Postovulatory aging of the rat oocyte: Cytologic changes

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The purpose of this study was to determine whether the morphologic changes associated with postovulatory aging of the rat oocyte represent a random crumbling of cell structure or an orderly process with a predictable sequence and timing. Unfertilized rat oocytes recovered from the oviducts at various intervals after ovulation were examined by light and electron microscopy to assess the occurrence of time related cytologic changes. Fertilized ova were also studied to compare the normal pattern of development with the process of aging. The majority of the oocytes examined within the first 10 h after ovulation had a pyriform shape, were devoid of polar body and contained a meiotic spindle in the protruding zone. The remaining oocytes during this period were round and in a few of them the first polar body was visible in the perivitelline space. At about 18 h after ovulation, most oocytes had changed from a pyriform to a round shape and the second polar body was present in the perivitelline space. A large proportion of the oocytes recovered at subsequent times had undergone fragmentation giving rise to cytoplasmic masses of various sizes. Nucleus and/or nucleolus were present in the majority of these late round oocytes as well as in many cytoplasmic masses of fragmented oocytes. The changes observed with the light microscope were paralleled by orderly ultrastructural modifications that affected the microvilli, the cortical granules, the chromosomes, and the location of some organelles. Collectively, these changes partially resemble the activation that follows fertilization and suggest that the rat oocyte undergoes spontaneous activation prior to its cytolysis.

It is concluded that the rat oocyte fulfills important requirements for studying morphological correlates of the loss of fertilizability and potential for normal development in delayed insemination experiments.

Key words: *Oocyte cytology, oocyte fragmentation, postovulatory aging.*

INTRODUCTION

When fertilization does not take place soon after ovulation, the secondary oocyte undergoes progressive changes that decrease its potential for fertilization and normal development and eventually lead to its disintegration. Among the consequences of the instability of the secondary oocyte is that fertilization of senescent eggs, resulting from delayed matings, is associated with a variety of anomalous patterns of fertility and development in mammals (Adams and Chang, 1962; Austin and Braden, 1953; Austin, 1970; Blandau and Young, 1939; Blandau, 1952; Chang, 1952; Hammond, 1934; Hunter, 1967; Odor and Blandau, 1956; Shaver and Carr, 1967; Vickers, 1969; Jongbloet, 1985; Sharav, 1991).

It is likely that specific structural changes occur due to postovulatory aging, interaction with the oviductal milieu or both, that change

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either the fertilizability or the capacity of the oocyte to undergo parthenogenetic development (Zernicka-Goetz, 1991). In both instances, those changes can be correlated to loss of fertilizability and/or increased rate of abnormal development (Szollosi, 1971; 1975; Maleszewski, 1992). Experimental testing of this proposition requires first to identify species in which the structural changes follow a reproducible sequence and timing. To this end, fertilized rat ova at different stages of development and unfertilized oocytes aged in the oviducts for varying periods "in vivo" were examined by light and electron microscopy to determine the morphological changes that take place following ovulation. The findings reported here provide a baseline with which to analyze the relationships between structural changes and loss of fertilizability and/or capacity for normal development in the rat.

METHODS

Sprague-Dawley virgin female rats weighing 220-260 g were housed under controlled temperature (24 ± 2° C). Lights were kept on from 0700 to 2100. Vaginal smears were examined every morning and animals were used after two or more consecutive 4 day cycles.

A total of 16 rats were sacrificed between 0500 and 0700 on the day of estrus, to determine the median ovulatory time. It was found that 50% of the animals had one or more cumulus encased oocytes in the ampullae at 0600 which was reckoned as time 0 of the experiment.

Other rats were used to collect oocytes or fertilized eggs.

Oocytes were collected from oviductal flushings from 64 rats at 8 different intervals between 0 and 36 h. Each oviduct was flushed separately with sterile PB1 medium at pH 7.2-7.4 (Whittingham and Wales, 1969).

Immediately after recovery, 2 oocytes randomly selected from each female were transferred quickly into 3% glutaraldehyde in sodium cacodylate buffer 0.1 M, pH 7.4. After 60 min in the fixative they were embedded separately in 2% agarose at 37° C. After cooling to room temperature, a block of approximately one mm^3 containing the egg was cut and fixation was continued for an additional 60 min. The blocks were then washed in the same buffer and postfixed in osmium tetroxide. The specimens were dehydrated in increasing grades of acetone and were embedded in spurr low viscosity media (Polysciences Inc., Warrington, Penn). Serial sections of each oocyte were obtained with a diamond knife. Semithin sections were treated with 1% sodium borate for 2 min and stained with toluidine blue. Thin sections were stained with saturated uranyl acetate and treated with lead citrate (Reynolds, 1963). The sections were examined and photographed with a Phillips 300 Electron Microscope.

Oocytes recovered early after ovulation were placed in a drop of 0.032% hyaluronidase (Difco) in PB1 medium, to disperse the cumulus cells and facilitate microscopic observation. Immediately after, they were examined in a drop of PB1 medium within a ring of silicone gel, between slide and cover slip, using Nomarsky interference optics. Following this examination, they were classified by their morphological features as round, pyriform or frag-mented (Figs 1A, C, D). Round oocytes exhibiting a polar body in the perivitelline space were treated as a subgroup of round oocytes (Fig 1B). A total of 374 oocytes were studied by light microscopy and 35 oocytes by electron microscopy.

In order to obtain fertilized eggs, females were caged in the evening of proestrus with males of proven fertility, and mating was verified by observation of spermatozoa in the vaginal smear on the following morning. Fertilized ova recovered from the oviducts at various intervals after ovulation were examined by light and electron microscopy to assess their morphology. A total of 114 fertilized ova were studied by light microscopy and 16 by electron microscopy.

RESULTS

I. Aging

Ia. Observations with the light microscope

Figure 1 shows representative examples of each class of oocytes and Figure 2 shows

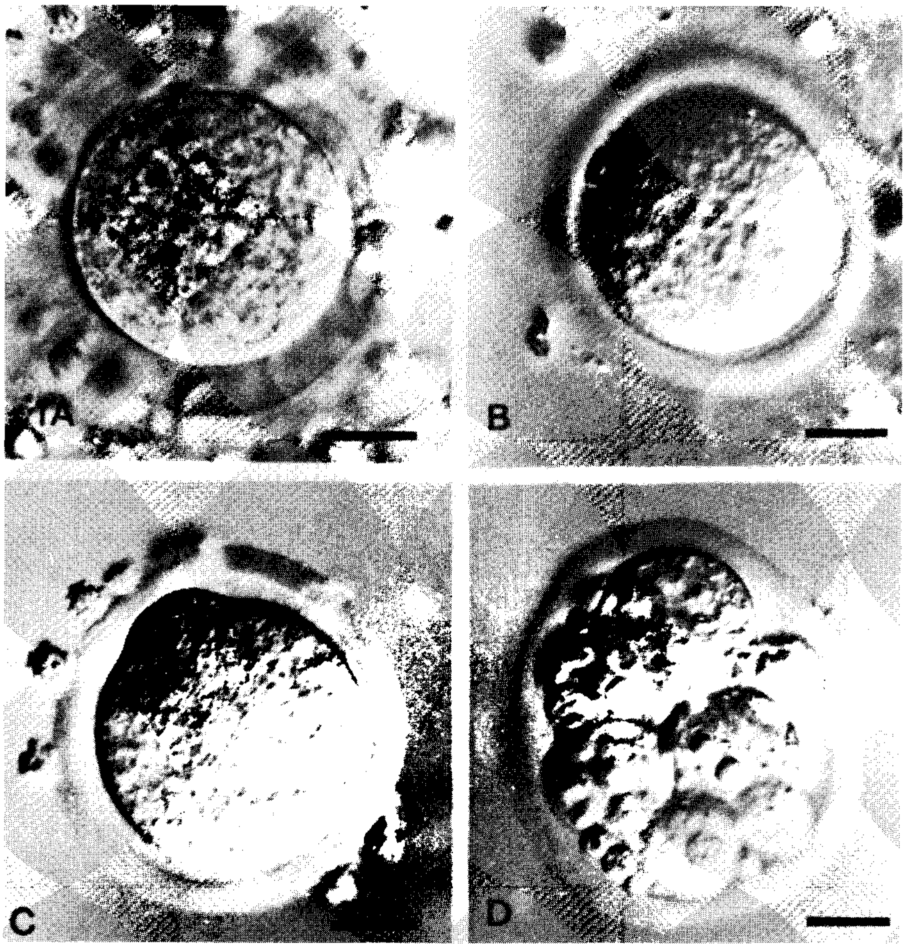

Fig 1. Different types of oocytes recovered from the rat oviduct. (A) Round oocyte in cumulus. (Bar = 25 μm). (B) Round oocyte with a polar body in the perivitelline space. (Bar = 21 μm). (C) Pyriform oocyte with a cone-shaped protuberance. (Bar = 32 μm). (D) Fragmented oocyte. A nucleus and nucleolus can be seen in focus in one of the cytoplasmic masses. (Bar = 26 μm).

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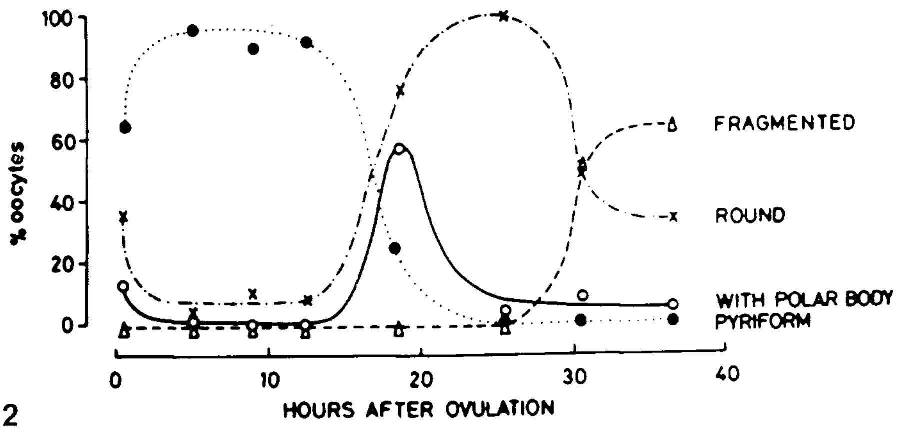

Fig 2. Frequency of various types of rat oocytes recovered from the oviducts at increasing intervals following ovulation. The category "with polar body" is a subgroup of round.

their frequency at each interval examined. The majority of oocytes recovered between 0 and 13 h were pyriform and the remaining were round. A polar body was visible in none of the pyriform and in 14% of the round oocytes recovered between 0 and 3 h. Variations in the contour of pyriform oocytes suggested different stages in the formation of a polar body and an example of this is shown in Figure 3. At 18 h the proportion of pyriform and round oocytes was reversed. At this time, 57.4% of the recovered oocytes were round and presented a polar body in the perivitelline space. At 25 h, 100% of the oocytes recovered were round, but a polar body was recognizable in very few of them. At 30 h, 51.5% of the oocytes had undergone fragmentation giving rise to cytoplasmic masses of various sizes (Fig 1D). Nucleus and/or nucleolus were present in 50% of the round oocytes and in many of the cytoplasmic fragments examined at this time or later (Figs 4, 10, 11). The proportion of fragmented oocytes and the number of fragments per oocyte increased with time.

Ib. Observations with the electron microscope.

Oocytes recovered 0-3 h after ovulation. Membrane residues and vesicles were present in the perivitelline space of some recently ovulated oocytes (Fig 5). Cortical granules were abundant in the cortex and short and thick microvilli lined the entire surface (Fig 6). Occasionally at the end of this period some cortical granules were observed releasing their contents to the perivitelline space (Fig 9A). Cisternae of endoplasmic reticulum, mitochondria, individual vesicles and vesicular complexes of varying dimensions were found within the subcortical region (Fig 6) and in the center of the oocyte. However, in the vicinity of the polar body or around the meiotic apparatus those structures were absent and a thin layer of granular material lay parallel to the cell membrane, which lacked microvilli and cortical granules (Figs 5, 7, 8).

Microtubules and condensed chromosomes were clearly distinguishable in the metaphase plate. The meiotic spindle was located within the cone shaped protuberance of the pyriform oocytes with the long axis oriented tangentially to the surface of the oocyte. Small vesicles were intermingled with the spindle fibers (Fig 7). In no instance was a centriole observed at the spindle poles even though several spindles were sectioned serially.

Lamellar structures which have been referred to as "yolk plates" (Szollosi, 1965; 1972) arranged in stacks of varying number, scattered throughout the cytoplasm in a random fashion, were the most abundant cytoplasmic component at all intervals (Figs 5-7).

Oocytes recovered 4-13 h after ovulation. In comparison with the earlier specimens, it was found that the cone shaped protuberance had enlarged and it still contained the

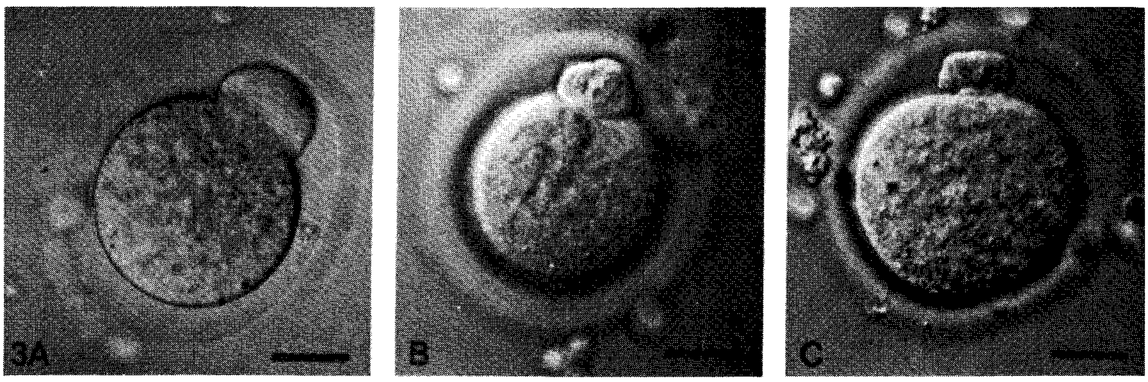

Fig 3. Three pyriform oocytes recovered from the oviducts at 19 h after ovulation probably representing different stages in the extrusion of the second polar body. Ultrastructural details of the protruding cytoplasm are shown in Figure 7B. Bars = 26.5 µm (A), 26.7 µm (B), 28 µm (C).

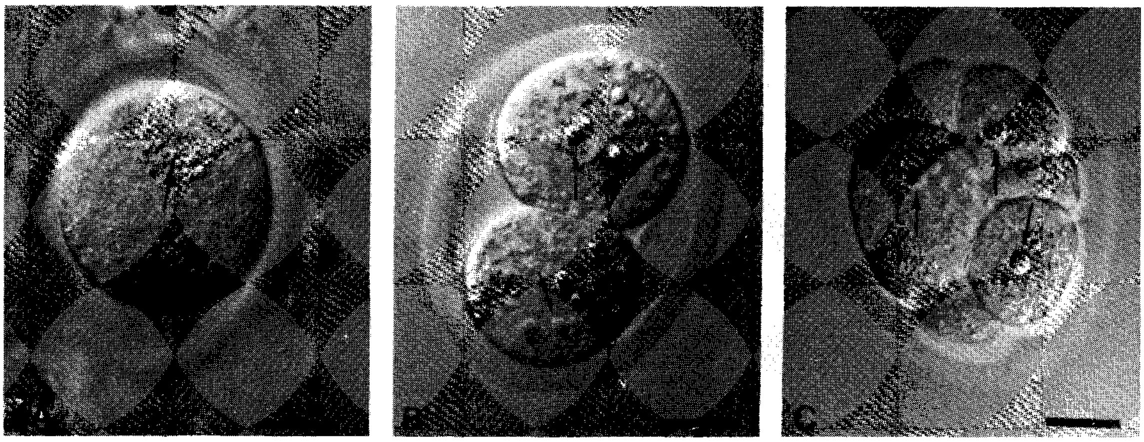

Fig 4. Round and fragmented oocytes recovered from the rat oviduct between 30 and 36 h after ovulation. Nucleus and nucleolus (arrows) can be seen in: (A) A round oocyte. (Bar = 28.6 μm). (B) Both cytoplasmic masses of a two-fragment oocyte. (Bar = 27 μm). (C) Some fragments of a multifragmented oocyte. (Bar = 28 μm).

meiotic apparatus within it (Fig 8). The organelles had increased in number and they were organized in clusters (Fig 8). At the end of this period some cortical granules had migrated towards the center of the egg and there was a decline in the number of microvilli and in the amount of organelles.

Oocytes recovered 18-26 h after ovulation. The surface of these oocytes was completely covered by microvilli. The thin layer of granular material and the meiotic apparatus were no longer present. At the end of this period a second wave of cortical granules were releasing their contents to the perivitelline space (Figs 9B, C). Some round oocytes showed one or more nuclei with nucleolus (Figs 4A, 10). Many Golgi-like multilaminar structures and clusters of vesicles were present in the vicinity of each nucleus.

Oocytes recovered 30-36 h after ovulation. The ultrastructure of the round oocytes obtained during this period appeared the same as that described above. Fragmented oocytes showed one or more nuclei with nucleolus (Fig 4B, C).

These oocytes had clusters of organelles between each nucleus and the surface of each cytoplasmic mass. Surface contacts between fragments were not observed (Fig 11).

II. Development

Observations with light and electron microscopes.

Table I shows the frequency of each type of egg and some of the events that characterized them at increased intervals after ovulation.

All the eggs recovered at 2 h were penetrated and had a pyriform shape; the tail of the sperm was visible in the fertilizing cone (Fig 12). At this time the second polar body was visible in 97% of them. The majority of the eggs recovered at 6 h were round and had at least one pronucleus. Later, between 10 and 15 h, all recovered ova were round and most of them had developed two pronuclei (Fig 13A,B). At 30 h, all the eggs examined had undergone the first cleavage and a nucleus was visible in each one of the blastomeres (Fig 14).

DISCUSSION

This study demonstrates that unfertilized rat oocytes undergo orderly and reproducible structural changes whose nature and sequence are reminiscent of those followed by fertilized oocytes. The changes include the extrusion of the second polar body, exocytosis of cortical granules, development of nuclei, cytoplasmic division and spatial reorganization of organelles. This sequence

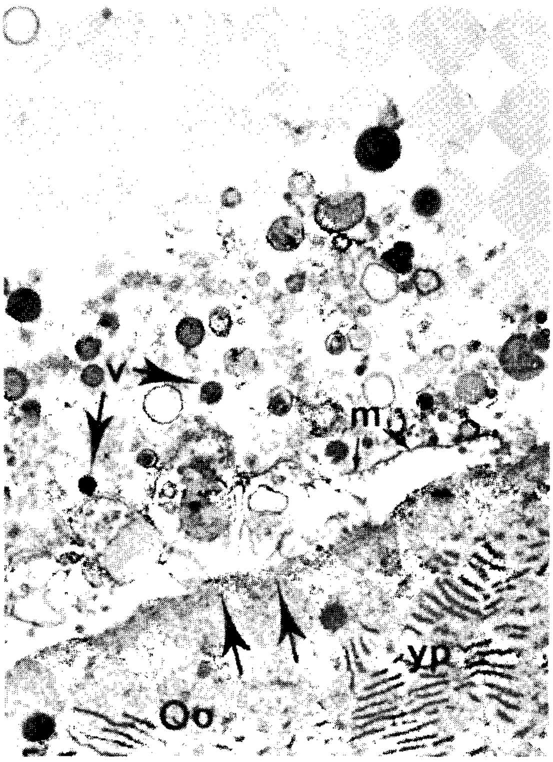

Fig 5. Round oocyte recovered 1 h after ovulation. The membranous structures seen in the perivitelline space most likely represent the remains of the first polar body. Oo: oocyte; m: polar body membrane; v: vesicles; yp: yolk plates; arrows: layer of granular material. (Bar = 1.33 μm).

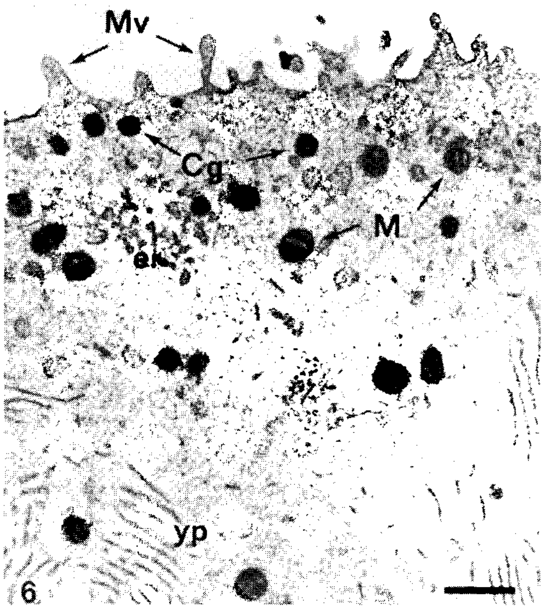

Fig 6. Cortex of a recently ovulated rat oocyte recovered 1 h after ovulation. Cg: cortical granules; Mv: microvilli; M: mitochondria; yp: yolk plates; er: endoplasmic reticulum. (Bar = 0.38 μm).

could well be a partial morphological expression of the same biological program activated by the fertilizing spermatozoon.

According to Odor (1955), Tsafriri and Kraicer (1972) and Keefer and Schuetz (1982), the first polar body is formed before ovulation and it is present in the perivitelline space of rat oocytes during a short period before its disintegration. Our observations are consistent with a rapid disappearance of the first polar body. A polar body was seen in a few specimens recovered during the first hour after ovulation and in none of those recovered in the next 12 h.

The shift from pyriform to round shape occurring at about 18 h was associated with the appearance of a polar body in nearly 60% of the oocytes. This observation is consistent with the formation of the second polar body at this time. Undoubtedly the transient cone-shaped protuberance with the meiotic apparatus in it corresponds to the initial step of the second polar body formation and its release into the perivitelline space returns the oocyte to its usual round shape. This cone-shaped protuberance can be considered the earliest morphological expression of spontaneous activation in the rat oocyte. The occurrence of nucleus-like structures in parthenogenetically activated rat oocytes has been used as criterion of full activation (Zernicka-Goetz, 1991). Nucleus-like structures morphologically similar to the rat pronuclei of fertilized ova appear also in round oocytes approximately 25 h after ovulation (compare Figs 10, 13). These nuclear-like structures add another element to the analogy with egg activation. In other species the relationship between the cone-shaped protuberance and the extrusion of the second polar body is not clear: in the unfertilized mouse oocyte a cone-shaped protuberance similar to the one observed in the rat oocyte has been described, but a second polar body formation has not been reported (Phillips and Shalgi, 1980; Szollosi, 1971). In hamster oocytes, the cone-shaped protuberance is not observed, but the extrusion of the second polar body has been reported (Yanagimachi and Chang, 1961; Longo, 1973). The second polar body, as the first one, must be a short-lived structure since it was absent in the majority of the oocytes recovered between 25 and 35 h.

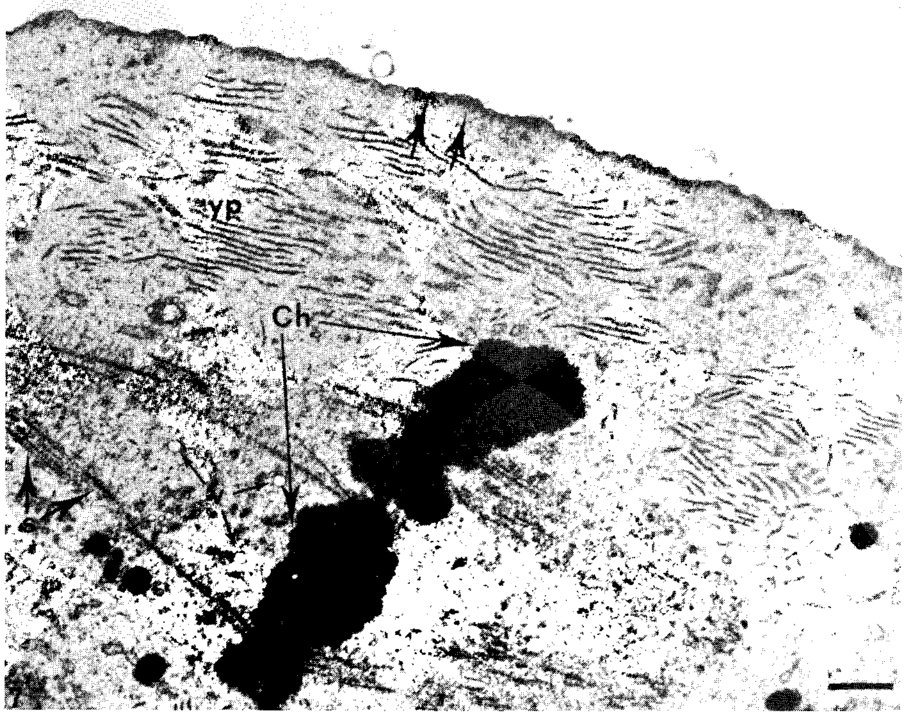

Fig 7. Pyriform oocyte recovered 3 h after ovulation. Meiotic spindle with its main axis oriented tangentially to the surface. Notice the absence of microvilli and the smooth appearance of the plasma membrane (arrows). **Ch**: chromosomes; **s**: spindle axis; **v**: small vesicles; **yp**: yolk plates; arrows: layer of granular material. (Bar = 1.0 μm).

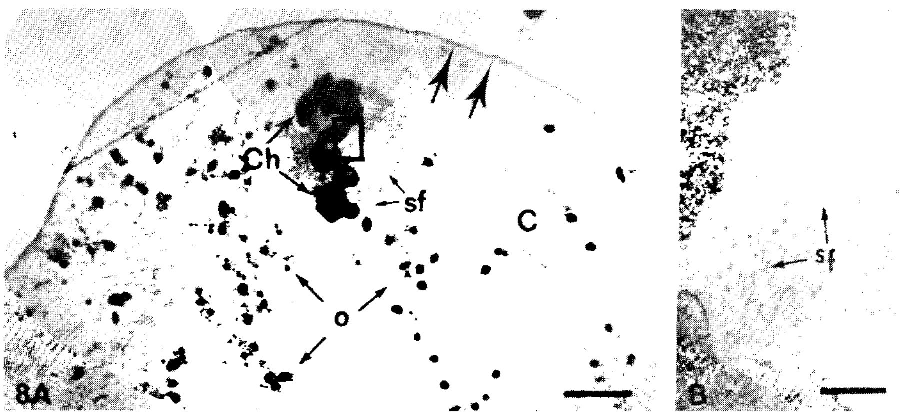

Fig 8. Pyriform oocyte recovered 10 h after ovulation. (A) An oblique section through the meiotic spindle located in the cone shaped protuberance is shown. **Ch**: chromosomes; **sf**: spindle fibers; **C**: cone; **o**: organelles; arrows: layer of granular material. (Bar = 0.83 μm). (B) High magnification of the structure framed in Figure 8A. Note the spindle fibers obliquely sectioned. **sf**: spindle fibers. (Bar = 1.25 μm).

Features of fragmentation, such as the presence of nucleus in many of the cytoplasmic masses and the clustering of organelles (Izquierdo and Vial, 1962) resemble the initial steps of preimplantation development. Thus, fragmentation may be regarded as a form of spontaneous activation in which the absence of syngamy leads to abnormal cell divisions rather than the orderly cleavage that follows fertilization.

Oocyte fragmentation has also been observed in mouse, rabbit and human, but not