increased significantly in Class C in which about 50% of the oocytes extruded the first polar body and 31% remained in metaphase I. About 76% of Class D oocytes completed nuclear maturation *in vitro*.

The percentage of degenerative oocytes with clumps of condensed chromatin was nearly the same (about 20%) in follicular Classes A, B and C.

The different stages of nuclear progression were analysed by electron microscopy. In 3 oocytes out of 6 from Class A, 3 out of 4 from Class B and 1 out of 4 from Class C arrested at the germinal vesicle stage after 48 h of culture, 2 or 3 small nucleoli were found instead of a single large nucleolus (Pl. 2, Fig. 6); these totally compact and agranular nucleoli were frequently adjacent to the nuclear envelope. Fragmentation of the nucleolus was probably due to a degenerative process. The germinal vesicle of these oocytes was round and more or less centrally located in the cytoplasm. In oocytes in which nuclear maturation was starting, the germinal vesicle was peripherally located and the nuclear envelope was markedly undulated; the chromatin was condensing close to the nuclear envelope and some large areas of Golgi were present in the cytoplasm close to the germinal vesicle (Pl. 2, Fig. 7). When metaphase I and II spindles were present (in 1, 3 and 2 oocytes from Classes A, B and C respectively) their organization according to chromosome distribution, kinetochores and microtubule arrangement (Pl. 3, Figs 8 & 9) was like that of oocytes from large antral follicles matured *in vitro* (unpublished data). Furthermore the cortex of these oocytes in metaphase I or II was almost empty of cytoplasmic organelles such as mitochondria, endoplasmic reticulum, Golgi and lipid droplets, but the cortical granules had migrated towards the oocyte periphery and were aligned along the plasma membrane as in preovulatory oocytes matured *in vitro*.

Discussion

The acquisition of meiotic competence in oocytes has often been correlated in the literature with the appearance of an antral cavity and oocyte size. It has been demonstrated for several rodent species that oocytes become competent to resume meiosis *in vitro* when the follicles enclosing them develop an antrum (Erickson & Sorensen, 1974; Iwamatsu & Yanagimachi, 1975; Bar-Ami & Tsafriri, 1981). However, the present study indicates that pig oocytes do not acquire this competence to resume meiotic maturation when the antrum is formed. In the pig, the antrum is fully differentiated in follicles 0·4–0·8 mm in diameter (Crozet *et al.*, 1981) but only 12% of the oocytes liberated from these follicles resume meiosis after 24 h. Successful germinal vesicle breakdown occurred in oocytes from follicles >1 mm in diameter. Conflicting results have been reported concerning the ability of oocytes from follicles 1–2 mm in diameter to progress beyond the germinal vesicle stage (McGaughey *et al.*, 1979; Tsafriri & Channing, 1975; Anderson & Hillensjö, 1982). The present work, using serial follicular size classes, partly elucidates these conflicting results because 28 and 6% of the oocytes from follicles 0·8–1·6 mm and 1·7–2·2 mm in diameter, respectively, remained in the germinal vesicle stage after 24 h of culture. A substantial difference in maturation rate (oocytes in metaphase II) was also noted in these follicular classes after 48 h of culture.

The majority of pig oocytes in follicles >1 mm in diameter acquired the competence to resume meiotic maturation *in vitro* but only those of follicles of about 2 mm in diameter completed the first meiotic division *in vitro*. These results confirm the previous findings of Tsafriri & Channing (1975) for pigs and of Sorensen & Wassarman (1976) for mice who concluded that the ability to undergo germinal vesicle breakdown and continue to metaphase I was acquired earlier during folliculogenesis than the ability to reach metaphase II.

The increase in the size of pig oocytes from 100 to 120 μm in small antral follicles appears to be related to their ability to resume meiosis *in vitro*. While oocytes with a mean diameter of about 100 μm cannot complete germinal vesicle breakdown, those with a mean diameter of 115 μm, which is nearly equal to that of full-sized pig oocytes (McGaughey *et al.*, 1979), seem to be able to complete meiotic maturation. In the same manner, hamster and mouse oocytes successfully mature *in vitro* only when they have reached full size (Iwamatsu & Yanagimachi, 1975; Sorensen & Wassarman, 1976).

When the antrum is forming in pig follicles 0·5 mm in diameter, the oocytes have a fibrillo-granular and vacuolated nucleolus and are intensively engaged in RNA synthesis (Crozet *et al.*, 1981). The present data indicate that, at this stage, their ability to resume nuclear maturation *in vitro* is very limited and none complete it. During further follicular development, the nucleolus is progressively compacted and oocyte rRNA synthesis significantly decreases (Crozet *et al.*, 1981). After 24 h of culture, a large number of these oocytes (66%) complete germinal vesicle breakdown; only 17·3% of them reach metaphase II after 48 h of culture. In Class C follicles (diam. 1·7–2·2 mm), the process of nucleolar compaction is completed in the majority of oocytes and a relatively high percentage (50%) of them progresses to metaphase II after 48 h of culture. This percentage increases to 76% in Class D follicles (diam. 3–5 mm). It is tempting to postulate that the morphological and functional changes that occur in oocytes from early antral follicles constitute one of the stages required for oocyte acquisition of meiotic competence.

The high rate of rRNA and hnRNA synthesis which characterizes pig oocytes from early antral follicles (Crozet *et al.*, 1981) is probably needed for the storage of some information necessary to the initiation and completion of the first meiotic division. Golbus & Stein (1976) proposed that the changes that take place in the pattern of protein synthesis during mouse oocyte meiosis are controlled by messenger RNAs that are already present and not by RNA synthesized during meiosis. It is possible that inability to resume meiotic maturation is due to the absence or insufficiency of messenger RNAs coding for a cytoplasmic factor, e.g. like the maturation-promoting factor in amphibians (Masui & Markert, 1971). The present experiments demonstrate that the majority of pig oocytes from follicles >1 mm in diameter can progress beyond germinal vesicle breakdown and that they probably already possess the aptitude to synthesize these cytoplasmic factors.

A significant decrease in rDNA transcriptional activity occurs during the process of nucleolar compaction at the time when the oocytes reach full size, although an active hnRNA synthesis seems to be maintained (Crozet *et al.*, 1981). Some RNA synthesis activity has been demonstrated up to resumption of meiosis in mouse oocytes from antral follicles (Rodman & Bachvarova, 1976; Wassarman & Letourneau, 1976). This late RNA synthesis in oocytes may also play a role in the final acquisition of their meiotic competence, as shown by the fact that oocyte ability to mature *in vitro* continues to increase in larger follicles (Tsafriri & Channing, 1975; present results).

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