

Cytoplasmic argyrophilic protein(s) in cumulus-oocyte complexes: a preliminary study *

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Summary — The presence of acidic argyrophilic protein(s) in cumulus cells and oocytes was examined in bovine, ovine, porcine and murine cumulus-oocyte complexes (COCs) using a specific silver-staining method. The COCs were isolated from small (1 mm) or large (3–5 mm) antral follicles; they were fixed immediately after slaughter (first group) or after transfer to the laboratory (ca 60 min, second group) and then silver stained according to Likovský and Smetana (1981). The argyrophilic proteins were accumulated mainly in foot processes of cumulus cells on oocytes isolated from large bovine follicles. The intensity of the reaction was less evident in small follicles. The intensity of staining gradually disappeared from cumulus cells as the maturation continued. Moreover, we were unable to detect similar strong labelling in mouse, pig and sheep COCs. Such a chronology suggests that argyrophilic protein(s) may play a role in the transition from meiotic arrest to resumption in bovine oocytes. Our results also suggest that preliminary events of maturation may occur just after slaughter, i.e. a relatively long time before the COCs are isolated and cultured *in vitro*.

cumulus oophorus / oocyte maturation / farm mammals / argyrophilic proteins

Résumé — **Protéines argyrophiles cytoplasmiques dans le cumulus oophorus : étude préliminaire.** La localisation ultrastructurale de protéines argyrophiles acides a été examinée dans des complexes cumulus-ovocytes (COCs) isolés de follicules à antrum de petit (1 mm) ou grand diamètre (3-5 mm). Les COCs ont été fixés immédiatement après l'abattage (premier groupe) ou après transport au laboratoire (environ 60 min après, deuxième groupe) et colorés à l'argent par la technique de Libosky et Smetana (1981). Excepté la localisation habituelle dans les nucléoles, des protéines argyrophiles ont été trouvées dans les «pieds» des cellules du cumulus d'ovocytes bovins isolés à partir de grands follicules. La réaction est plus faible dans les COCs provenant de petits follicules ; d'autre part, elle disparaît progressivement du cytoplasme des cellules du cumulus au cours de la reprise de la méiose. Enfin, elle n'est pas détectable dans les COCs de souris et de truie et à peine dans ceux de brebis. Les protéines mises en évidence jouent peut-être un rôle dans la régulation de la reprise de la méiose des ovocytes bovins. Nos observations suggèrent aussi que des changements préliminaires à l'achèvement de la maturation peuvent commencer dès l'abattage, bien avant l'isolement et la mise en culture des COCs.

cellules du cumulus oophorus / maturation ovocytaire / Mammifères domestiques / protéines argyrophiles

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INTRODUCTION

Although bovine oocytes are currently matured, then fertilized and cultured up to the blastocyst stage *in vitro*, the mechanisms regulating the onset of maturation, *ie* germinal vesicle breakdown (GVBD) are only insufficiently understood. Apparently, mRNA and protein synthesis are involved in the processing of GVBD (Kastrop *et al*, 1991). Follicular components also play an important role in the maintenance of the GV stage (Sirard *et al*, 1992). On the other hand, the cyclic nucleotides, which are known as potent inhibitors of GVBD in rodents (Törnell *et al*, 1991), do not block GVBD in bovine oocytes (Fulka *et al*, 1993).

The role of cumulus cells has long been considered as vital for oocyte maturation, and for acquisition of cytoplasmic ability to transform the sperm head into pronucleus after fertilization, thus allowing the eggs to develop normally (Thibault *et al*, 1987). Indeed, the cumulus-oocyte contact and metabolic coupling are detected a relatively long time after the GVBD occurred (Laurinčík *et al*, 1992; deLoos *et al*, 1991). On the other hand, the interruption of contact between cumulus and oocyte is an absolute prerequisite for GVBD in sheep (deSmedt and Szöllösi, 1991). Obviously, some aspects of the role of cumulus cells are still unclear.

During the last decade, silver staining of nucleolar proteins has received increasing attention. This technique, adapted to the electron microscopic level, has allowed the visualization of the silver deposits mainly associated with nucleolar structures. It has been shown that reducing proteins are responsible for the staining (Olert *et al*, 1979), these proteins are highly phosphorylated acidic proteins containing thiol and disulfide groups. Two of the major silver-staining proteins have been identified as

nucleolar proteins B23 and C23 (Lischwe *et al*, 1979). Evidence has been obtained that these phosphorylated non-histone proteins are involved in regulation of the nucleolar function (synthesis, packaging, and maturation of pre-rRNA).

In this paper we describe the presence of argyrophilic (or related) protein(s) in the bovine cumulus-oocyte complexes (COCs). Although we were unable to relate the decrease of these proteins to the GVBD, our results may bring some new insights into the complexity of oocyte maturation in the bovine species and explain species-specific differences.

MATERIALS AND METHODS

Bovine oocytes were collected at the slaughterhouse from small (1 mm) or large (3–5 mm) follicles. Ovaries were obtained from 2–5-yr-old cows of unknown stage of the estrous cycle. Oocytes were aspirated from follicles using a 21-gauge needle and carefully selected. Only those with compact cumuli were used. Some of the oocytes were mounted on the slide immediately after their isolation. They were fixed with acet-alcohol (1:3 v/v) and, after staining with orcein (2% w/v), and were observed under a phase-contrast microscope to evaluate the stage of their nuclear maturation. The remaining oocytes were fixed for silver staining immediately after slaughter (approx 20 min *post mortem*) or immediately after the transfer of ovaries to the laboratory (approx 60 min *post mortem*) or after different times of culture *in vitro*. Before the culture, oocytes were washed 3 times in the culture TC 199 medium supplemented with 0.22 mM Napyruvate (Sigma, USA), 25 µg/ml Gentamicin (Sigma, USA) and 25% fetal calf serum (Vysoká škola veterinární, Brno, Czech Republic). Oocytes were cultured for 6, 12, 18 or 24 h. At the end of each time interval, some of oocytes were checked for the stage of their nuclear maturation as described above and the remainder were fixed for silver staining. At the end of 6 h in the culture almost all the oocytes remained at the GV stage. After 12 h in the culture all oocytes reached metaphase I. After 18 h in the culture bovine oocytes were observed to complete their

meiotic maturation and they transitioned from the telophase I stage to the metaphase II stage. After 24 h in the culture all oocytes were observed to reach the metaphase II stage.

Similarly, porcine oocytes were isolated from the ovaries of prepubertal gilts obtained at slaughterhouse. Some of selected oocytes were checked for the stage of nuclear maturation and the remaining oocytes were processed for silver staining. All checked oocytes were observed at the GV stage.

Ovine oocytes were obtained from sheep which were primed with 1 500 IU of PMSG (Bioveta Ivanovice na Hane, Czech Republic) 14 d after they were given intravaginal sponge containing 40 mg Cronolone (Intervet International BV, Boxmeer, the Netherlands). Forty-eight hours after the PMSG injection, the sheep were slaughtered and their ovaries were removed. Oocytes were aspirated immediately after slaughter. Some of them were checked for the stage of their nuclear maturation. They were all at the GV stage. The remaining oocytes were fixed for silver staining.

Mouse oocytes were obtained from superovulated females (ICR) which were given 10 IU of PMSG. Forty-eight hours after PMSG injection, the females were killed and their ovaries were placed in a drop of culture medium. The follicular wall was opened using the tip of a 20-gauge needle. Some of the isolated oocytes was checked for the stage of meiotic maturation; they were all found at the GV stage. The remaining oocytes were fixed for silver staining.

After fixation (2.5% glutaraldehyde, 30 min, 4°C), the COCs were briefly rinsed in PBS alone, washed in distilled water for 10 min and postfixated with a mixture of methanol/glacial acetic acid 3:1 for 20 min at room temperature, washed in 50% methanol for 10 min and in distilled water 3 x 10 min, after which a silver-staining solution was applied for 8-10 min at 60°C. The silver-staining solution was prepared according to the original method described by Likovsky and Smetana (1981). COCs were then washed in distilled water 3 x 10 min, dehydrated with ascending concentrations of alcohol and embedded in Epon 812. The specimens were cut with a diamond knife on LKB Nova Ultratome III. Some ultrathin sections were contrasted with uranyl acetate and lead citrate, the remaining sections were left for the control of the staining reaction.

COCs of all studied animal species were processed in parallel by a routine electron microscopic technique after embedding in Epon 812. All ultrathin sections were viewed and photographed in a JEOL JEM CX II 100 electron microscope at 80 kV.

RESULTS

In this study, argyrophilic proteins, which are known to be associated with ribosomal genes (or related phosphoproteins), were surprisingly found in the cumulus cells, mainly in the foot processes which are in close contact with the oocyte membrane (fig 1), or cross the zona pellucida (fig 2). Some labelling was also detected in the cumulus cell cytoplasm close to the nucleus (fig 3).

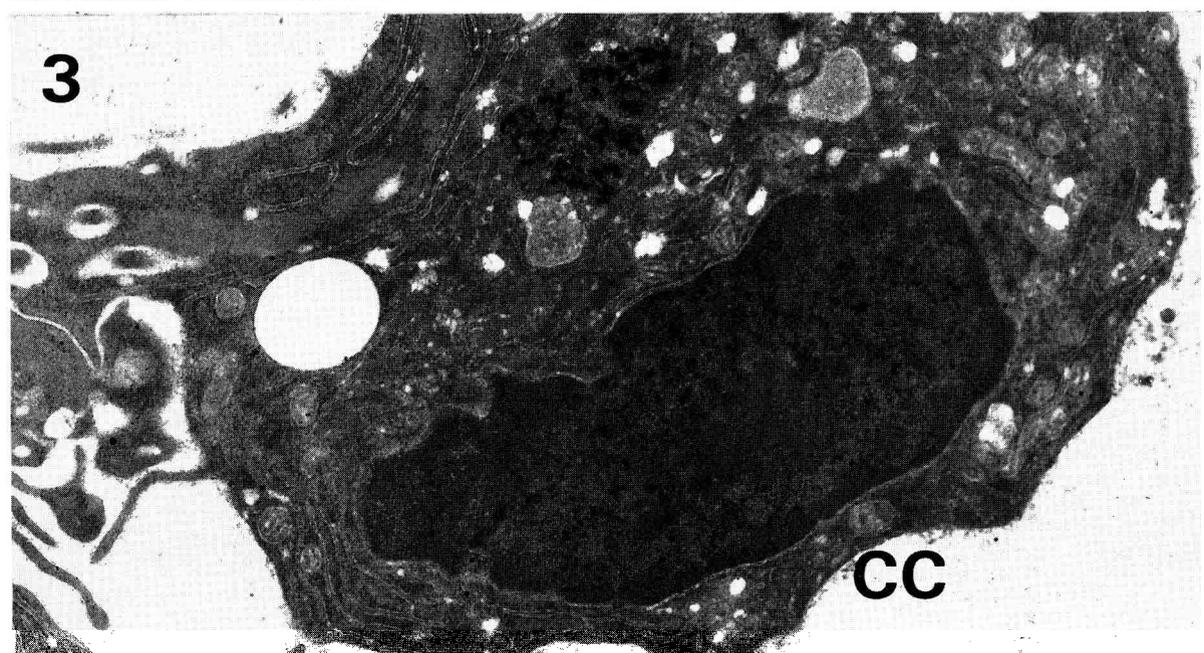
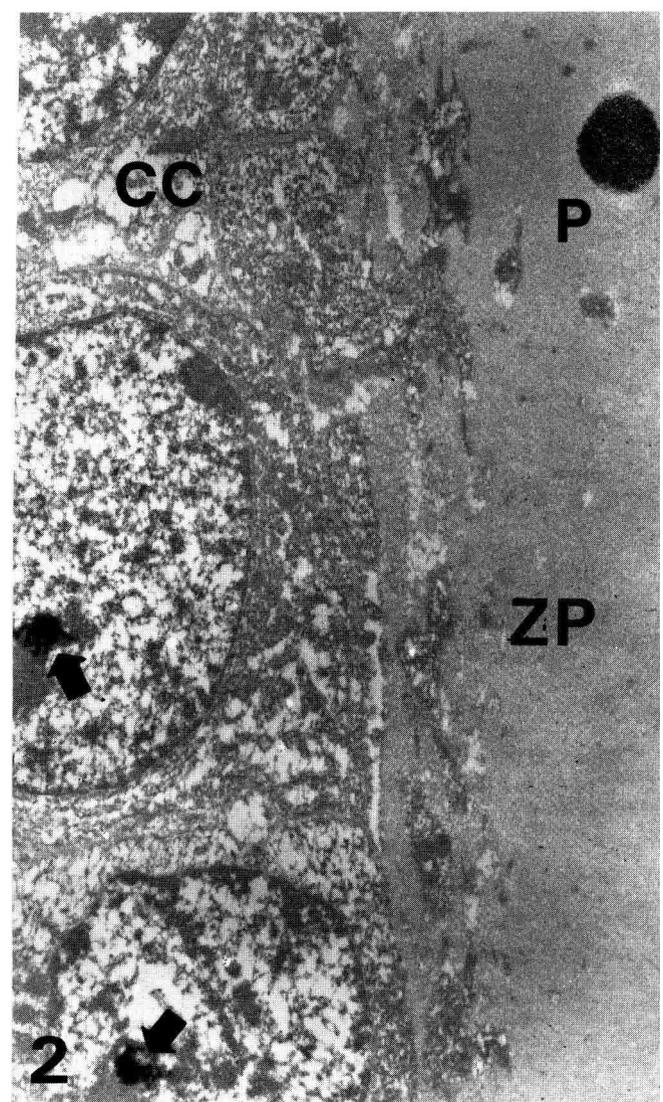
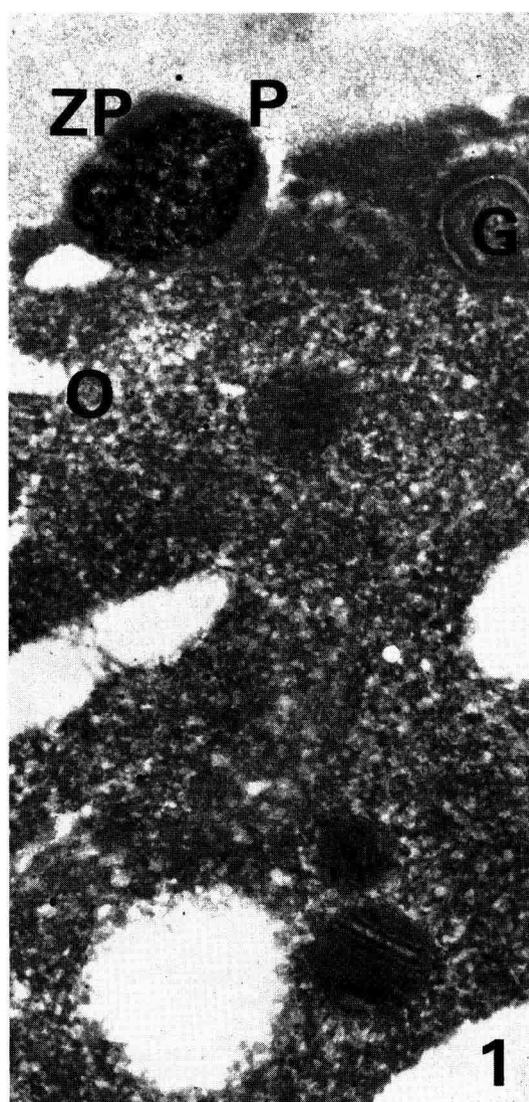
The labelling was much more intense in the cumulus cells which were fixed just after slaughter (not shown). We found a very strong labelling only in the COCs coming from large follicles (compare figs 4 and 5). The density of silver staining gradually decreased as the oocyte maturation proceeded (fig 6).

Very low intensity labelling was observed in sheep COCs and practically no foot processes were labelled in pig or mouse cumulus cells (fig 7).

The nucleoli of follicular cells of all species examined always showed the typical labelling pattern (fig 8) with a strong reaction within the fibrillar centres and a dense fibrillar component.

DISCUSSION

Using a silver-staining procedure which is generally accepted as specific for argyrophilic proteins, we were able to detect a very strong intensity of labelling mainly in the foot processes of bovine cumulus



cells. This phenomenon is very difficult to explain as these argyrophilic proteins are generally nucleolar proteins (Antalíková and Fulka Jr, 1990). However, the labelling does not seem to be an artifact because: (i) we were able to detect it repeatedly in bovine material; (ii) it has not been detected in parallel experiments in pig and mouse COCs; and (iii) the typical nucleolar structures were labelled at the same time.

The incidence of only high labelling in bovine oocytes may correspond to species-specific differences in the regulation of meiotic arrest in mammals. It is well known that the bovine oocytes can be blocked at the GV stage by protein synthesis inhibitors; mouse GVBD occurs even when these inhibitors are present (see Thibault *et al*, 1987 for review). On the other hand, cyclic nucleotides are very efficient blockers of rodent GVBD but in bovine, pig, and sheep oocytes their role has not been proven (Törnell *et al*, 1991).

Although the involvement of 'small' protein(s) in the regulation of oocyte maturation was suggested some years ago (Tsafiriri, 1978), such a protein has not yet been isolated and defined. The phosphoproteins detected in our experiments could be good candidates for a regulators of maturation. Two facts, however, suggest not. It is generally accepted that the phosphoproteins labelled with silver have a high molecular weight (75 000 and more)

Table I. Distribution of argyrophilic proteins in cumulus cells.

Species	Cumulus cell cytoplasm	Cumulus cell nucleolus (FC + DFC)	Cumulus cell foot process
Cow	++	+++	+++
Sheep	+	+++	-
Pig	-	+++	-
Mouse	-	+++	-

FC = fibrillar centre; DFC = dense fibrillar component; - to +++: increasing intensity of silver-staining reaction.

and are mostly present within nucleolar structures, where they are involved also in an essential metabolic process and can therefore be considered as so-called housekeeping proteins (Bourbon *et al*, 1988).

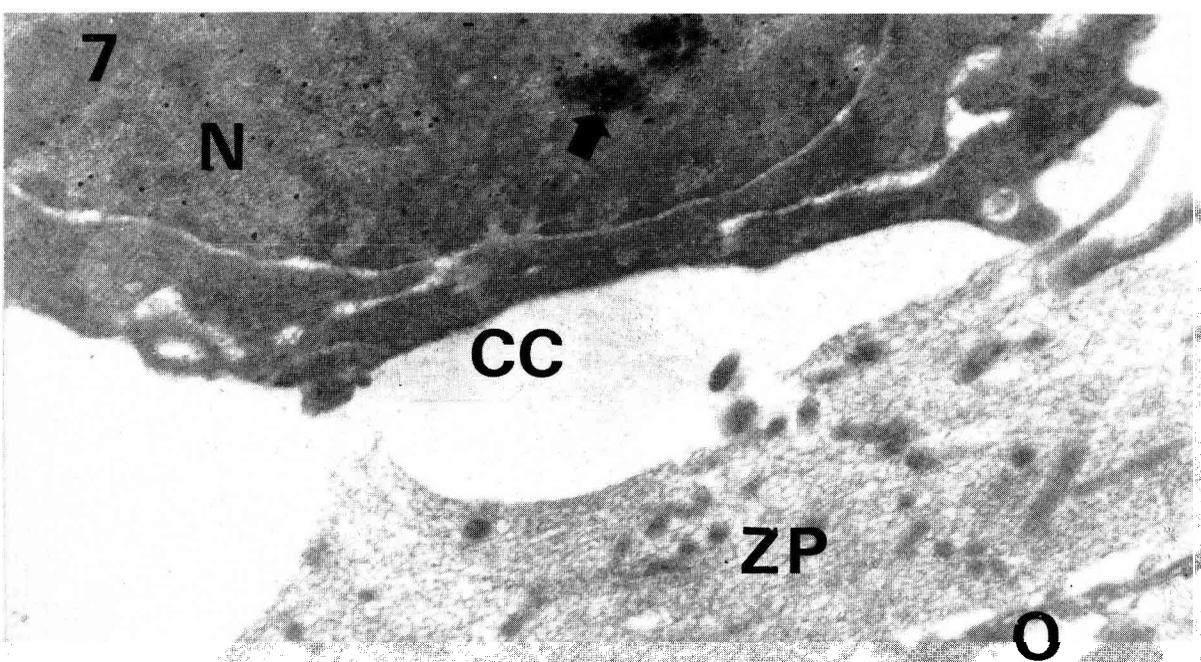
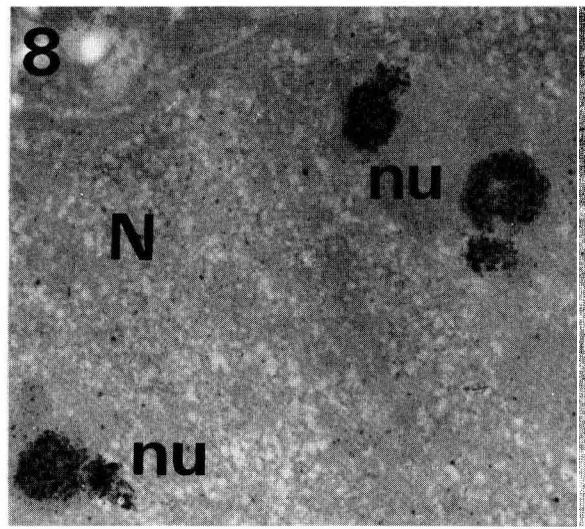
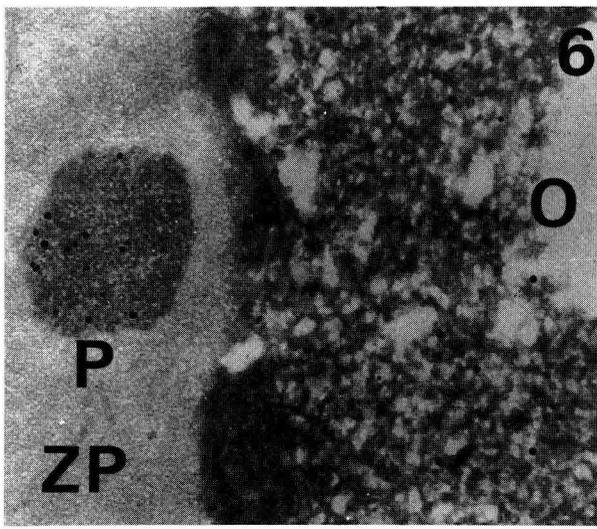
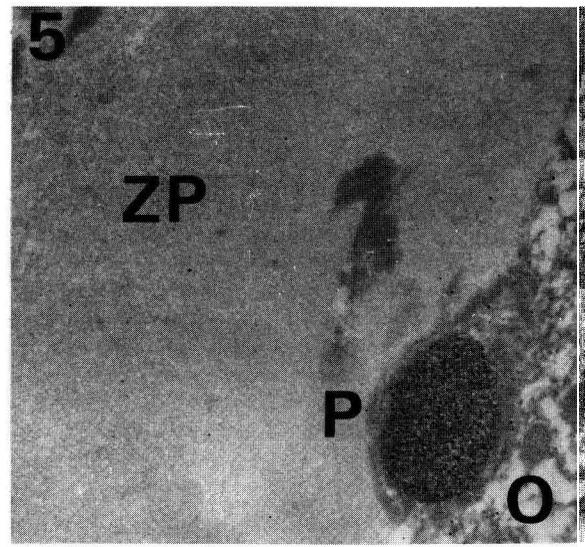
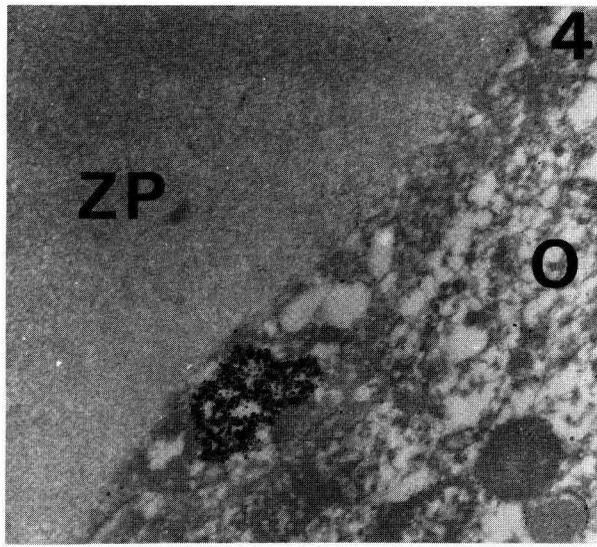
The localization of the argyrophilic proteins in the foot processes suggests a transport to the oocyte. It is accepted that only very small molecules can pass through the gap junctions between the oocyte and cumulus cells (Lawrence *et al*, 1978). In fact, we are presently unable to detect the transport of labelled material into the oocytes. The possibility of degradation and subsequent transfer is at

Fig 1. Bovine COC; part of cumulus cell cytoplasmic process in the perivitelline space, filled with Ag⁺ material, in close contact with oolemma; x 36 000.

Fig 2. Bovine COC; cumulus cells in close vicinity of the zona pellucida containing a cytoplasmic process with a strong positive reaction. Arrows: nucleoli of follicular cells; x 11 000.

Fig 3. Bovine COC; large aggregates of silver positive material in cytoplasm of a cumulus cells; x 23 000.

Abbreviations: ZP = zona pellucida; CC = cumulus cell; P = cytoplasmic process of cumulus cell; N = nucleus; O = peripheral part of oocyte; N = nucleus; nu = nucleolus.



present too speculative. The argyrophilic proteins in the COCs may also be smaller to those in the nucleolus.

However, it is important to note the following observations, which may indicate an indirect role of these phosphoproteins in the regulation of oocyte maturation. First, the intensity of labelling was much higher when the oocytes were fixed in the slaughterhouse compared with after transfer to the laboratory. This may indicate an intense degradation by endogenous proteases or phosphatases, which are known to take part in oocyte maturation in mammals (Jagiello *et al*, 1978; Kubelka *et al*, 1988; Rime and Ozon, 1990). A certain amount of protein transport to the oocyte could also occur after slaughter. Both hypotheses are in agreement with the observation that the intensity of labelling further decreases during maturation.

The lower intensity of the labelling after the transfer to the laboratory may suggest that in fact the commitment of meiosis (or at least part of this process) started at the animal death. The steps that are blocked by protein synthesis inhibitors may well represent much more distant processes in that cascade. Clearly further experiments are necessary to characterize which components are in fact labelled and their eventual role in the process of bovine oocyte maturation.

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Fig 4. Bovine COC; very intense labelling in a cell process after fixation just following slaughter (large follicle); x 26 000.

Fig 5. Bovine COC; removed from a small follicle and fixed after 45 min, a weaker labelling occurs; x 25 000.

Fig 6. Bovine COC; very low intensity of silver staining in a cumulus cell process after 24 h culture; x 36 000.

Fig 7. Murine COC; cumulus cell processes penetrating the zona pellucida and cytoplasm were unlabelled with silver; labelling is present only within the nucleoli (arrow); x 30 000.

Fig 8. Ovine COC; only nucleoli of follicular cells show typical labelling pattern; x 30 000.

Abbreviations: as for figs 1-3.

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