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Timing of Nuclear Progression and Protein Synthesis Necessary for Meiotic Maturation of Bovine Oocytes[1]

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ABSTRACT

In cows, protein synthesis is required for germinal vesicle breakdown (GVBD). This study examines more closely the need for protein synthesis and the nuclear changes in the bovine oocyte during 24 h of culture. Bovine oocytes with compact and complete cumulus were washed and incubated in groups of 10 for up to 24 h in 50-μl drops of TCM-199 supplemented with follicle-stimulating hormone (NIAMADD, 0.5 μg/ml), luteinizing hormone (LH) NIAMADD, 5 μg/ml), estradiol-17β (1 μg/ml), pyruvate (20 μM), and 10% heat-treated fetal calf serum. Medium was overlaid with paraffin oil. Oocytes (n = 891) were fixed at the end of each 3-h interval from 0 to 24 h of culture, or at 24 h after addition of cycloheximide (10 μg/ml at 10 different times during maturation (0, 1, 2, 3, 6, 9, 12, 15, 18, 21 h; n = 175). At each time point, the chromosomal status of oocytes was evaluated, frequencies were computed, and the time spent on each step was determined. The germinal vesicle (GV) was present from 0 to 6.6 h, GVBD at 6.6 to 8.0 h, chromatin condensation at 8.0 to 10.3 h, metaphase I at 10.3 to 15.4 h, anaphase I at 15.4 to 16.6, telophase I at 16.6 to 18.0 h, and metaphase II at 18.0 to 24 h. Cycloheximide blocked oocyte maturation at GVBD, if added from 0 to 3 h; at chromatin condensation, if present from 6 to 24 h; and at metaphase I, when present from 9 to 12 h. When cycloheximide was present from 12 to 24 h an increasing number of oocytes reached metaphase II (19% for 12 h, 36% for 15 h, 51% for 18 h, 90% for 21 h, 94% for 24 h), but many abnormalities were noted: formation of one or two "pronucleus-like" structures with or without polar bodies, multiple metaphases, and fragmentation of chromatin. Therefore, a certain amount of protein synthesis is still needed after 12 h for the majority of oocytes to complete meiosis I. In conclusion, protein synthesis is needed at four different steps in bovine oocyte maturation.

INTRODUCTION

In most mammals, oocytes enter into the early stages of meiosis during fetal life and become arrested at the dictyate stage of prophase I until they are committed to ovulation or atresia. Each oocyte is surrounded by a layer of granulosa cells and remains in meiotic arrest for many years depending on the species (reviewed by Wassarman, 1988). Each oocyte at the end of its growth period contains a single large nucleus or germinal vesicle (GV). In response to a surge in the secretion of luteinizing hormone (LH) with each ovarian cycle, a limited number of oocytes in selected follicles will resume meiosis, resulting in the dissolution of the nuclear membrane, or germinal vesicle breakdown (GVBD), formation of the first polar body, and rearrest at metaphase II.

When cumulus-enclosed or denuded oocytes from a number of mammalian species are isolated from ovarian follicles and cultured in vitro, they resume meiosis spontaneously and undergo GVBD (Edwards, 1965). Oocytes that have recommenced meiosis spontaneously in vitro are also physiologically competent, as indicated by their ability to undergo normal development if the proper conditions are applied (mice: Schroeder and Eppig, 1984; sheep, Staigmiller and Moor, 1984; rats, Fleming et al., 1985; cattle, Critser et al., 1986; Sirard et al., 1988). GVBD of the oocyte in vitro is believed to depend on the removal of inhibitors contained in the follicular complex in vivo (Channing et al., 1980). In rodents, candidates for these inhibitors are cyclic adenosine monophosphate (Dekel et al., 1984), hypoxan-

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thine (Downs et al., 1985), and adenosine (Downs et al., 1986), all of which will transiently prevent meiotic resumption in cows (Sirard and First, 1988). The mechanism whereby putative inhibitors suppress meiosis and the basis of spontaneous meiotic resumption in vitro are poorly understood (for review: Schultz, 1986).

In a number of species, specific stages of meiosis require protein synthesis. For example, in amphibians (Maller et al., 1985) and domestic species such as swine (Motlik and Fulka, 1986), sheep (Moor and Crosby, 1986), and cattle (Hunter and Moor, 1987), protein synthesis inhibitors can prevent GVBD. In contrast, in invertebrates (Masui and Clarke, 1979) and in mice (Schultz and Wassarman, 1977), protein synthesis is not required for meiosis to resume. Mouse oocytes are blocked at later stages of meiosis when translational inhibitors are present (Schultz and Wassarman, 1977). Rats may have proteins with short half-lives necessary for GVBD and thus are not inhibited by translational blockers unless GVBD is delayed past decay of the proteins (Eckholm and Magnusson, 1979).

In this study, we have examined the timing of progression of bovine oocytes through meiosis I using maturation conditions that are known to result in normal development (Critser et al., 1986; Sirard et al., 1988). We also evaluated nuclear changes in relation to translational inhibition during specific time periods.

MATERIALS AND METHODS

Media and Chemicals

Cumulus oocyte complexes were washed in 4-(2-hydroxyethyl)-piperazineethanesulfonic acid (HEPES)-buffered Tyrode's medium (TALP-HEPES, Bavister et al., 1983) supplemented with 3 mg/ml bovine serum albumin (BSA), 0.2 mM sodium pyruvate, and 50 μg/ml of gentamycin sulfate (Sigma Chemical Co., St. Louis, MO). Incubation medium was TCM-199 with $NaHCO_3$ (Sigma Chemical Co.) supplemented with 10% heat-treated fetal calf serum (Gibco, Grand Island, NY; one lot was aliquoted and maintained at –70°C before use), pyruvate, and gentamycin as above. Gonadotropins and steroids were also added as described by Critser et al. (1986): follicle-stimulating hormone (0.5 μg/ml, NIAMDD), LH (5 μg/ml, NIAMDD), and 17β-estradiol (1 μg/ml; Sigma Chemical Co.). The other chemical added to the incubation (maturation) medium when specified was cycloheximide (10 μg/ml; Sigma Chemical Co.)

Oocyte Recovery and Culture

Oocytes were obtained by aspirating small (1–5 mm) follicles on bovine ovaries obtained after slaughter. The ovaries were transported in saline at 37°C from the slaughterhouse to the laboratory (2 h); the number of ovaries available ranged from 100 to 300 each day. Aspiration was performed with an 18-gauge needle and a 10-ml syringe. Ovaries were pooled and used at random. Follicular contents were transferred to 50-ml conical tubes and allowed to sediment for 15 min. The bottom part was then aspirated and observed under a stereomicroscope for cumulus-oocyte complexes (COC). The COC were selected according to the criteria defined by Leibfried and First (1979); only oocytes surrounded by a complete corona cell layer and 2–6 compact cumulus layers were used in this study. Dark-looking cumuli were also discarded from this study. The COC were washed three times in TALP-HEPES and then cultured in 50-μl drops of maturation medium under paraffin oil at 39°C with 5% CO_2 in air and a high humidity atmosphere for 0–24 h. The processing time from aspiration to culture was 1 h.

Experimental Design

In the first experiment, cumulus-enclosed oocytes (10 COC/50-μl drops) were cultured in the maturation medium alone for 0, 3, 6, 9, 12, 15, 18, 21, or 24 h and fixed for light microscopic analysis of their nuclear status. In the second experiment, groups of 10 COC were transferred to cycloheximide-containing medium after 0, 1, 2, 3, 6, 9, 12, 15, 18, or 21 h. All groups were cultured a total of 24 h before being processed for fixation and staining. Each experiment was repeated three times.

Evaluation of Nuclear Status

At the end of incubation, the COC were transferred to 1-ml conical tubes and vortexed for 2 min in Ca^{2+}- and Mg^{2+}-free Hanks' salt solution. The naked oocytes were put on slides and covered by coverslips supported by a vaseline/paraffin mixture retained with epoxy glue. The slides were then immersed in ethanol:acetic acid (3:1) for a minimum of 24 h before they were stained with 1% aceto-orcein and examined under a phase-contrast microscope at 200× and 500× magnification. Results are expressed as percentage of oocytes at each stage of maturation for each time point. To simplify the

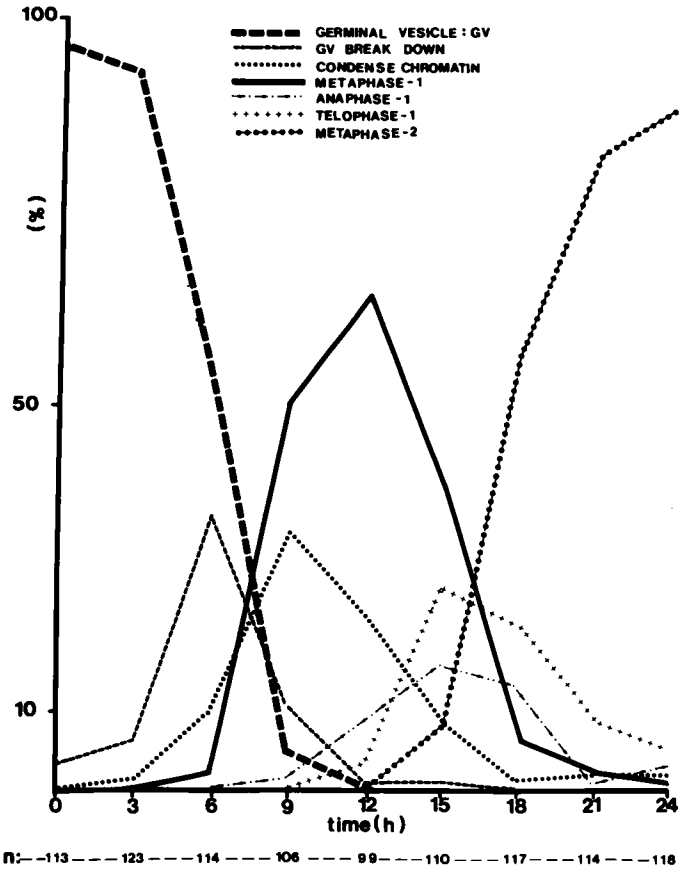


FIG. 1. Changes in nuclear status of oocytes throughout maturation. Results are expressed as percentage of oocytes at each stage of maturation at each time point. To simplify the analysis, seven stages were chosen: germinal vesicle *(GV)*; germinal vesicle breakdown *(GVBD)*, defined by the absence of visible nuclear membrane; chromatin condensation, characterized by a cluster of DNA material without individual chromosomes; metaphase I; anaphase I telophase I; and metaphase II. The total number of oocytes fixed at each time point from three replicates is shown at the *bottom* of the *Figure*.

analysis, seven stages were chosen: GV stage, GVBD defined by absence of visible nuclear membrane, chromatin condensation characterized by a cluster of DNA material without individual chromosomes, metaphase I, anaphase I, telophase I, and metaphase II.

RESULTS

Changes in nuclear status of oocytes throughout maturation are depicted in Figure 1 and 2. The total number of oocytes fixed at each time point from three replicates is shown at the bottom of Figure 1 and ranges

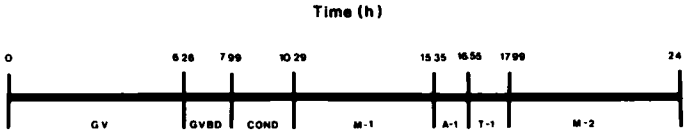


FIG. 2. The *areas* under the *seven curves* depicted in *Figure 1* were added to represent 24 h. The *area* under each *curve* in *Figure 1* is expressed as a fraction of 24 h. This computation allows an estimate of the mean time that an oocyte spent at each nuclear stage.

from 99 to 123. The areas under the curves of Figure 1 were computed with the purpose of simplifying the observation of nuclear changes; these are shown in Figure 2. The total area under the seven curves represents 24 h, and the area under each one is expressed as a fraction of 24 h. This computation allowed an estimate of the mean time that oocytes spent at each nuclear stage. This varied with each oocyte, including the few that did not reach metaphase II in the 24-h incubation time (12%; Fig. 1). Oocytes that were degenerated (resulting in a different reaction to the stain) were omitted from computation but represent a small fraction (3–4%) at each time point.

The results of the second experiment are shown in Table 1. All oocytes were fixed 24 h after the beginning of maturation and were therefore cultured in the presence of cycloheximide for 0–24 h. The total number of oocytes at each time point (100–128) are from three replicates. When a protein synthesis inhibitor was added at the beginning of the maturation period, GVBD occurred in less than 10% of the oocytes and none of the oocytes reached metaphase I. Up to 3 h of protein synthesis was required for most of the oocytes to proceed to chromatin condensation. COCs in cycloheximide for a significant length of time from the start of incubation did not undergo cumulus expansion either.

When cultured for less than 6 h in inhibitor-free medium, the majority of oocytes (406/409) could not reach metaphase I, but when cultured for 9 h under these conditions, 33% were able to attain this configuration. When half of the maturation period transpired without inhibition (12 H), a small percentage of oocytes (14%) reached a normal configuration of mature oocytes; but, before 18 h of inhibitor-free culture had transpired, many abnormalities were observed. Structural abnormalities of the chromatin were classified in five groups to simplify evaluation: one "pronucleus-like" structure with or without a polar body, two "pronuclei-like" structures, two metaphase plates with diverse degrees of chromatin condensation, and fragmen-

TABLE 1. Nuclear status after 24 h of incubation when cycloheximide was added at different times during meiotic maturation.

Nuclear configuration[#]	Time (h) of cycloheximide addition (10 μg/ml)										
	0	1	2	3	6	9	12	15	18	21	(24 h)*
Germinal vesicle intact	97	83	77	63	30	4	1	--	--	--	--
Germinal vesicle breakdown	7	12	14	25	14	5	--	--	--	--	--
Chromosome condensation	3	6	8	11	59	53	7	3	2	1	1
Metaphase I	--	1	1	1	2	33	28	--	--	2	2
Anaphase I, Telophase I	--	--	--	--	--	--	--	1	7	4	5
Metaphase II	--	--	--	--	--	1	19	38	51	109	120
One pronucleus	--	--	--	--	--	1	8	6	2	--	--
One pronucleus + one polar body	--	--	--	--	--	--	14	49	35	2	--
Two pronuclei	--	--	--	--	1	--	12	6	1	--	--
Two metaphases	--	--	--	--	--	2	9	3	6	3	--
Chromatin fragmentation	--	--	--	--	--	1	4	--	--	--	--
Total	107	102	100	100	105	100	102	106	104	121	128

*Control, no addition.

[#]Chromatin status of cycloheximide-treated oocytes at different periods after the beginning of maturation (Time 0). This experiment was done in triplicate. The first six rows describe the configurations classified as normal. The last five rows describe the chromatin abnormality observed when the oocytes were fixed at 24 h of maturation after various exposure periods to cycloheximide.

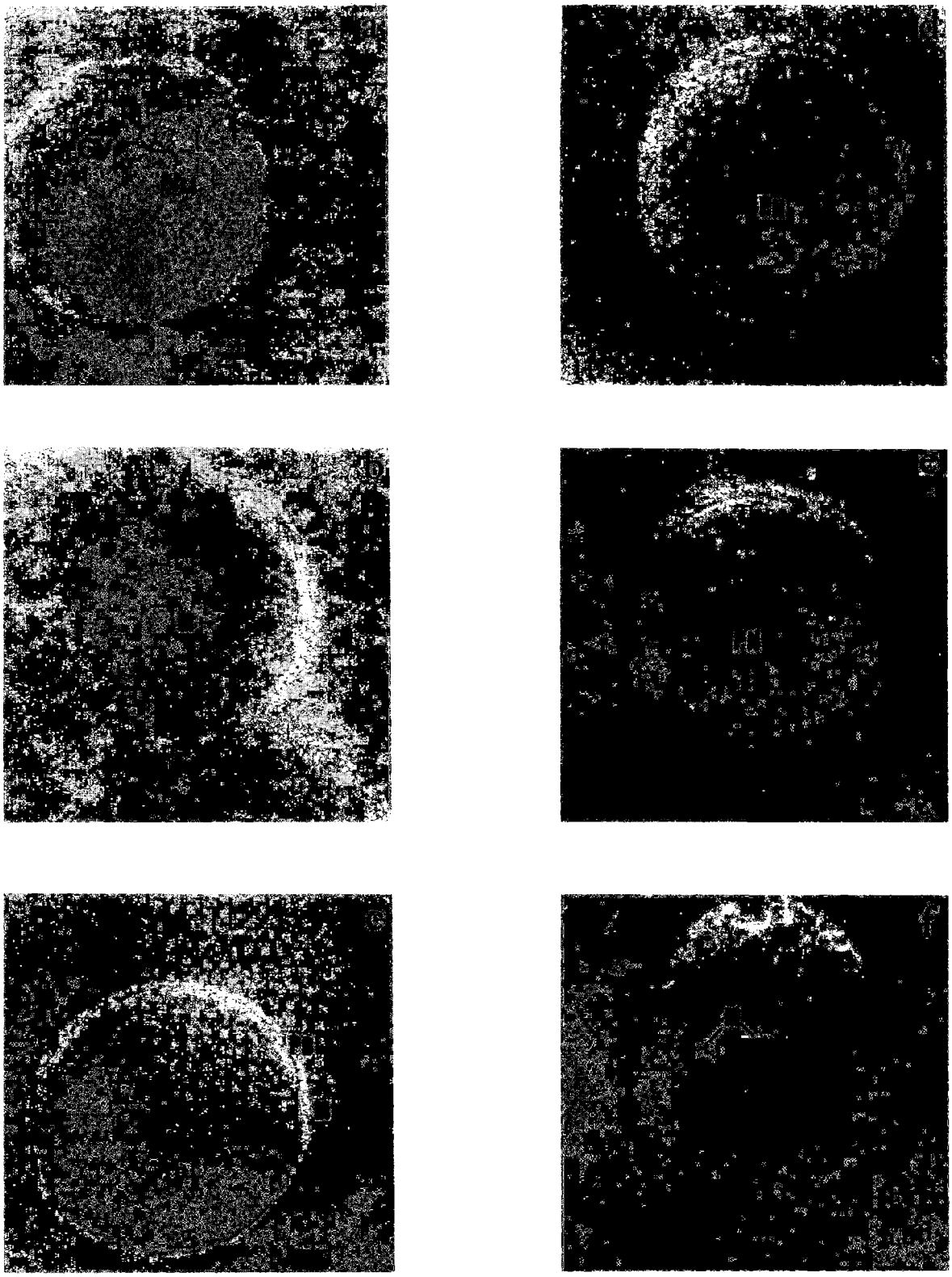
tation characterized by three or more chromatin groups with diverse degrees of condensation. Those morphological alterations are shown in Figure 3d–f. Figure 3a–c represents normal configuration of GV and metaphase I and II stages. The most common abnormality observed was the formation of one pronucleus-like structure along with one polar body, indicating that the first meiotic division had occurred. Surprisingly, the chromatin included in the polar body in those cases remained mostly in a condensed form, as shown in Figure 3c. Most of the oocytes (109/121) that were allowed 21 h in the absence of cycloheximide were not affected by a subsequent 3 h of incubation with the inhibitor. Although data were not recorded since evaluation of this end point is difficult, no expansion of cumulus cells was seen when cycloheximide was present for most of the incubation period.

DISCUSSION

The results presented here delineate the timing of nuclear events in the bovine oocyte during maturation in vitro with this culture system. In the majority of oocytes, the nuclear membrane underwent breakdown after 6.5 h of culture. The time at which each event occurred confirms previous studies (Sreenan, 1970; Motlik et al., 1978; King et al., 1986). The use of more time points and oocytes during the complete period of meiotic maturation allowed us to define more precisely the configurational changes involved and the relative amount of time spent at each stage. Since the maturation conditions used here can also result in normal fertilization and development (Critser et al., 1986; Sirard et al., 1988), their physiological significance can be considered. During in vivo maturation, GVBD occurs between 4 and 8 h after the LH peak (Kruip et al., 1983) or between 6 and 9 h, in superovulated animals according to Hyttel et al. (1986). In this later study, a polar body was observed 19 h post-LH surge compared to 18 h in the present in vitro study. It is therefore possible to conclude that nuclear maturation in vitro proceeds at a similar rate to that in vivo if removal from the follicle is compared to time with the LH surge.

The precise timing used in this first experiment was also necessary to assess the effect of translational inhibition (cycloheximide). The results of Table 1 indicate that the inhibitory effect was rapid and was maintained throughout the incubation time. In cattle, as in sheep (Moor and Crosby, 1986), protein synthesis is required

FIG. 3. Photomicrographs of fixed and stained whole mounts of bovine oocytes representing the various nuclear configurations seen in these studies. *(a)* Bovine oocyte at the germinal vesicle stage after culture in maturation medium for 2 h followed by 22 h in the same medium to which cycloheximide (10 μg/ml) had been added. *GV*: Germinal vesicle, *Nu*: nucleolus. ×200. *(b)* Bovine oocytes showing a metaphase I plate *(M)* after 9 h of incubation in control medium followed by 15 h in medium with cycloheximide (10 μg/ml). ×200. *(c)* Bovine oocyte at the metaphase II stage after incubation in control medium for 24 h. *M*: metaphase; *PB*: polar body. ×200. *(d)* Bovine oocyte showing one pronuclear-like *(PN)* structure and a polar body after 12 h of maturation in control medium followed by 12 h in medium with cycloheximide (10 μg/ml). ×200. *e* Bovine oocyte showing two pronuclear-like *(PN)* structures after 12 h maturation in control medium followed by 12 h in medium with cycloheximide (10 μg/ml). ×200. *f* Bovine oocyte showing two metaphase plates *(M)* after 18 h of maturation in control medium followed by 6 h in medium with cycloheximide (10 μg/ml). ×200.



for the GV to undergo breakdown. This was shown previously by Hunter and Moor (1987). However, protein synthesis apparently is needed to proceed to metaphase I since addition of inhibitor after GVBD did not prevent chromatin condensation but did prevent the formation of a normal spindle. This result is in contrast to studies in sheep where metaphase I is reached after GVBD, even in the presence of inhibitors (Moor and Crosby, 1986).

Bovine oocytes were arrested in metaphase I in the presence of inhibitors, but this third block seemed to depend on a short period of protein synthesis–when the duration of the block was compared to the time oocytes normally spend at metaphase I (Fig. 1). Indeed, as soon as 12 h after removal from follicles, a few oocytes acquired the factors necessary to complete meiosis I, since some oocytes formed a normal metaphase II when cycloheximide was added after 12 h of culture. This could indicate that a short period of translation is needed for this step.

In amphibians (Maller, 1985), mice (Clarke and Masui, 1983), and cattle, a certain amount of protein synthesis is needed to maintain the metaphase II configuration, otherwise chromatin decondensation occurs (Fig. 3) and a pronucleus-like structure appears. This effect has also been observed in sheep (Moor and Gandolfi, 1987). In mice, the missing proteins may be related to normal somatic cell cycle proteins (Howlett, 1986). In cattle, in contrast to mice (Clarke and Masui, 1983), inhibition of protein synthesis does not prevent the extrusion of the first polar body in oocytes that can proceed beyond metaphase I.

It seems that new proteins are crucial for meiotic maturation, yet this is not the sole means of regulating meiotic progression. In other species, post-translational modification of already existing proteins is also involved in nuclear changes and cell cycle events (sheep, Crosby et al., 1984; mice, Howlett, 1986). Although we do not have this information in cattle, substances that are capable of regulating protein phosphorylation can inhibit GVBD in the bovine oocyte in a dose-dependent way (Sirard et al., 1988). Protein phosphorylation is one method of post-translational regulation. The need for protease activity is also important in bovine oocytes, as shown by Jagiello et al. (1979), since trypsin-like inhibitors can prevent meiotic resumption. Post-translational modifications and/or protease activity may occur early in a sequence of molecular events leading to resumption and completion of meiosis. Delineating the temporal sequence of molecular changes and the causal relationships necessary for meiosis I in the bovine oocyte is the goal of future work.

REFERENCES

Bavister BD, Leibfried ML, Lieberman G, 1983. Development of preimplantation embryos of the golden hamster in a defined culture medium. Biol Reprod 28:235–47

Channing CP, Schaerf FW, Anderson LD, Tsafriri A, 1980. Ovarian follicular and luteal physiology. In: Greep RO (ed.), International Review of Physiology, Reproductive Physiology III, Vol. 22. Baltimore: University Park Press; pp. 117–201

Clarke HJ, Masui Y, 1983. The induction of reversible and irreversible chromosome decondensation by protein inhibition during meiotic maturation of mouse oocytes. Dev Biol 97:291–301

Critser ES, Leibfried-Rutledge ML, First NL, 1986. Influence of cumulus cell association during in vitro maturation of bovine oocytes on embryonic development. Biol Reprod 34 (Suppl. 1):192

Crosby IM, Osborn JC, Moor RM, 1984. Changes in protein phosphorylation during maturation of mammalian oocytes in vitro. J Exp Zool 229:459–66

Dekel N, Aberdam E, Sherizly I, 1984. Spontaneous maturation in vitro of cumulus-enclosed rat oocytes is inhibited by forskolin. Biol Reprod 31: 244–50

Downs SM, Coleman DL, Eppig JJ, 1986. Maintenance of murine oocyte meiotic arrest: uptake and metabolism of hypoxanthine and adenosine by cumulus cell-enclosed and denuded oocytes. Dev Biol 117:174–83

Downs SM, Coleman DL, Ward-Bailey PF, Eppig JJ, 1985. Hypoxanthine is the principal inhibitor of murine oocyte maturation in a low molecular weight fraction of porcine follicular fluid. Proc Natl Acad Sci USA 82: 454–58

Eckholm C, Magnusson C, 1979. Rat oocyte maturation: effect of protein synthesis inhibitors. Biol Reprod 21:1287–93

Edwards RG, 1965. Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. Nature (Lond) 208:349–51

Fleming AD, Evans G, Walton EA, Armstrong DT, 1985. Developmental capability of rat oocytes matured in vitro in defined medium. Gamete Res 12: 255–63

Howlett SK, 1986. A set of proteins showing cell cycle dependent modification in the early mouse embryo. Cell 45:387–96

Hunter AG, Moor RM, 1987. Stage-dependent effects of inhibiting ribonucleic acids and protein synthesis on meiotic maturation of bovine oocytes in vitro. J Dairy Sci 70:1646–52

Hyttel P, Callesen H, Greve T, 1986. Ultrastructural features of preovulatory oocyte maturation in superovulated cattle. J Reprod Fertil 76:645–56

Jagiello G, Ducayen M, Goonan W, Downey S, 19789. Inhibition of bovidae oocyte meiosis by serine protease inhibitors. Proc Soc Exp Biol Med 157: 550–52

King WA, Bousquet D, Greve T, Goff AK, 1986. Inhibition of bovidae oocyte meiosis by serine protease inhibitors. Proc Soc Exp Biol Med 157:550–52

Kruip TAM, Cran DG, Van Beneden TH, Dielemann SJ, 1983. Structural changes in bovine oocytes during final maturation in vivo. Gamete Res 8: 29–47

Leibfried L, First NL, 1979. Characterization of bovine follicular oocytes and their ability to mature in vitro. J Anim Sci 48:76–86

Maller JL, 1985. Regulation of amphibian oocyte maturation. Cell Differ 16: 211–21

Masui Y, Clarke HJ, 1979. Oocyte maturation. Int Rev Cytol 57:185–282

Moor RM, Crosby IM, 1986. Protein requirement for germinal vesicle breakdown in ovine oocytes. J Embryol Exp Morphol 94:207–20

Moor RM, Gandolfi F, 1987. Molecular and cellular changes associated with maturation and early development of sheep eggs. J Reprod Fertil 34: 55–69

Motlik J, Fulka J, 1986. Factors affecting meiotic competence in pig oocytes. Theriogenology 25:87–96

Motlik J, Koefoed-Johnson HH, Fulka J, 1978. Breakdown of the germinal vesicle in bovine oocytes cultivated in vitro. J Exp Zool 205:377–84

Schroeder AC, Eppig JJ, 1984. The developmental capacity of mouse oocytes that matured spontaneously in vitro is normal. Dev Biol 102:393–97

Schultz RM, 1986. Molecular aspects of mammalian oocyte growth and maturation. In: Rossant J, Pederson RA (eds). Experimental Approaches to Mammalian Embryonic Development. Cambridge, England: Cambridge University Press, pp. 195–237

Schultz RM, Wassarman PM, 1977. Biochemical studies of mammalian oogenesis: Protein synthesis during oocyte growth and meiotic maturation in the mouse. J Cell Sci 24:167–94

Sirard MA, First NL, 1988. In vitro inhibition of oocyte nuclear maturation in the bovine. Biol Reprod 39:229–34

Sirard MA, Leibfried-Rutledge ML, Parrish JJ, Ware CM, First NL, 1988. The culture of bovine oocytes to obtain developmentally competent embryos. Biol Reprod 39:546–52

Sreenan J, 1970. In vitro maturation and attempted fertilization of cattle follicular oocytes. J Agric Camb 75:313–96

Staigmiller RB, Moor RM, 1984. Effect of follicle cells on the maturation and developmental competence of ovine oocyte matured outside the follicle. Gamete Res 9:221–29

Wassarman PM, 1988. The mammalian ovary. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction. New York: Raven Press, pp. 69–102