BIOLOGY OF REPRODUCTION 17, 370–379 (1977)

Hydrolysis of the Hen's Perivitelline Layer by Cock Sperm in vitro

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ABSTRACT

The intact perivitelline layer (PL) at ovulation was composed of a meshwork of anastomosing fibers uniformly enveloping the hen's ovum. When incubated under appropriate conditions with fresh cock sperm areas of this reticulum were hydrolyzed presumedly by acrosin. Light microscopy and scanning and transmission electron microscopy revealed PL breakdown to be considerably more extensive over the animal pole. The sperm observed embedded in this hydrolyzed matrix did not exhibit a typical mammalian or invertebrate acrosome reaction. The plasmalemma overlying the nucleus in most sperm was distended and the acrosomal membranes appeared fenestrated. The plasmalemma overlying the acrosome in a few sperm was discontinuous. Under identical experimental conditions, ova removed from the distal infundibulum or proximal magnum of the oviduct fail to show PL hydrolysis. It appears that the tertiary layers may mask PL sperm receptor sites, act as physical barriers not rendered penetrable by cock sperm acrosin or contain acrosomal enzyme inhibitors.

INTRODUCTION

Light microscopic examination of fertilization of the avian ovum has been limited to the pigeon (Harper, 1904; Bartelmez, 1912; van Durme, 1914), sparrow (van Durme, 1914), hen (Olsen, 1942) and turkey (Olsen and Fraps, 1944). The mechanism of sperm penetration was not discussed until Bellairs et al. (1963) assessing the fine structure of the perivitelline layer (PL) suggested that sperm may simply pass between the fibers comprising this investment. This was recently given added support by Fujii (1976) who claimed to have observed such spaces containing sperm during *in vitro* fertilization of ova obtained from the infundibulum.

The PL uniformly covers the hen's ovum in a manner analogous to the mammalian zona pellucida. The PL overlying the animal pole (which consists of the blastodisc and underlying nucleus of Pander) is preferentially hydrolyzed *in vitro* by cock sperm (Howarth and Digby, 1973) or by partially purified cock sperm acrosin (Ho and Meizel, 1975). With either agent the PL overlying the remainder of the ovum requires longer incubation time for hydrolysis.

Ova removed from the distal infundibulum or proximal magnum (the albumin secreting region of the hen's oviduct) are enveloped by two tertiary layers (Bellairs et al., 1963; Bain and Hall, 1969; Gilbert, 1971). Intimately associated with the outer surface of the PL is a 50–100 nm thick sheet, the continuous membrane. External to it is the outer perivitelline layer (OPL) which ranges from 3 to 8 μm in thickness. The synthesis and assimilation of these additional investments are thought to be initiated in the distal infundibulum and continued throughout the magnum (Bain and Hall, 1969). The presence of these tertiary layers prevented the hydrolysis of the ovum's PL when incubated with cock sperm *in vitro* (Howarth and Digby, 1973).

The present paper describes the fine structure of sperm interaction with ova recovered from the body cavity and oviduct of the chicken, *Gallus domesticus*. Emphasis is placed on events occurring in the animal pole region.

MATERIALS AND METHODS

The classification of the yolk membranes was done in accordance with that suggested by Wyburn et al. (1965) and Gilbert (1971) with some modifications. The secondary membrane, formed during oocyte maturation, will be referred to as the perivitelline layer (PL). The tertiary investments, which are formed by

Accepted April 29, 1977.
Received December 16, 1976.

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the oviduct, are the continuous membrane and the outer perivitelline layer (OPL). In the infundibulum, the latter has a filamentous appearance and will be referred to as the expanded OPL. This is modified by the time the ovum reaches approximately 5 cm into the magnum to a concentrically arranged, dense fibrous reticulum. This will be referred to as the condensed OPL. The latter is also known as the chalaziferous layer.

Approximately 30 min postoviposition hens were killed and their ovaries and oviducts examined for signs of ovulation. Only ova found in the body cavity, the infundibulum or proximal 5 cm of the magnum were used. Individual ova were quickly placed into 15 ml of warm (41°C) modified Ringer's solution (Olsen and Neher, 1948) and 0.1 ml of fresh, undiluted, pooled cock semen was added (approximately 2×10^7 sperm after dilution). After 15–18 min, the incubation media was replaced with 20 ml of 2 percent glutaraldehyde in 0.15 M cacodylate buffer, pH 7.2 for 2 h. Afterwards the animal pole and other regions of the ovum were removed and placed in the same fixative for an additional hour. Following an overnight wash with several changes of 0.15 M cacodylate buffer plus 5 percent sucrose, the samples were further fixed in 2 percent osmium tetroxide in 0.15 M cacodylate buffer for 2–3 h. After dehydration with ethanol they were embedded in Spurr Low-Viscosity Embedding Media (Polyscience, Inc., Warrington, PA). Thin sections were stained with aqueous uranyl acetate for 15–20 min followed by lead citrate for 4 min at 60°C. These were examined with a Philips 200 Electron Microscope operated at an accelerating voltage of 60 or 80 kv. Thick sections (1–2 μm) were stained with Paragon Multiple Stain (Paragon C. and C. Co., Inc., Bronx, N.Y.) or a solution of 1 percent Azure II and 1 percent methylene blue to which had been added a few grains of sodium borate at the time of staining. These were examined with a Zeiss Photomicroscope II.

For scanning electron microscopy specimens were fixed and dehydrated as above, critical point-dried and mounted on aluminum stubs. These were coated with gold/palladium and examined with a Kent Cambridge Stereoscan operated at an accelerating voltage of 10 kv.

RESULTS

The PL overlying the animal pole and remaining regions of the ovum was structurally identical and exhibited similar variations in thickness (2–4 μm). Microvillus-like extensions originating from the follicular cells or the vitelline membrane were observed on the surface of the PL (Fig. 14). Some appeared to emerge from the pores between the fibers (Fig. 14). In thin section these extensions contained microfilaments, tubular profiles and glycogen-like granules (Figs. 9, 10).

Light microscopy (LM) of body cavity ova incubated with sperm clearly demonstrated areas of hydrolysis. In the nonblastodisc regions the lateral extent of the hydrolyzed PL was 7 μm or less and was visualized as a nonstaining concavity which usually extended the full width of the PL (Figs. 3, 4). This reticulum was extensively hydrolyzed when overlying the central aspect of the animal pole (Figs. 1, 2). Sperm were observed in the hydrolyzed matrix and in the subjacent perivitelline space (Fig. 1).

The fine structure of the hydrolyzed matrix was identical in all areas examined, however, more sperm were associated with the PL overlying the animal pole than in other areas of the ovum. Individual electron dense fibers with no discernible substructure prior to the introduction of sperm underwent dissolution that transformed them into a matrix composed of flocculent and filamentous materials (Figs. 7, 8). The flocculent component was predominantly localized to the outer surface of the hydrolyzed matrix whereas the filamentous material was uniformly distributed throughout the hydrolyzed layer (Fig. 8). High magnification of individual, partially hydrolyzed fibers revealed them to be the source of the filamentous material (Fig. 8).

Sperm in the hydrolyzed matrix exhibited some distention of the plasma membrane particularly over the region of the nucleus. The acrosome remained intact although the inner and outer acrosomal membranes appeared fenestrated (Fig. 11). The plasma membrane over-

FIG. 1. Hydrolysis of the perivitelline layer (arrows) is evident. Note the sperm in the perivitelline space (curved arrow). Body cavity ovum. X570.

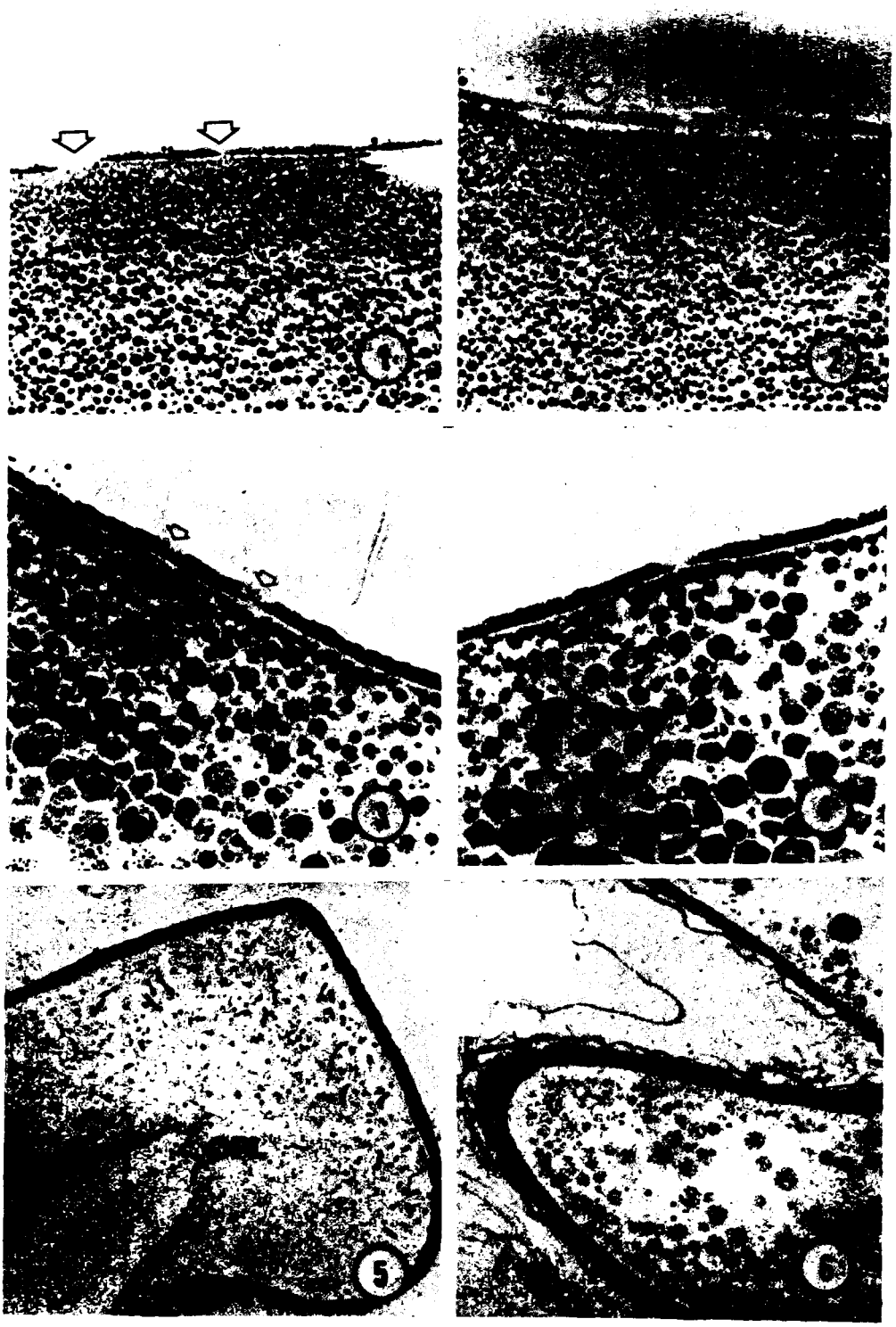
FIG. 2. This light micrograph shows extensively hydrolyzed perivitelline layer (arrows). Body cavity ovum. X570.

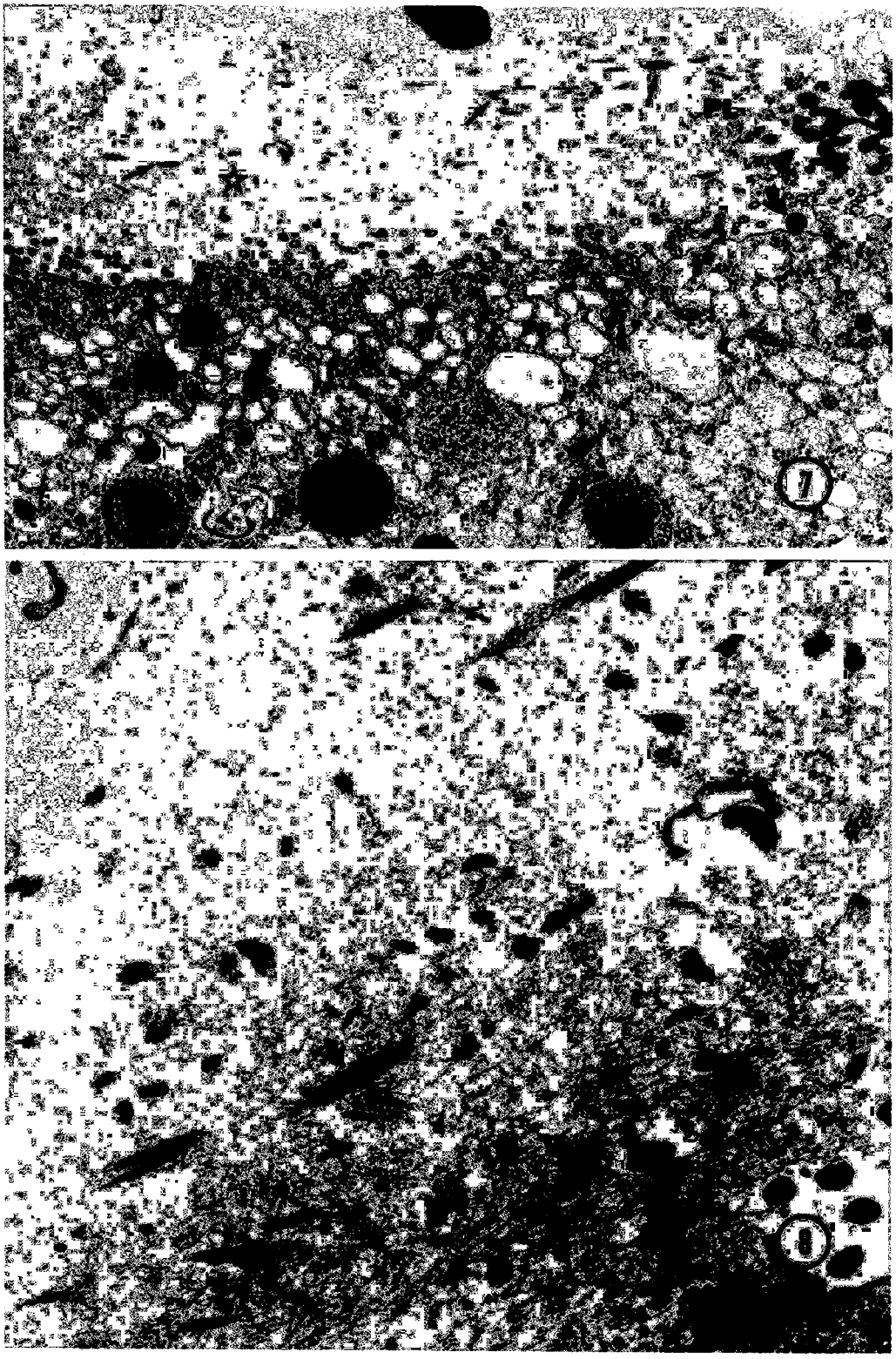
FIG. 3. Approximately 3 cm away from the animal pole small areas of hydrolyzed perivitelline layer (arrows) are observed. Body cavity ovum. X570.

FIG. 4. A single area of hydrolyzed perivitelline layer is observed approximately 3 cm from the animal pole. Body cavity ovum. X570.

FIG. 5. Numerous sperm are embedded in the expanded outer perivitelline layer (OPL). Distal infundibulum ovum. X570.

FIG. 6. Sperm are observed between the fibers of the condensed outer perivitelline layer (arrows). Mid-magnum ovum. X510.





lying the acrosome of some of these sperm was discontinuous. No spatial relationship existed between these two irregularities and no membrane vesiculation was observed. In transverse section the midpiece of most sperm were nearly circular, however, several appeared angular with as many discrete sides as there were mitochondria viewed.

Scanning electron microscopy (SEM) revealed sperm in various degrees of PL penetration. Unhydrolyzed areas of the ovum's surface appeared as a meshwork of anastomosing fibers with a slightly porous ground substance occupying the interstices (Figs. 13, 14). Sperm observed on the surface of the ovum exhibited either curvature of the whole head region (Fig. 17) or an acute bending of the anterior portion of the nucleus (Fig. 14). By observing various degrees of sperm penetration it was apparent that the head initially entered the PL (Fig. 15). The tails of partially embedded sperm were either on the surface or appeared "sinking" into the hydrolyzed material. The spongy appearance of the latter gave no indication of its former surface structure.

Ova recovered from the infundibulum and proximal magnum possessed two tertiary layers, the continuous membrane and the OPL (Figs. 9, 10). Those ova removed from the infundibulum were enveloped by the expanded OPL which resembled the hydrolyzed PL matrix previously described (Figs. 5, 9, 10). It was 6 μm or more in width. After traversing the infundibulum and approximately 5 cm into the magnum, a concentric array of close-knit fibers, the condensed OPL, was observed around the ovum (Figs. 6, 12, 18). When incubated with cock sperm under the identical conditions as body cavity ova, these oviductal ova failed to show PL hydrolysis (Figs. 5, 6, 12). With light microscopy the unstained expanded OPL highlighted the embedded sperm and underlying intact PL (Fig. 5). Fibers of the condensed OPL were readily stained as were the sperm observed between them (Fig. 6). The distribution of sperm embedded in the tertiary layers was not related to the position of the animal pole.

Sperm were observed in the expanded OPL in the vicinity of the continuous membrane but not penetrating it. A path either created by the physical penetration of the sperm or by its hydrolytic activity was associated with some sperm in the expanded OPL. The sperm between the fibers of the condensed OPL rarely penetrated the inner two or three layers of this investment (Figs. 6, 12). Except for the fenestrated appearance of the acrosomal membranes and for some distention of the plasma membrane, the fine structure of the sperm was not altered (Figs. 11, 12).

A single ovum was recovered from the midportion of the infundibulum and incubated with cock sperm under the described conditions. The continuous membrane as well as some filamentous elements of the expanded OPL were present around the ovum. Sperm hydrolyzed these as well as the underlying PL. This was the only instance where sperm penetration of the continuous membrane was observed.

DISCUSSION

We presume that the hydrolysis of the PL of body cavity ova was due to the activity of the trypsin-like enzyme acrosin (Buruianna, 1956;

FIG. 7. A survey micrograph showing the hydrolyzed perivitelline layer (PL) overlying the animal pole. Note the partially intact fibers (arrows) and vitelline microvilli. Body cavity ovum. Curved arrow, sperm. X9,400.

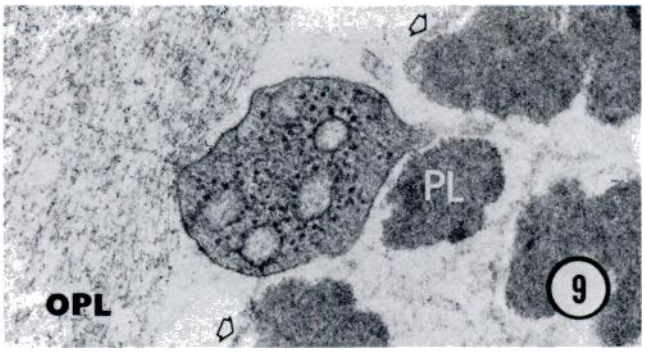
FIG. 8. This figure is an enlargement of the area corresponding to the region around the star shown in Fig. 7. The flocculent (arrows) and filamentous components and bits of partially hydrolyzed fibers (curved arrows) of the perivitelline layer are observed in the matrix overlying the animal pole. The vitellus is observed in the lower right corner. Body cavity ovum. X25,000.

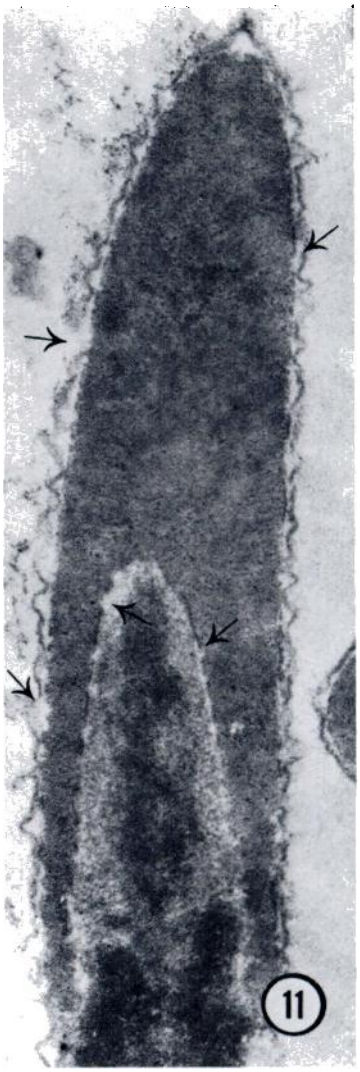
FIG. 9. The bulbous process on the surface of the perivitelline layer (PL) contains spherical profiles and glycogen-like granules. The two tertiary investments, the continuous membrane (arrows) and the expanded outer perivitelline layer (OPL) are present. Animal pole region of a distal infundibulum ovum. X38,600.

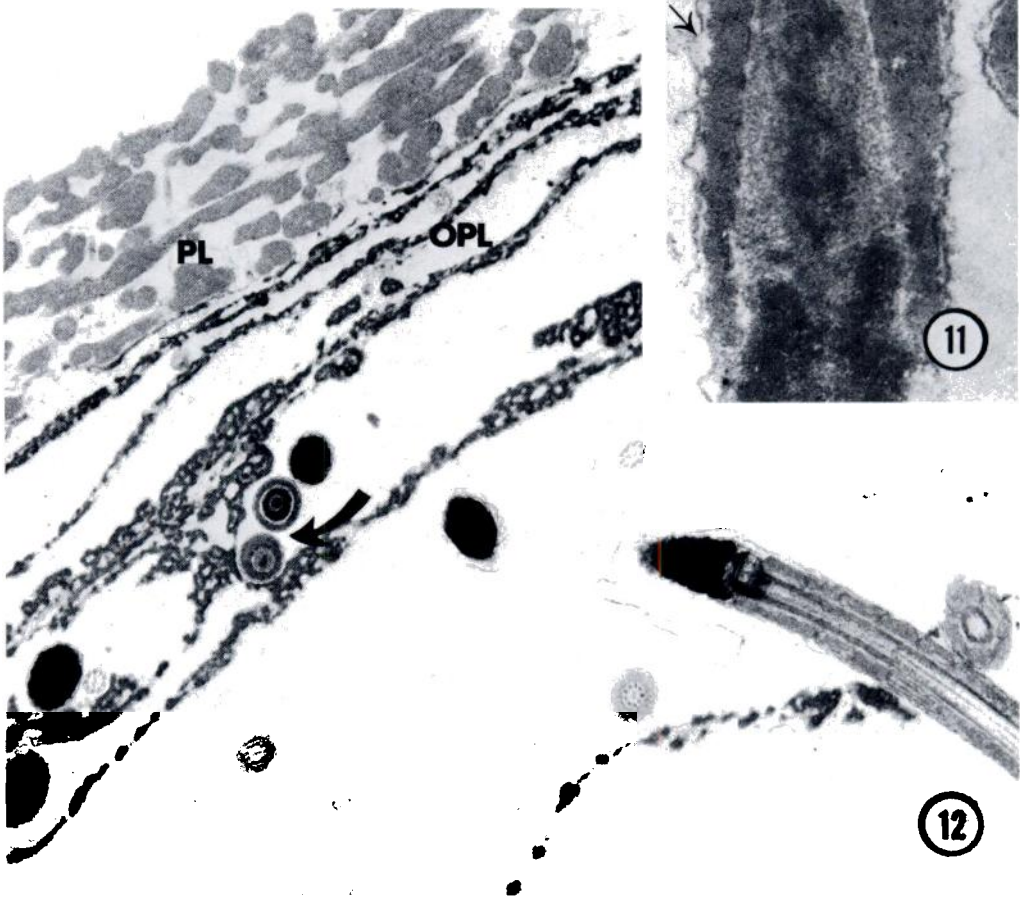
FIG. 10. An elongated process is observed on the surface of the perivitelline layer (PL) as well as in the PL reticulum (arrow). Note the filamentous and osmiophilic, granular components in the expanded outer perivitelline layer (OPL). Animal pole region of a distal infundibulum ovum. X27,500.

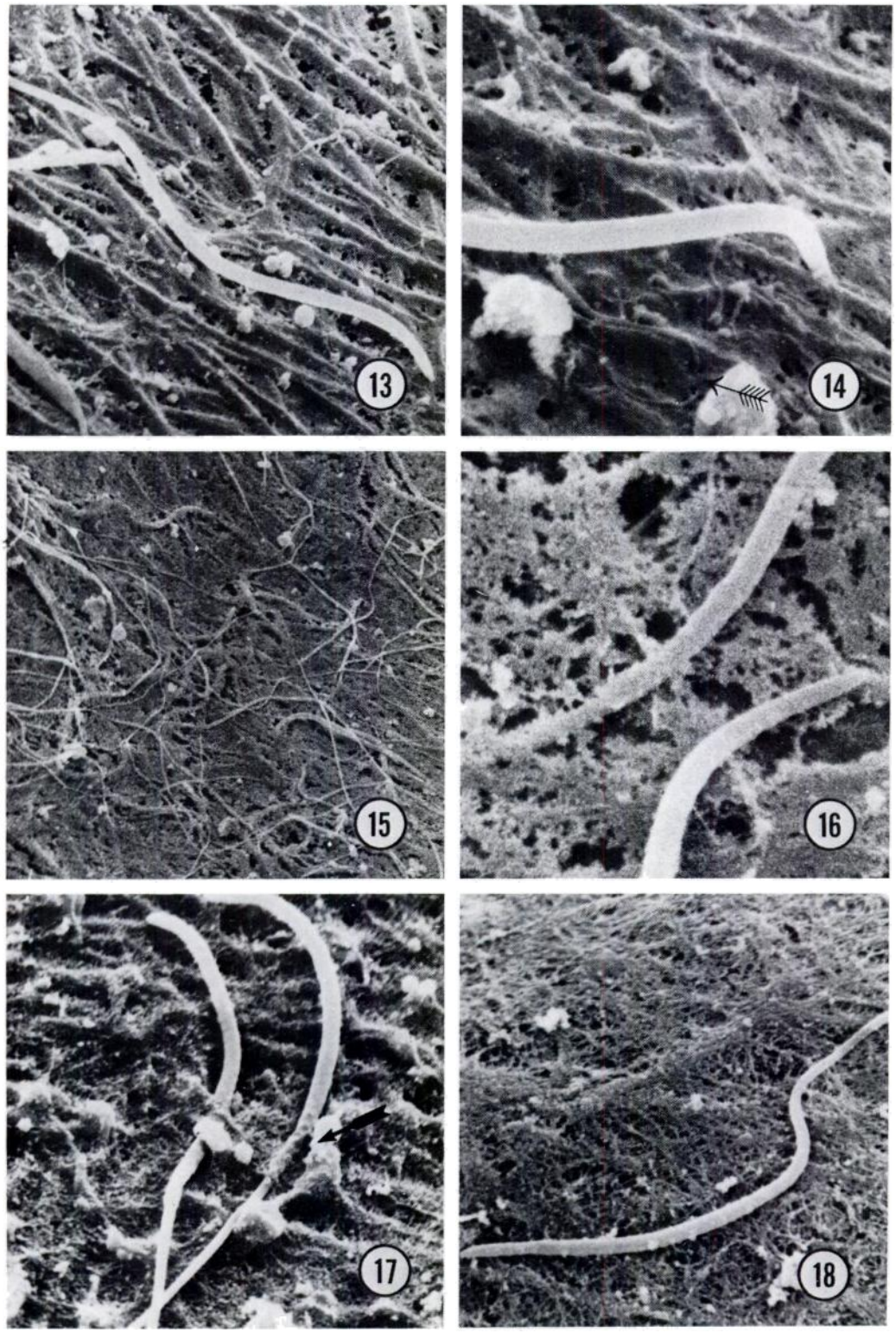
FIG. 11. A ruffled plasmalemma and a fenestrated acrosomal membrane (arrows) characterizes this sperm observed embedded in the hydrolyzed perivitelline layer. Body cavity ovum. X77,900.

FIG. 12. Sperm incubated with an ovum removed from the magnum do not deeply penetrate the fibers of the condensed outer perivitelline layer (OPL). Note the intact acrosomes (curved arrow), PL, perivitelline layer. X18,000.









Ho and Meizel, 1970; Polakoski, 1972; Howarth and Digby, 1973; Brown and Hartree, 1976). This acrosomal enzyme reduced the fibers and ground substance of the PL to a spongy-looking matrix composed of filamentous and flocculent materials.

The suggestion of Bellairs et al. (1963) on the ability of cock sperm to traverse the PL "without having to break it down" was recently given support by Fujii (1976). By use of SEM he claimed to have observed sperm in the open spaces between the PL fibers of infundibular ova in the act of penetration. He subsequently assigned a minor role to acrosin. Our observations revealed that a void does not exist between the PL fibers but that these spaces are actually occupied by a ground substance. The latter as well as the fibers of the PL of oviductal ova are concealed by the tertiary layers when viewed by SEM. What Fujii observed may have been sperm in these investments. In any case, his micrographs did not compare favorably with any observations made by us during the course of this study. In this instance verification of his observations with transmission electron microscopy would prove informative.

Evidence of a mammalian or invertebrate type of acrosome reaction as reviewed by Yanagimachi (1973) and Franklin (1970), respectively, was not observed in the sperm seen in the hydrolyzed matrix. However, the acrosomal membrane appeared fenestrated. Some of the sperm were also seen to have a discontinuous plasma membrane over the acrosome. These changes coincided with the hydrolytic activity observed and taken together may be analogous to the acrosome reaction of other species.

Howarth and Digby (1973) observed a preferential hydrolysis of the animal pole and suggested a chemotaxic mechanism might be involved. Our observations lend support to this proposal. However, the role of chemotaxis in the fertilization process in the animal kingdom has been questioned by Rothschild (1956). The possibility of a concentrated array of sperm receptor sites associated with the surface of the animal pole, although not attracting sperm, would allow for sperm recognition and attachment to this region. Immediately following ovulation these are exposed allowing for extensive sperm interaction over the animal pole. The deposition of the tertiary investments effectively masks these receptor sites. We are presently examining the distribution of concanavalin A and cationized ferritin receptor sites on the PL to evaluate such a possibility. Additional factors may influence the activation of acrosin over the animal pole. For example, Ca^{++} and Mg^{++} have been shown to stimulate the activity of extracted cock sperm acrosin (Ho, 1974). Their distribution may be such that only the animal pole can promote extensive acrosin activity.

Ho and Meizel (1975) applied partially purified cock sperm acrosin to pieces of filter paper and placed them on different areas of the ovum. In 63 percent of the trials the filter paper over the animal pole sank first, signifying PL hydrolysis. These authors stated that chemotaxis need not be a factor in the preferential hydrolysis observed by Howarth and Digby (1973) and suggested several other possibilities. These included regional differences in the biochemical composition of the PL and differences in the structure and composition of the underlying yolk-plasm. Preliminary data gathered in this laboratory on the amino acid composition of the PL over the animal pole and other regions of follicular ova were identical. In addition, we did not observe variation in the fine structure in the intact or in the hydrolyzed PL of those regions. However, the morphology of the blastodisc, the portion of the animal pole immediately underlying the PL, was consider-

FIG. 13. A sperm rests on the surface of the perivitelline layer of a body cavity ovum. Note the fine microvillus-like extensions on the surface of the perivitelline layer and its porous nature. ×4,500.

FIG. 14. The anterior portion of the sperm head exhibits an acute bending. Note the microvillar extensions eminating from the porous ground substance (arrow). ×9,500.

FIG. 15. Sperm are observed in various stages of perivitelline layer penetration. Animal pole region of body cavity ovum. ×1,500.

FIG. 16. The intact fibers of the perivitelline layer seen in Figs. 13 and 14 are transformed into a spongy matrix when hydrolyzed by cock sperm acrosin. Several sperm are partially embedded in the hydrolyzed matrix. ×9,100.

FIG. 17. Sperm are observed on the expanded outer perivitelline layer. Note the contoured appearance of the midpiece of one sperm (arrow). ×9,100.

FIG. 18. A sperm atop the condensed outer perivitelline layer. Note the latter's fine dense reticulum. ×4,900.

ably different from the remaining ovum (Bakst and Howarth, 1977). These differences may have influenced the data of Ho and Meizel (1975).

Several factors could be responsible for the failure of cock sperm to fully penetrate the ovum's tertiary layers and hydrolyze the PL. The fibers of the condensed OPL, which are predominantly ovomucin (Conrad and Philips, 1938; Sugihara et al., 1955), may constitute an effective barrier not rendered penetrable by cock sperm acrosin. In addition, this sialoprotein (Feeney et al., 1960) could have a similar antifertility effect on cock sperm as the sialoproteins of Cowper's gland mucin and fetuin had on capacitated rabbit sperm (Srivastava and Gould, 1973). These authors observed a dose-response between the amount of sialoprotein present and the degree of inhibition of fertilization. This may relate to the inability of the undeveloped OPL found on an ovum removed from the midportion of the infundibulum to prevent sperm penetration.

The condensed OPL probably contains other albumin proteins (Bellairs et al., 1963) including ovomucoid and ovoinhibitor, which are known trypsin (Lineweaver and Murray, 1947) and trypsin and chymotrypsin inhibitors (Matsushima, 1958), respectively. Ho (1974) demonstrated some inhibition of partially purified cock sperm acrosin with ovomucoid although it was the least effective following soybean and lima bean trypsin inhibitors. It is not known whether ovoinhibitor has an inhibitory effect on cock sperm acrosin. The polyanion poly-α-L-glutamic acid, which is found only in the infundibulum and uterovaginal junction of the hen's oviduct (Harrison and Heald, 1966), was also found slightly inhibitory to extracted cock sperm acrosin (Ho, 1974).

The ability of the condensed OPL to prevent sperm penetration *in vitro* is unequivocal. Whether it has the same function *in vivo* is questionable. The hen's megalecithal ovum necessitates physiological polyspermy in order to maximize the chances of syngamy. Thus by virtue of its large surface area the ovum should be able to accommodate the "several hundred sperm" observed on its surface (Bobr et al., 1964). The limited damage confined to the animal pole, if preferentially hydrolyzed by sperm *in vivo*, is literally patched-up by the rapid deposition and assimilation of the expanded OPL and later the condensed OPL. That the ovomucin fibers protect against sperm-induced rupture and give strength to the yolk investments was first suggested by McNally (1943). The temporal relationship between the initial sperm egg encounter and the formation of an effective barrier to sperm penetration would also negate the role of the tertiary investments as a block to pathological polyspermy. It takes approximately 30 min for the ovum to reach the magnum after ovulation (Warren and Scott, 1935), yet sperm pronuclei formation was observed in a body cavity ovum estimated to have ovulated 15 min prior to its fixation (Olsen, 1942). The presence of greater numbers of sperm in the infundibulum after intrauterine insemination increased fertility but simultaneously resulted in increased embryonic mortality (Kamar and Hafez, 1975). An excess of sperm penetrating the PL prior to the formation of an effective sperm barrier (i.e., the tertiary layers) could result in pathological polyspermy and subsequent embryonic death. Under natural conditions the tertiary investments may serve as a block to pathological polyspermy if sperm are present in the distal infundibulum and magnum.

ACKNOWLEDGMENTS

We would like to thank Dr. John Munnell (Department of Anatomy and Radiology, School of Veterinary Medicine, University of Georgia) for his generous assistance and Dr. Walter J. Humphreys, Director of the Central Electron Microscopy Laboratory, for the use of its facilities.

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