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Changes in the Spermatozoon During Fertilization in Hydroides Hexagonus (Annelida): I. Passage of the Acrosomal Region through the Vitelline Membrane

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CHANGES IN THE SPERMATOZOON DURING FERTILIZATION IN *HYDROIDES HEXAGONUS* (ANNELIDA)

I. Passage of the Acrosomal Region through the Vitelline Membrane

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ABSTRACT

In the previous paper the structure of the acrosomal region of the spermatozoon was described. The present paper describes the changes which this region undergoes during passage through the vitelline membrane. The material used consisted of moderately polyspermic eggs of *Hydroides hexagonus*, osmium-fixed usually 9 seconds after insemination. There are essentially four major changes in the acrosome during passage of the sperm head through the vitelline membrane. First, the acrosome breaks open apically by a kind of dehiscence which results in the formation of a well defined orifice. Around the lips of the orifice the edges of the plasma and acrosomal membranes are then found to be fused to form a continuous membranous sheet. Second, the walls of the acrosomal vesicle are completely everted, and this appears to be the means by which the apex of the sperm head is moved through the vitelline membrane. The lip of the orifice comes to lie deeper and deeper within the vitelline membrane. At the same time the lip itself is made up of constantly changing material as first the material of the outer zone and then that of the intermediate zone everts. One is reminded of the lip of an amphibian blastopore, which during gastrulation maintains its morphological identity as a *lip* but is nevertheless made up of constantly changing cells, with constantly changing outline and even constantly changing position. Third, the large acrosomal granule rapidly disappears. This disappearance is closely correlated with a corresponding disappearance of a part of the principal material of the vitelline membrane from before it, and the suggestion is made that the acrosomal granule is the source of the lysin which dissolves this part of the vitelline membrane. Fourth, in the inner zone the fifteen or so short tubular invaginations of the acrosomal membrane, present in the normal unreacted spermatozoon, lengthen considerably to become a tuft of *acrosomal tubules*. These tubules are the first structures of the advancing sperm head to touch the plasma membrane of the egg. It is notable that the surface of the acrosomal tubules which once faced into the closed acrosomal cavity becomes the first part of the sperm plasma membrane to meet the plasma membrane of the egg. The acrosomal tubules of *Hydroides*, which arise simply by lengthening of already existing shorter tubules, are considered to represent the acrosome filaments of other species.

INTRODUCTION

The vitelline membrane or other coverings of the eggs of some species would appear to present a formidable barrier to the penetration of the spermatozoon. It has long been supposed that removal of this barrier is made possible by lysins contained in the sperm, and, indeed, extracts of sperm of certain species contain egg membrane lysins (reviewed in 18). It has been suggested (19) that the acrosome or the acrosome reaction (20) might release the lytic material. In *Hydroides* it is known that sperm extracts contain material which lyses the principal layer of the three-layered vitelline membrane and that at fertilization the spermatozoon makes its own particular hole in this membrane (5, 8, 9). Little, however, is known about how the lytic agent is structurally related to the acrosome, although it is often assumed that the acrosomal granule is its source. Even though a lytic agent plays a role in clearing a path for the passage of the spermatozoon through the vitelline membrane, this in itself does not account for the actual inward movement of the sperm head. There is some evidence, to be presented below, which indicates that certain of the acrosomal structures may actively effect the inward movement of the spermatozoon and thus bring the plasma membranes of the two gametes into contact. This paper will be concerned with the variety of changes which take place in the acrosomal region. A subsequent paper will deal with incorporation of the spermatozoon with the egg (7).

The observations to be reported below were made on moderately polyspermic material and possibly none of the eggs was monospermic. It is not known to what extent the events of sperm entry may differ between monospermy and polyspermy, but from observations of the living specimens it is presumed that in the main aspects, to be described below, they would be essentially the same.

Explanation of Figures

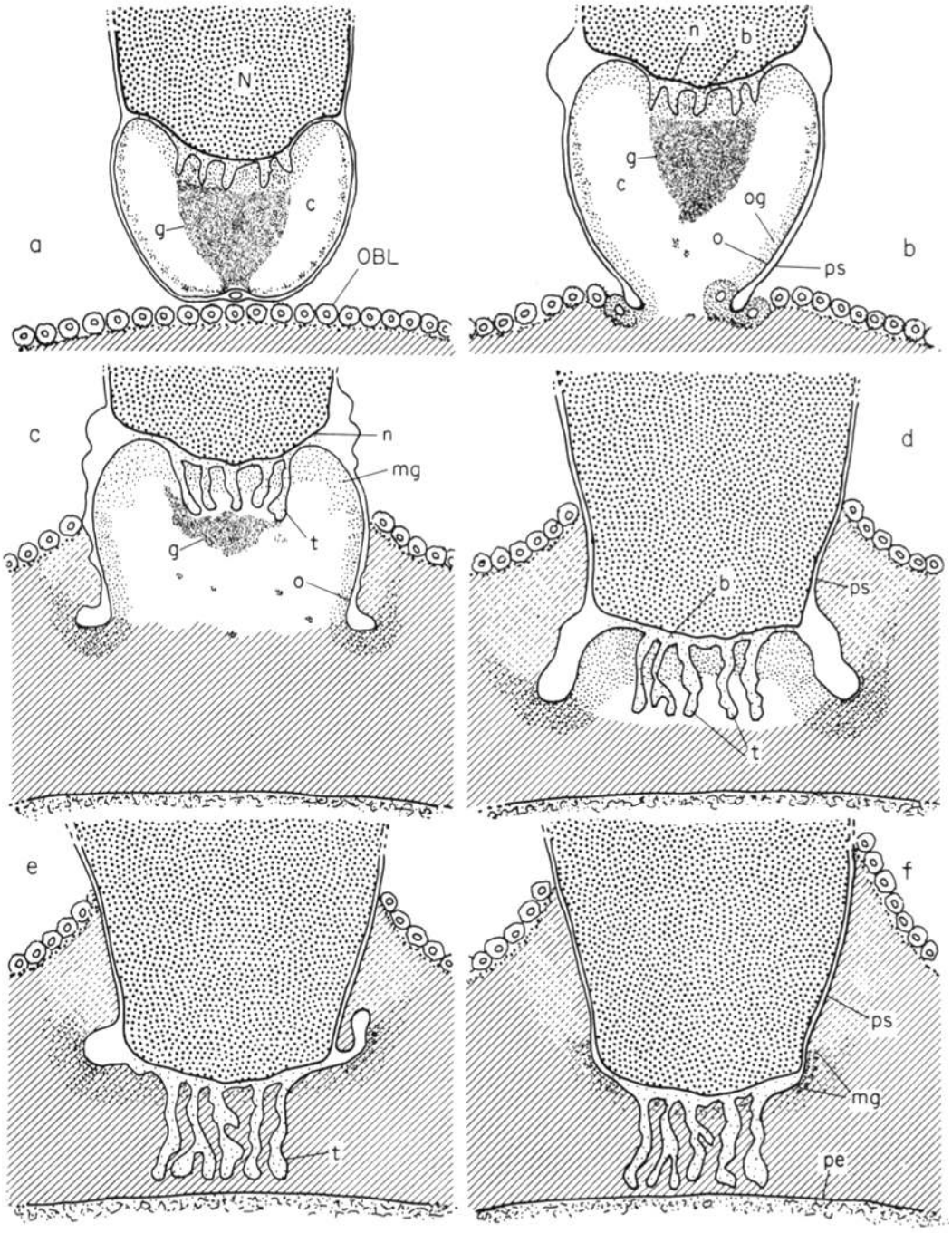
Unless otherwise indicated, all sections are approximately longitudinal with respect to the spermatozoon. The sperm head is approximately circular in cross-section, and the peripheral longitudinal sections are narrower than the central ones; however, the *extreme* divergences in length or width seen in certain comparable specimens are a reflection of compression during sectioning.

With the exception of those shown in Figs. 3, 31, and 32, all specimens were fixed 9 seconds after insemination.

b, material lying between nuclear envelope and base of acrosome
c, cavity of acrosomal vesicle
g, acrosomal granule
i, inner zone of acrosomal membrane
ig, granules of inner zone
m, intermediate zone of acrosomal membrane
mg, granular layer of intermediate zone
n, nuclear envelope
o, outer zone of acrosomal membrane
og, granular layer of outer zone
pe, plasma membrane of egg
ps, plasma membrane of spermatozoon
t, acrosomal tubule
IBL, inner border layer of vitelline membrane
M, mitochondrion
ML, middle layer of vitelline membrane
N, nucleus
OBL, outer border layer of vitelline membrane
Y, yolk granule in egg cytoplasm

FIGURE 1

Diagrams of successive stages in passage of sperm head into vitelline membrane. *a*, presumed position at time of contact with OBL of vitelline membrane; *b*, earliest observed stage in penetration of ML; *c* to *e*, while granular material of outer and intermediate zones everts, membranous wall of region shortens, acrosomal granule disappears, and acrosomal tubules lengthen; *f*, acrosomal tubules are apical part of sperm plasma membrane as apex of nucleus nears egg plasma membrane.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MATERIALS AND METHODS

The sperm and eggs used for this study were of the annelid *Hydroides hexagonus*. The animals were obtained in the vicinity of Woods Hole, Massachusetts.

Sperm was collected as shed and suspended in sea water or in a 0.001 M solution of Versene in sea water. Freshly shed eggs were collected and washed several times in sea water. Insemination was accomplished with a sperm suspension of sufficient concentration to assure a low to moderate percentage of polyspermy, as determined by developmental studies of unfixed portions of the cultures. The spermatozoa show little motility until they have been in sea water for a period of up to 30 minutes (14). Only motile suspensions of sperm were used. It is probable that all the eggs described in this paper were polyspermic. Fixations were made at 9 seconds after insemination and at other intervals thereafter.

Fixation was performed by mixing equal volumes of inseminated eggs and 4 per cent osmium tetroxide in sea water; thus the final concentration of fixative was 2 per cent. After approximately ¾ hour in the fixative the material was washed successively in sea water, diluted sea water, and distilled water, and dehydrated in increasing concentrations of ethyl alcohol. The material was then infiltrated in three changes of a mixture of 85 per cent *n*-butyl and 15 per cent methyl methacrylate monomer containing 2 per cent Luperco as catalyst and polymerized in an oven at 63°C. No centrifugation was employed in these preparations.

Sections were cut with a Porter-Blum microtome and spread by the method of Satir and Peachey (17); some effects of sectioning compression remained. The sections were stained with lead hydroxide by the method of Watson as modified by Dalton and Zeigel (10) and examined with an RCA model EMU-3C electron microscope. All structures described were observed in serial sections of many specimens. The original magnifications of the micrographs ranged from 11,000 to 30,000; the final magnifications of the figures were obtained by photographic enlargement.

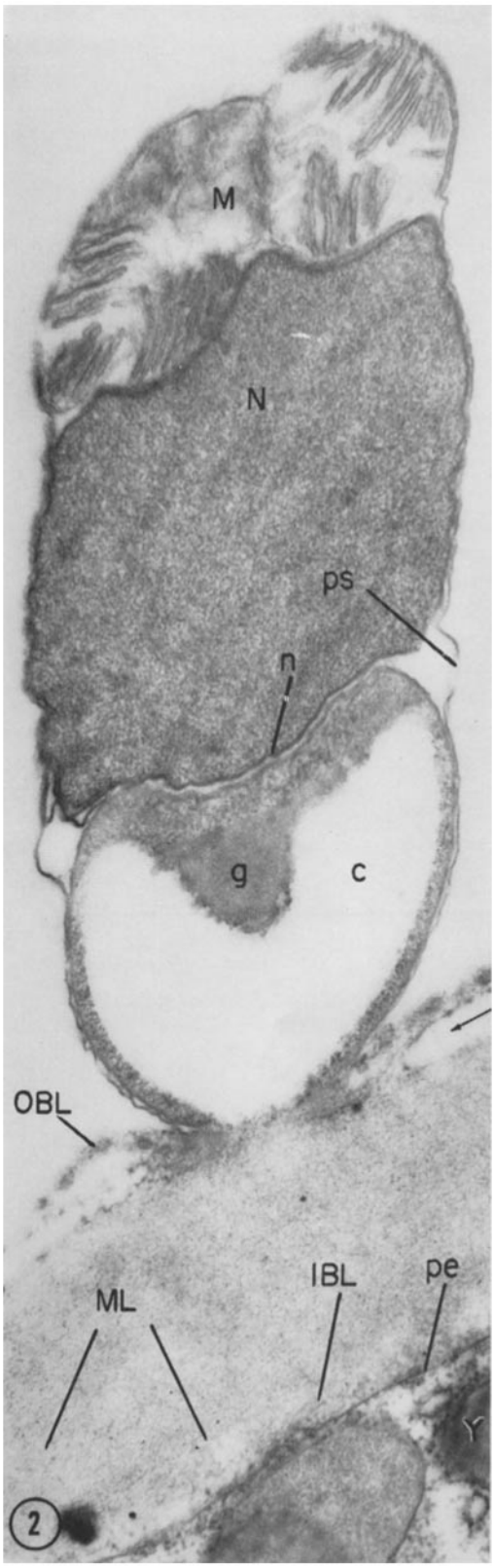
OBSERVATIONS

The structure of the vitelline membrane and that of the acrosome of the unattached spermatozoon which is capable of entering the membrane may be described briefly as follows.

The vitelline membrane consists of a thin granular *outer border layer* (to be referred to below as the OBL), a thick *middle layer* (ML) of rather amorphous material which constitutes the main body of the vitelline membrane, and a thin *inner border layer* (IBL), also amorphous, which adjoins the plasma membrane of the egg (Fig. 2). Microvilli which are extensions of the surface of the egg proper project into the full thickness of the vitelline membrane (Fig. 23). More extensive descriptions of the membrane may be found elsewhere (5, 8, 9).

The hexagonally packed granules of the OBL are the first elements of the egg to confront an approaching spermatozoon (Figs. 3 and 4). An irregular sheet of much smaller particles not hitherto described *per se* lies beneath the OBL and possibly serves to attach the OBL to the ML. Except in areas which are immediately involved with sperm entry, the OBL and its underlying particles do not change in general appearance following fertilization. Typical unaffected areas are shown in Figs. 4 and 23.

The acrosome consists of an acrosomal vesicle which is bounded by a single continuous membrane. Its wall is distinguishable into *inner*, *intermediate*, and *outer zones* (6). Each zone consists of its respective zone of the continuous acrosomal membrane together with the finely granular material which adjoins the membrane. A large acrosomal granule extends from the inner zone as a base to the outer zone of the acrosomal membrane at the apex of the acrosomal vesicle. The cavity of this vesicle is continuous peripherally but not centrally, since it is spanned by the attached acrosomal granule. In the inner zone the acrosomal membrane is invaginated into about fifteen short tubules. In the outer zone this membrane is closely surrounded by the plasma membrane. At the apex of the acrosomal region an *apical vesicle* is sandwiched between the plasma membrane and the acrosomal membrane. *These two membranes lie close together* where they meet at the apical vesicle. This line of junction constitutes a natural "fracture line" or rim of dehiscence. When the apex of the acrosomal region breaks open, as it sometimes does, the apical vesicle with its surrounding membranes comes off like a lid, opening the acrosomal vesicle to the exterior. When the acrosomal vesicle is open it appears that the edges of the plasma and acrosomal membranes are fused at the rim of dehiscence. A detailed account of these structures may be found in (6). Successive stages in passage of the acrosomal region



through the vitelline membrane are shown diagrammatically in Fig. 1.

A. *Earliest Observed Stage*

Principal Changes in the Acrosome: In the earliest stage of vitelline membrane penetration thus far observed, the apex of the formerly closed acrosomal region is open (Figs. 2, 4, and 5). The profile of the acrosome has changed from a somewhat flattened one, characteristic of the unattached spermatozoon, to an elongated one. The acrosomal granule is no longer attached to the outer zone of the acrosomal membrane, and the apex of this granule appears to be torn or shredded (Fig. 4). The disposition of the small apical vesicle which formerly lay outside the apex of the acrosome has not been determined. At the rim or lip of the newly formed orifice the edge of the acrosomal membrane appears to meet and be fused with the edge of the sperm plasma membrane. The two membranes, in effect, are now one continuous plasma-acrosomal membrane of mosaic origin. A slight thickening at the rim may indicate the presumed area of fusion (Fig. 4).

Changes in the Vitelline Membrane: At this early stage the tip of the acrosome has breached only the OBL of the vitelline membrane. The tip occupies a shallow crater rimmed by a low fold of the adjacent part of the OBL. The few OBL granules which touch the lip of the new acrosomal orifice appear to be swollen and perhaps to have fused together (Figs. 4 to 6). Beneath them the smaller underlying particles of the OBL sometimes appear to have fused into a small continuous sheet or patch. The granules, and the particles too, show greater opacity than in the areas farther away from the sperm head.

Other Aspects: The outer edge of the intermediate zone of the acrosome curves away from the nucleus instead of hugging it as in the unattached spermatozoon. In the region of this separation the sperm plasma membrane bulges loosely (Figs. 2, 4, 7 to 9). Occasional vesicles, both large and small, are seen in the bulged area, and the nuclear

FIGURE 2

Spermatozoon in very early stage of penetration of vitelline membrane. Section slightly lateral to mid-line; somewhat compressed along diagonal from upper left to lower right of figure. Line pointing to OBL of vitelline membrane also points to rim of crater in which apex of acrosome lies. Arrow at right lies in space caused by artificial separation of OBL from ML. × 54,000.

envelope sometimes is pulled out here or shows thread-like projections. In the outer zone the layer of granular material next to the acrosomal membrane appears to be thicker but less opaque than in unattached sperm heads. In the inner zone the invaginated tubules of the acrosomal membrane (Figs. 7 to 9) still appear essentially as they do in the unattached sperm head. As before attachment, an ill defined, moderately dense material lies between the nuclear envelope and the base of the acrosome (Figs. 4 and 8). This material persists throughout subsequent stages (Figs. 10, 27, and 32).

B. *Later Stages*

After the OBL of the vitelline membrane has been breached, the spermatozoon moves on through the remainder of the vitelline membrane and as it does so the acrosomal region undergoes three major changes: (1) the entire acrosomal vesicle becomes everted, (2) the acrosomal granule diminishes and disappears, and (3) the invaginated tubules of the inner zone of the acrosomal membrane become considerably lengthened (Fig. 1). All these are gradual changes and some occur simultaneously, but in the following account each will be considered separately so as to follow one change continuously from beginning to end. It is appropriate to emphasize that *all these changes are found in cultures fixed 9 seconds after insemination,* and all micrographs shown in this paper, with the exception of Figs. 3, 31, and 32, are from such cultures.

1. *Eversion of the Acrosomal Vesicle:* a) BEHAVIOR OF GRANULAR MATERIAL. Figs. 10 and 11 show a specimen in the next stage of penetration. A small amount of the material adjoining the outer zone of the acrosomal membrane has become everted and turned back outside the lip of the acrosomal orifice. On the right side in these figures this reflected portion seems to lie between the ML and the OBL of the vitelline membrane, but on the left side, which has begun to penetrate, it seems simply to intermingle with the material of the ML.

As the process of eversion continues (Figs. 12 to 21), the reflected granular material can be seen as a trailing streak behind the advancing acrosome. The impression is that each portion of material which is turned out remains approximately in the region where it was first reflected; hence it is *left behind* as the spermatozoon penetrates more deeply. The streak becomes fainter and wider as its distance from the advancing acrosomal orifice increases (Fig. 18) and it gradually merges with an area in which the material of the ML of the vitelline membrane is more diffuse than elsewhere. The impression is that this diffuse area is merely one in which

FIGURE 3

Outer tangential section of OBL of vitelline membrane. Note close, regular spacing of OBL granules. × 42,000.

FIGURES 4 AND 5

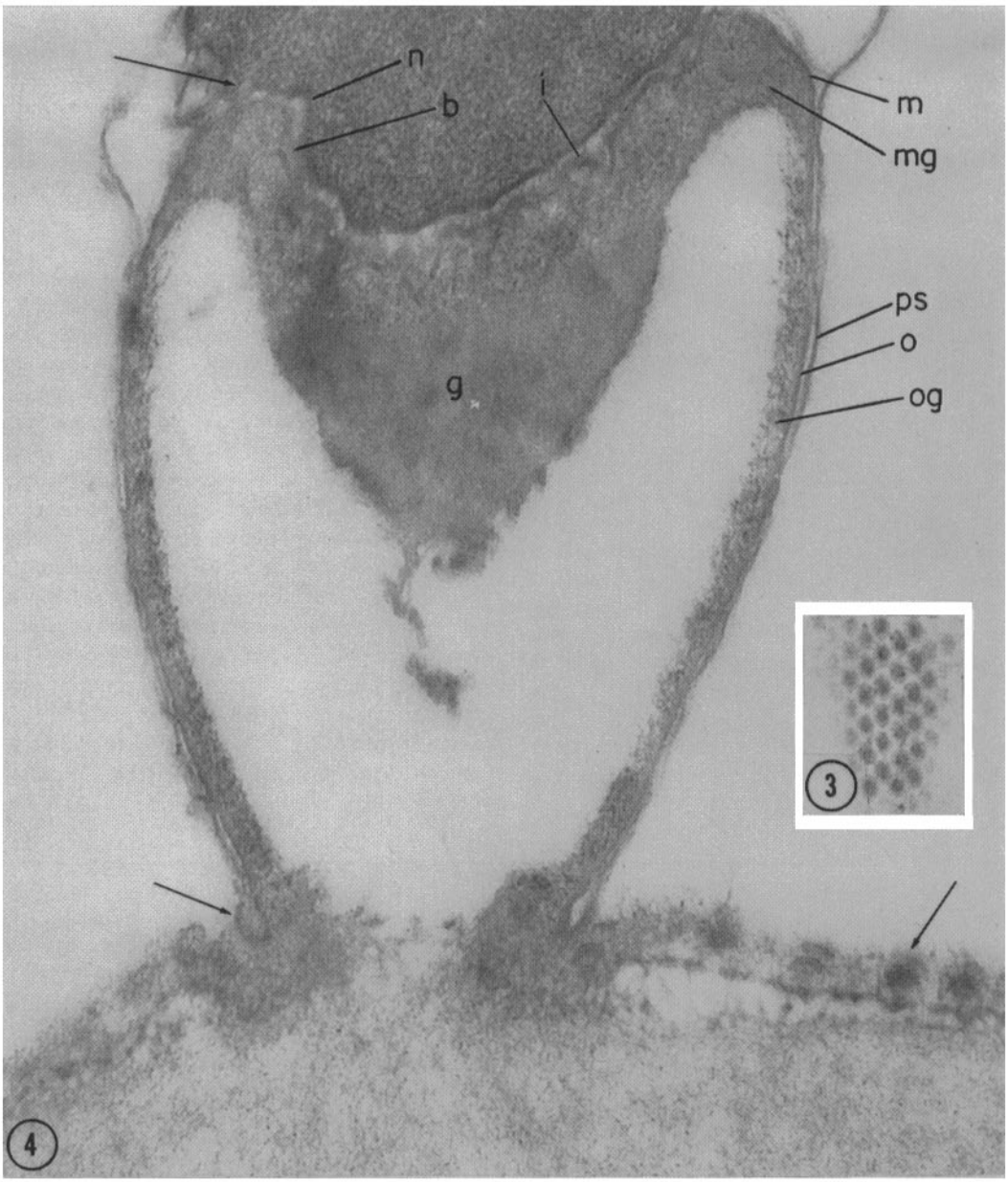
Adjacent serial sections of acrosomal region. × 109,000.

FIGURE 4

Lower left arrow points to lip of acrosomal orifice; note thickened area marking fusion between sperm plasma membrane and acrosomal membrane. Below lip, note swollen granules and affected underlying particles of OBL of vitelline membrane. Right arrow: normal OBL granule of region unaffected by penetrating spermatozoon; note smaller particles underlying OBL granules in this area. Apex of acrosomal granule appears shredded. Part of intermediate zone of acrosome has turned away from nucleus; in that area plasma membrane bulges loosely. Upper left arrow points to one of several thread-like projections of nuclear envelope (*n*).

FIGURE 5

Tangential section of lip of acrosomal orifice showing fused and swollen OBL granules.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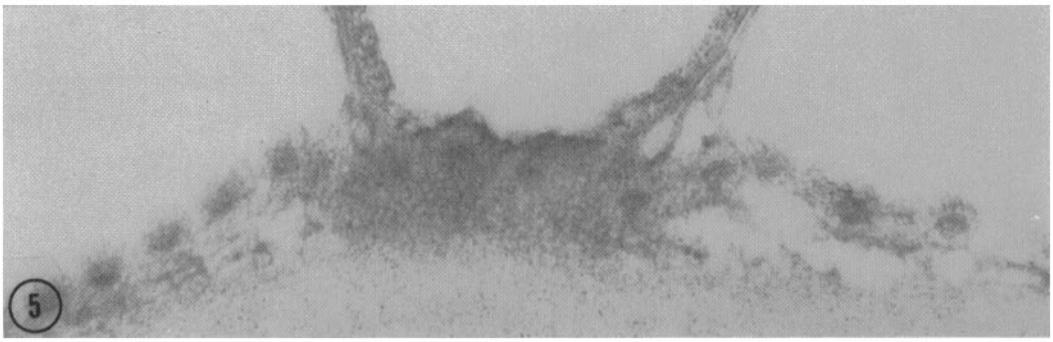
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the reflected granules and the material of the vitelline membrane have become more thoroughly intermingled than in the area closer to the lip. Eventually the entire granular layer of the outer zone of the acrosome becomes everted.

Next, the granular layer of the intermediate zone also becomes completely everted (Figs. 20 to 24, and 26). The acrosomal orifice enlarges greatly. As with the outer zone, the material becomes reflected around the lip of the orifice and gradually mingles with the material of the ML of the vitelline membrane; it is continuous with the earlier streak of intermingled material which trails back to the outer edge of the vitelline membrane.

Some of the micrographs which have been examined suggest inconclusively that in the everting material of both zones the granules are disposed in irregular lines in positions approximately normal to the acrosomal membrane.

Eventually little of the granular material remains to be recognized (Fig. 27). In some sections, however, a small remnant persists even in later stages. It appears as a few small masses of granules and a thin line or sheet lying very close to the membrane (Fig. 31). Remnants of this kind sometimes remain with the acrosomal membrane even when the apex of the sperm head is no longer closely surrounded by material of the vitelline membrane (Fig. 32).

b) Behavior of Plasma and Acrosomal Membranes. The membranous wall of the everting acrosomal region consists of a seemingly continuous sheet which is in fact a mosaic made up of plasma membrane, which is outside, and acrosomal membrane, which is inside; the two are fused at the rim of the acrosomal orifice and their line of junction is no longer apparent. During the process of eversion this wall gradually shortens until it is merely a small bulge encircling the apical end of the sperm head (Figs. 12 to 24 and 26). Next, this slightly bulging membrane folds back beside the sperm head (Figs. 24 left side, 25, and 27). Within the area enclosed by the membrane small vesicles frequently lie at the tip of the inner end of the fold and others lie scattered (Fig. 30). Cross-sections show the new relationships engendered by this folding (Figs. 28 to 30). The fold becomes smaller and often appears to be pressed closely against the sperm head. Finally, by means unknown, it disappears. The site of its disappearance contains the approximate line of junction of the two components of the now mosaic plasma-acrosomal membrane. This site remains identifiable as long as the above mentioned remnant of intermediate zone granular material stays with the membrane (Figs. 31, 32). In the membrane itself, however, no distinguishing mark whatsoever remains to show that *the part of the plasma*

Figure 6

Part of a section through the acrosomal orifice. OBL of vitelline membrane has been breached. Arrow: OBL granule which closely adjoins lip of acrosomal orifice is swollen. × 109,000.

Figures 7 to 9

Relationships in basal region of acrosomal vesicle of spermatozoon at very early stage of attachment. Granular material may be seen both within and between the tubular invaginations (*t*) of the inner zone membrane (*i*). The acrosomal granule is not visible in the lateral, slightly oblique sections shown in Figs. 7 and 8.

Figure 7

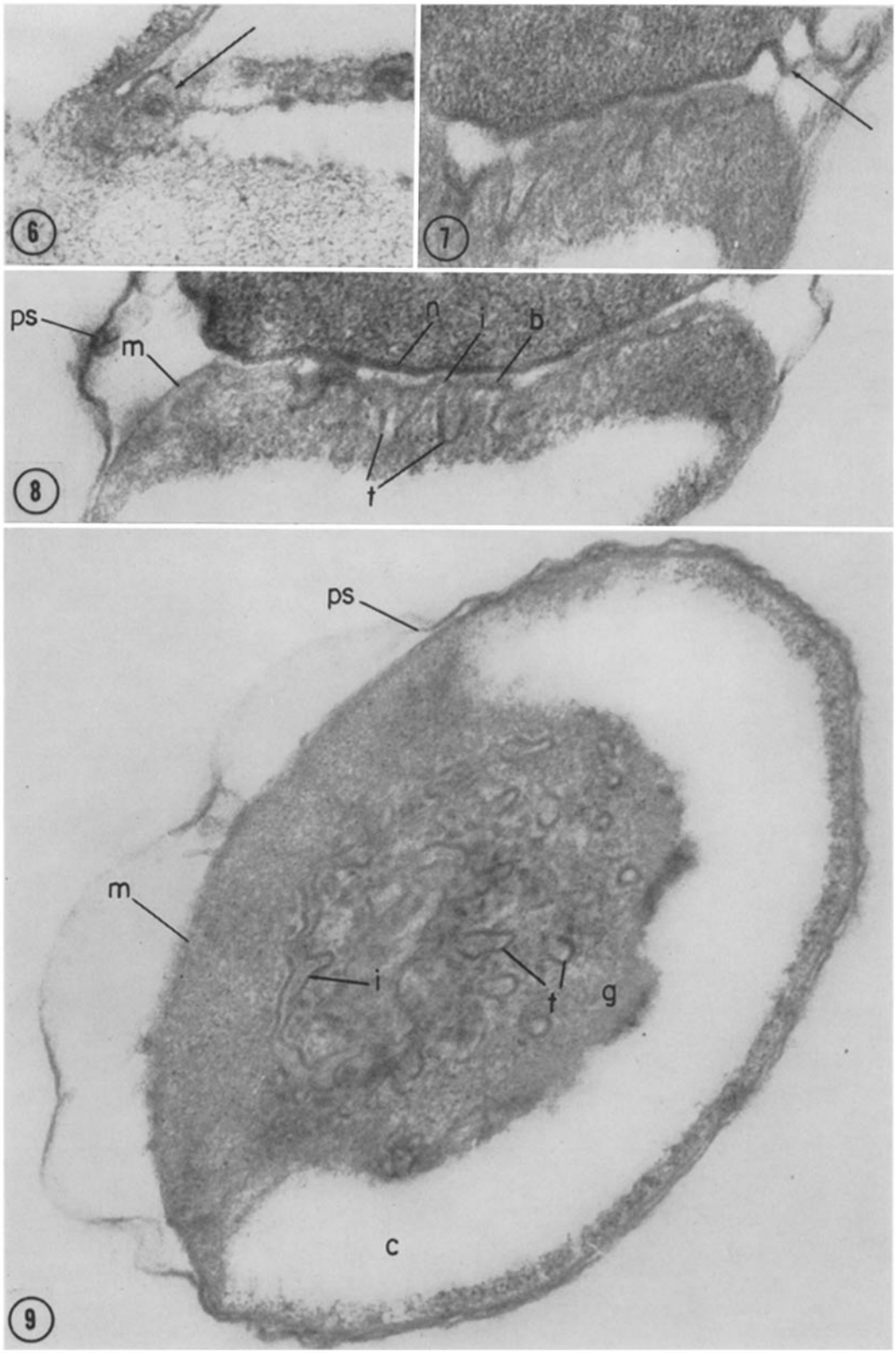
Arrow points to part of nuclear envelope pulled out in area formerly occupied by intermediate zone. × 118,000.

Figure 8

At left, note small vesicles in area inside bulging plasma membrane. × 118,000.

Figure 9

Slightly oblique cross-section. × 109,000.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membrane at the apex of the sperm nucleus is in fact the everted inner zone of the acrosomal membrane.

2. *Disappearance of Acrosomal Granule:* At the earliest stage described above (Fig. 4) the large acrosomal granule is widely separated from its former apical area of attachment with the acrosomal membrane. The granule is often frayed at its apex, or bits of its substance project irregularly from various points on its surface (Fig. 2).

Later the basal end of the granule, too, loses connection with the acrosomal vesicle. The separation begins soon after the sperm head starts moving into the ML of the vitelline membrane (Fig. 14). Often the separation (or dissolution?), which is from the inner zone of the vesicle, begins near the center of the broad flat base of the granule (Fig. 15). While the granule may have some slight attachment to the granular layer of the intermediate zone, it is only in the inner zone that its base ever comes into contact with the acrosomal membrane. In this zone the invaginated tubules of the membrane lengthen and seem almost to push the granule out toward the ML of the vitelline membrane (Figs. 16 and 17). The separating granule gradually decreases in size (Fig. 20) until only a few small fragments of its material can be seen (Fig. 18). These finally disappear at about the time when the intermediate zone of the acrosome begins to evert (Figs. 21 and 22).

Returning to the specimen shown in Fig. 15, it can be seen that a few separated fragments are free within the acrosomal cavity. Indeed, even in very early stages a few small particles of this same appearance are already scattered against that part of the ML of the vitelline membrane which adjoins the newly opened acrosomal orifice. As the sperm head continues to penetrate, the ML material progressively becomes diffuse and disappears from the area facing the orifice (Figs. 11, 13, 14, 16, 18, and 20), and regularly the scattered particles lie near the site of disappearance.

3. *Enlargement of Tubules of Inner Zone of Acrosomal Membrane:* There are about fifteen of these blind invaginations of the acrosomal membrane. At the earliest stage shown above (Figs. 2, 4, 7 to 9) these tubules still show the relationships which obtain in spermatozoa before fertilization. The tubules, which are approximately round in cross section, are simple invaginations of the inner zone of the acrosomal membrane (Figs. 7 to 9). The surface within a tubule, then, is continuous with what is elsewhere the outer surface of the acrosomal membrane, and the openings of the tubules join the potential space between the acrosome and the apical face of the nuclear envelope.

The tubules begin to lengthen at about the time when the acrosomal granule begins to separate from the inner zone (Figs. 14 and 15). Lengthening continues, and by the time the granule has disappeared, the blind ends of the tubules have reached the level of the orifice of the partially everted acrosome (Figs. 16 to 18 and 20). Eventually, with eversion of the intermediate zone, their blind ends come to be the apical elements of the advancing sperm head (Figs. 22 to 25). Their disposition is well shown in the oblique cross section in Fig. 28. The tubules may be slightly convoluted or branched (Figs. 21, 24, 25, and 27) and slightly distended toward the tip. Finally, as described elsewhere (7), they make contact with the plasma membrane of the egg.

It may be mentioned that finely granular material surrounds these invaginations while they are still short tubules within the original inner zone of the acrosome (Figs. 9 and 10). Most of this material later becomes dissipated into the material of the vitelline membrane. However, a few small masses usually remain close to the inner zone of the acrosomal membrane much as was the case with the remaining granules of the intermediate zone (Fig. 31).

DISCUSSION

1. *Initial Attachment of Spermatozoon to Egg Membrane*

The mechanism of initial attachment of the sperm head to the vitelline membrane cannot be

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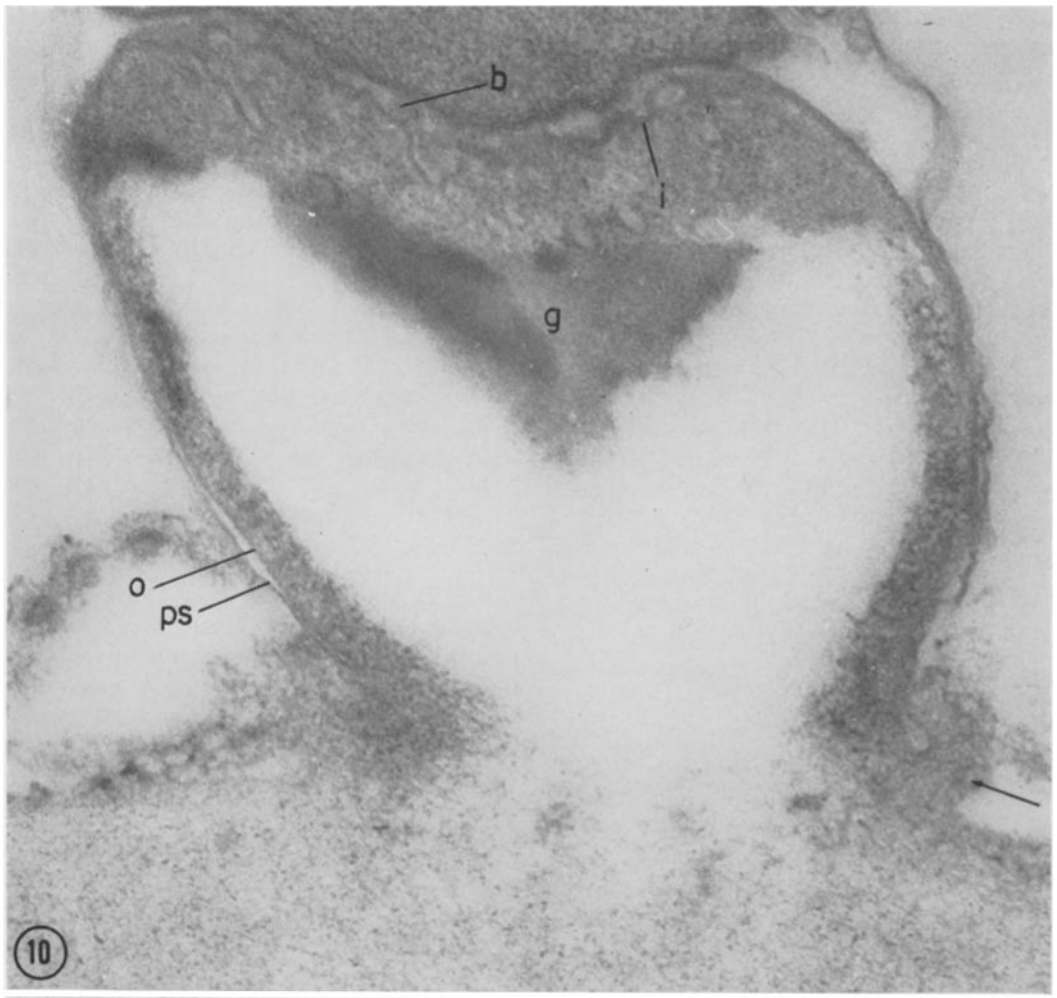
FIGURES 10 AND 11

Serial sections 1 and 5, respectively. Acrosomal region just starting to invade ML of vitelline membrane. Very early stage of eversion of outer zone material. At right, material everted around lip of orifice (arrow) lies *between* ML and OBL of vitelline membrane, but at left, everted material has entered ML. × 118,000.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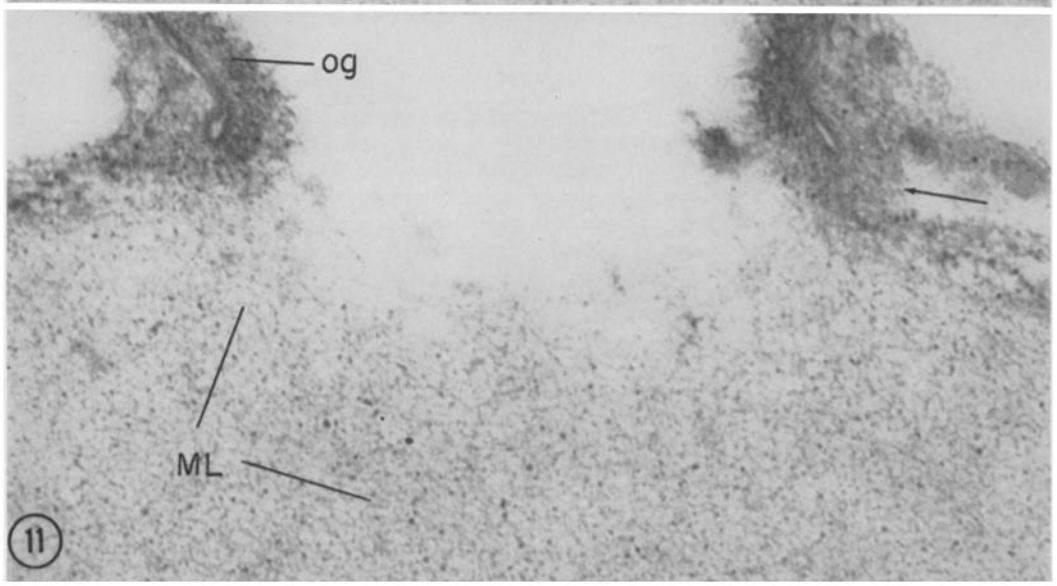
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said to be understood. At the very earliest stage available, the sperm head is already firmly attached by its apex and the acrosomal vesicle possesses an orifice. Formation of the orifice is not unique to sperm entry, since samples of unattached spermatozoa from sea water suspensions, and especially from frozen-thawed preparations, contain specimens in which the acrosome has opened (6). In these specimens, too, the plasma and acrosomal membranes appear to be fused around the orifice. Therefore, this fusion also is not unique to sperm entry; indeed, it has been suggested that the membranes may be fused at this site even before the acrosome opens. Presumably the acrosomal orifice forms by a kind of dehiscence which results in the removal of the lid of the acrosomal region, a lid formed by a sandwich of apical vesicle between plasma and acrosomal membranes (6). The mechanism which actuates the dehiscence of the apex of the acrosomal region is not known. Even though occasionally, as just mentioned, spermatozoa in no proximity to eggs may form these orifices, it seems most unlikely that this formation at fertilization is merely mechanical. One might postulate that there is a specific chemical interaction between the lid of the acrosomal region on the one hand, and the outer surface of the OBL granules on the other hand. Initially this interaction may be in the nature of mutual adsorption between these specific structures of spermatozoon and egg. One is reminded somewhat of the adsorption which is said to occur between virus and host cell (15).

The fate of the apical vesicle is undetermined and its role unknown. Possibly its contents help to breach the OBL of the vitelline membrane. As shown, the very limited portion of this layer nearest the rim of the acrosomal orifice appears to swell and become fused, while from the area within the perimeter of this orifice a few OBL granules seem to be missing. Surprisingly, sperm extracts which rapidly dissolve the broad expanse of the ML of the vitelline membrane in *Hydroides* do not affect the OBL (5). Possibly the apical vesicle carries a substance which does affect the OBL. Studies of preparations fixed earlier than 9 seconds after insemination may clarify this point.

2. *Eversion of Outer and Intermediate Zones of the Acrosomal Vesicle*

The outer and intermediate zones of the vesicle appear to be involved in the transportation of the spermatozoon through the vitelline membrane. The lip of the orifice of the vesicle comes to lie deeper and deeper within this membrane. At the same time the lip itself is made up of constantly changing granular material, as first the material of the outer zone and then that of the intermediate zone everts. One is reminded of the lip of an amphibian blastopore, which during gastrulation maintains its morphological identity as a *lip* but is nevertheless made up of constantly changing

FIGURES 12 AND 13

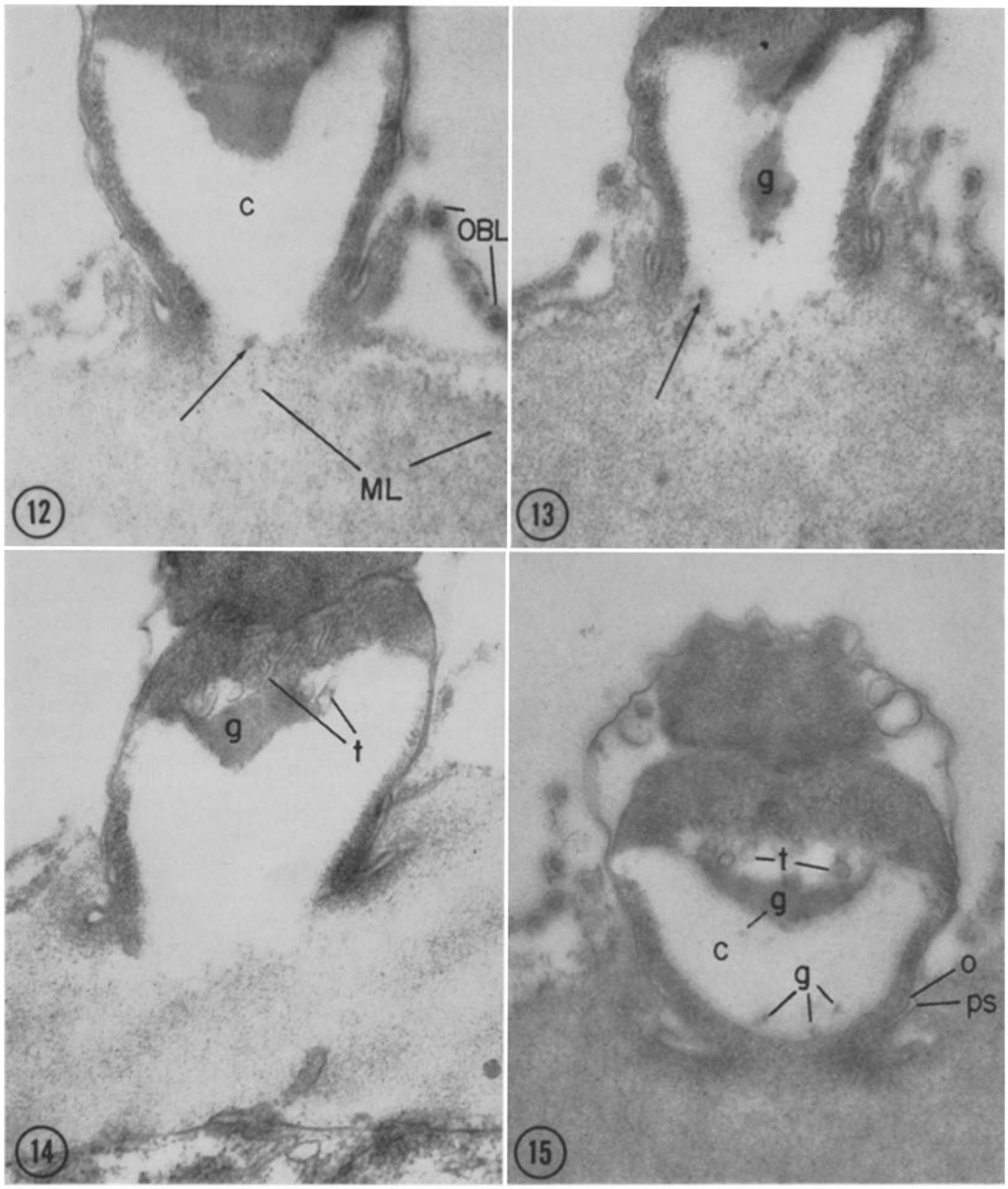
Serial sections 1 and 4, respectively, through the acrosomal region of a specimen during early invasion of the ML of the vitelline membrane. Apex of acrosome lies in crater formed by circular fold of OBL. Material of ML beginning to disappear before orifice of acrosome. Arrow: presumed fragment from acrosomal granule near area where vitelline membrane lies exposed to acrosomal orifice. In Fig. 13 a part of the acrosomal granule (*g*) probably was displaced by the knife during sectioning. × 54,000.

FIGURE 14

Penetrating acrosomal region. Partial separation of base of acrosomal granule from inner zone of acrosomal vesicle. Invaginated tubules of acrosomal membrane have lengthened a little. × 54,000.

FIGURE 15

Tangential section of acrosomal region. Wide separation between acrosomal granule and inner zone of acrosomal vesicle; detached fragments of the granule are free in cavity of vesicle; invaginated inner zone tubules are round in cross-section. × 59,000.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cells, with constantly changing outline and even constantly changing position. It is not known what causes the granular material to evert. Possibly as each portion meets the material of the ML of the vitelline membrane it reacts in some way so as to cause the next contiguous portion also to evert. But whatever the mechanism of eversion may be, it seems almost certain that *eversion of the acrosome is the means by which the apex of the sperm head is drawn through the vitelline membrane.*

3. *Role of the Acrosomal Granule*

The role of egg membrane lysis in fertilization has been discussed elsewhere (5). It has been demonstrated that in *Hydroides* a lytic agent from sperm can completely erode the material of the broad ML of the vitelline membrane while leaving the OBL and IBL apparently unaffected. It is clear, too, that in *Hydroides* lysis is involved in sperm penetration and that the individual spermatozoon is responsible for the lysis of the particular part of the membrane intervening between itself and the egg proper (5, 8, 9).

The acrosomal granule has long been suspected to be the source of the lytic agent, when evidence indicated that such an agent played a role in sperm entry (13). In *Hydroides*, the disintegration and disappearance of the acrosomal granule within a few seconds after attachment of the spermatozoon, simultaneously with the erosion of the ML of the vitelline membrane in its vicinity, seems to support the concept that the granule is the source of the lytic agent. Nevertheless, the possibility cannot be excluded that some electron transparent material contained within the cavity of the vesicle, or some part of the finely granular material which lines the vesicle, may also be sources of or contributors to lytic activity.

There have been numerous biochemical studies to characterize the composition of "the acrosome" (reviewed in 16). In *Hydroides* it is evident that the acrosome consists of a number of morphologically distinct elements (6) which include at least three zonal modifications of the acrosomal periphery, as well as the acrosomal granule. These appear to have different functions and probably are of different chemical composition. Histochemical methods in combination with electron microscopy may be expected to analyze and localize more precisely the chemical substances of the acrosome. The acrosome of *Hydroides* would seem to lend itself well to such a study.

In several sea urchin species studied by Afzelius (1, 2, 3) there was no evidence that the vitelline membrane of the egg had been altered in the vicinity of the ejected acrosomal granule. Afzelius considers these observations not conclusive.

Based on electron micrographs of the starfish acrosome, Dan (12) describes a "top-shaped mass of homogeneously electron dense material . . . which appears in all sections to be lacking a bounding membrane." From its appearance one might suppose that this structure might be an acrosomal granule of the kind just described for *Hydroides*. When the starfish spermatozoon is fixed after being exposed to egg water to induce the acrosome reaction, the whole complex of acrosomal substances and structures, including the above mentioned dense mass, is profoundly altered. Dan concludes that in the starfish there seems to be no role for a lysin in the series of events which she conjectures are involved in fertilization.

In some spermatozoa, for example the human spermatozoon (13), an acrosomal granule may

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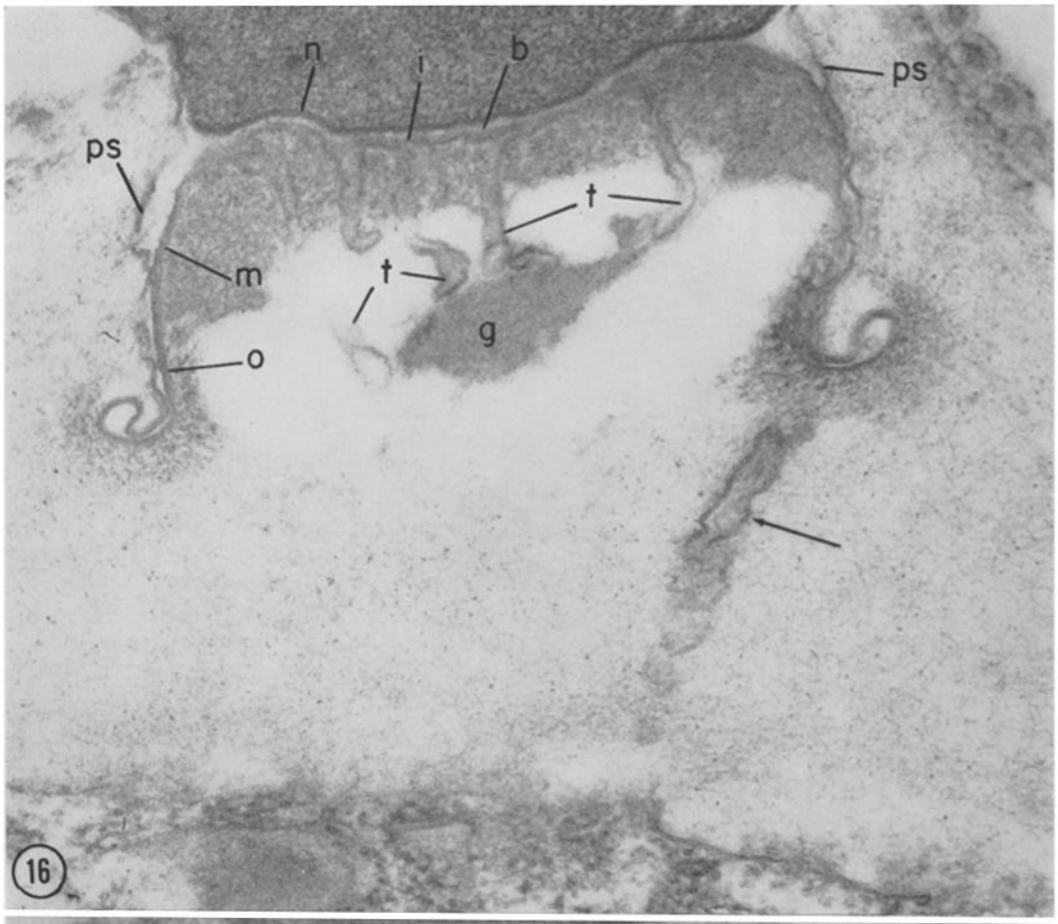
FIGURE 16

Spermatozoon with partly everted acrosome occupies area from which material of vitelline membrane has been lysed away. Note that the everted material merges gradually with the material of the ML. Acrosomal granule, now much reduced in size, is completely detached from inner zone. Invaginated inner zone tubules elongating. Arrow: microvillus of egg. × 92,000.

FIGURE 17

Acrosomal region of a stage later than that shown in Fig. 16. Further eversion of outer zone granules. The acrosomal granule is completely separated from the inner zone and in contact with ML material. × 92,000.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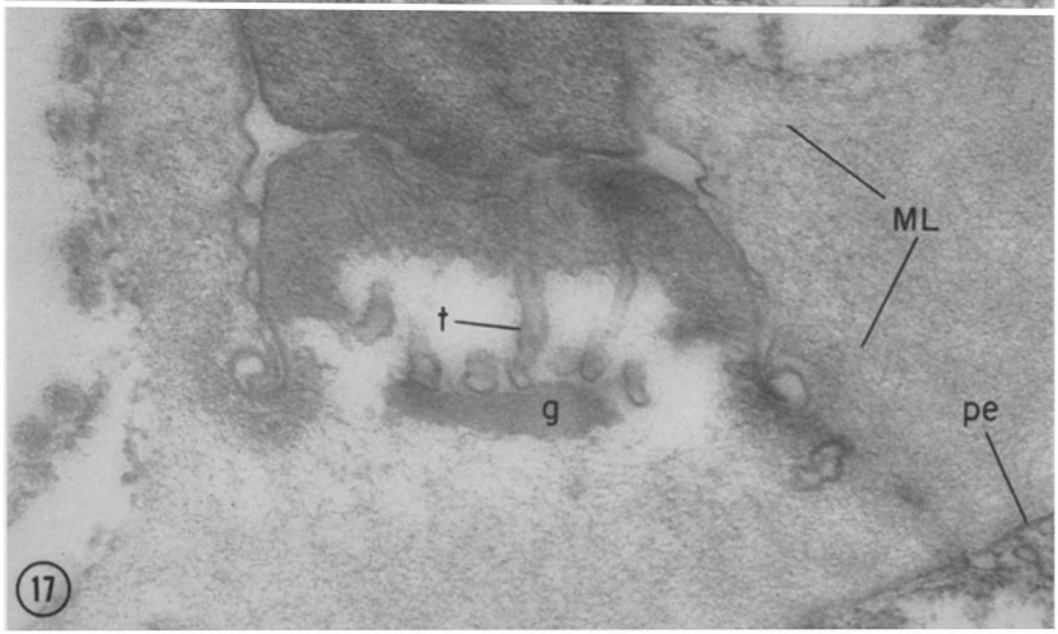
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be recognized during developmental stages but then disappears or at least ceases to be identifiable as such. Whether this signifies that in such a spermatozoon, as perhaps also in the starfish spermatozoon, the presumed lytic activity of the granule has ceased to play a part in sperm entry can, of course, be ascertained only by appropriate studies.

The occurrence of an acrosomal granule in a spermatozoon would lead one to suspect a lytic requirement for some aspect of its passage through the egg barriers.

4. *Acrosomal Tubules*

In *Hydroides* the inner zone membrane of the acrosomal vesicle is invaginated into fifteen or more blind tubules. Shortly after the sperm head attaches to the egg these tubules elongate considerably, and increase in diameter as well. The question arises whether they represent the "acrosome filament" which has been described for quite a number of species (reviewed in 4, 11). There appears to be no difference in the function of the two, namely, to make contact with the egg proper. Structurally, both arise as changes of the acrosome. Both become the most apical part of the penetrating sperm head. On the basis of these similarities it is concluded that the tubules of *Hydroides do* represent the acrosome filaments of other species. But since the structure in *Hydroides* is tubular and not filamentous, hereafter the term *acrosomal tubule* will be used in *Hydroides*. In *Hydroides*, however, instead of referring to *the* acrosomal tubule it is more accurate to refer to the *tuft* of acrosomal tubules.

Earlier evidence (8) had already indicated what the present paper clearly establishes, that the "acrosome filament" of *Hydroides* is tubular. On much scantier evidence the same earlier paper indicated that the acrosome filament of *Saccoglossus*, too, is tubular. In respect to this tubular structure, the "filaments" of these species differ from the only other acrosome filaments thus far reported in studies which have been based on electron microscopy of sections. These are the filaments of several species of sea urchin (1) and of the starfish (12). In both groups the acrosome filament is reported to be a structure which is not tubular but which shows indications of an internal longitudinally disposed fibrillar structure.

The formation of the acrosome filament has been considered to result from two possible alternatives (12): either it is completely formed during spermatogenesis and under appropriate conditions released explosively, like a coelenterate nematocyst, or it is formed rapidly *de novo* from precursor substances, like the ciliate trichocyst. Dan (12) considers that the acrosome filament of starfish "is not preformed . . . but rather formed very rapidly from precursor substances at the time of the acrosome reaction."

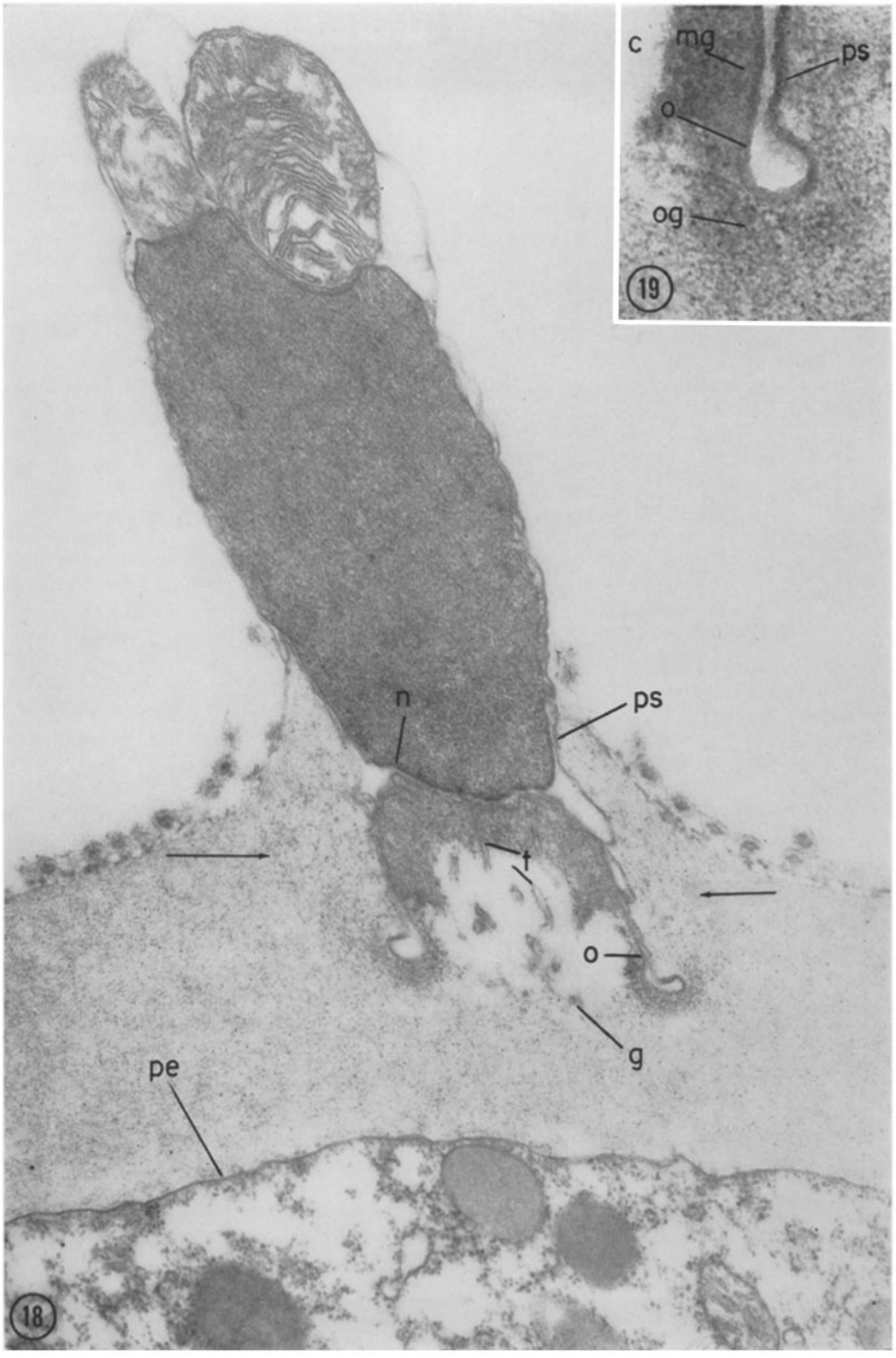
In *Hydroides* the acrosomal tubules are formed in a manner not included in either of the above mentioned alternatives. *The fifteen or more short tubular invaginations of the acrosomal membrane of the inner zone, already present in the unreacted spermatozoon, are the direct structural antecedents of the tuft of much longer acrosomal tubules present in the reacted spermatozoon. These tubules, therefore, neither are formed de novo from amorphous acrosomal substances like a trichocyst, nor exist preformed like a nematocyst. They form as*

FIGURE 18

Spermatozoon penetrating the vitelline membrane. Acrosomal granule, as such, has disappeared, but a few fragments remain at junction of vitelline membrane with orifice of acrosome. Continued eversion of outer zone of acrosome. Arrows point to edge of widening streak which trails behind advancing orifice. Streak consists of material of vitelline membrane intermingled with previously everted outer zone granules. × 74,000.

FIGURE 19

Enlarged detail of right side of acrosomal orifice shown in Fig. 20. Plasma and acrosomal membranes are continuous but line of fusion of the two original parts of this membrane is not distinguishable. Outer zone material mingles imperceptibly with material of vitelline membrane. × 139,000.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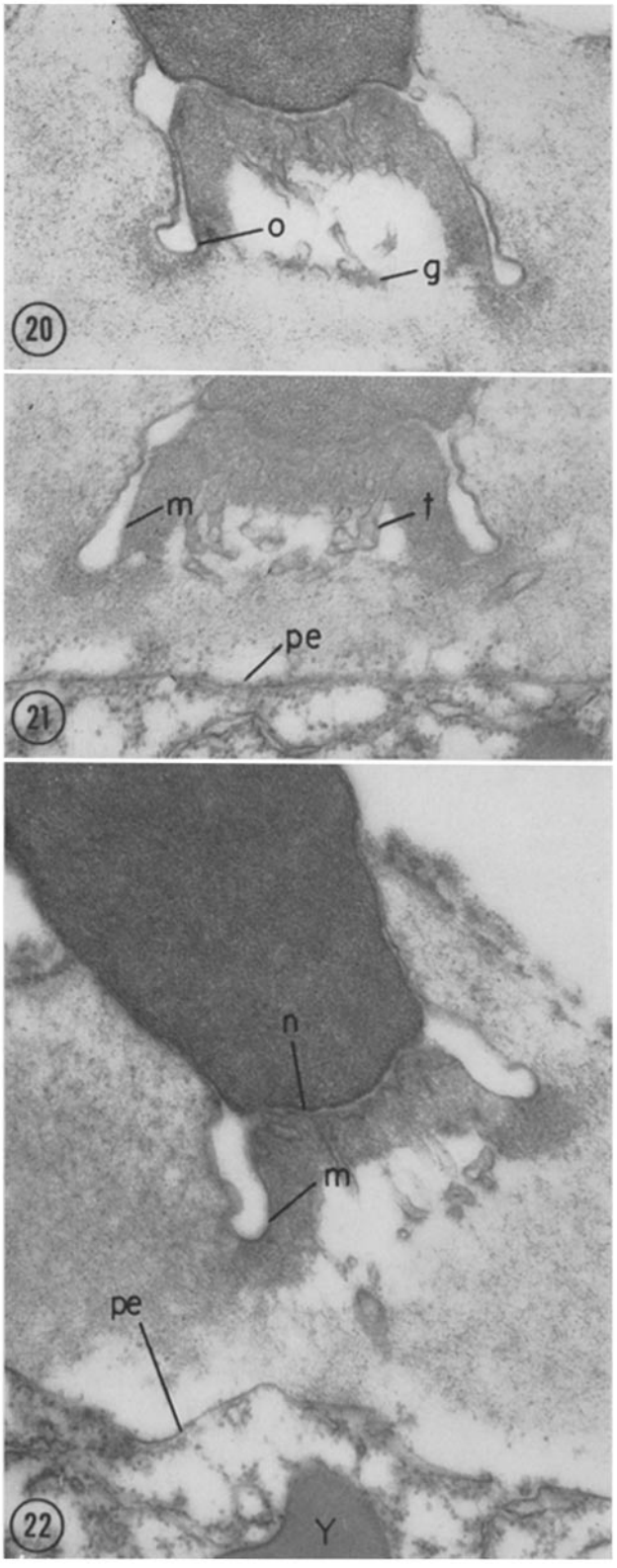

FIGURES 20, 21, AND 22

Successive stages of widening of acrosomal orifice as everison of outer zone is completed and eversion of intermediate zone begins.

FIGURE 20

Very late stage of eversion of outer zone. Small remnants of acrosomal granule (*g*) still visible at junction of acrosomal orifice with material of ML of vitelline membrane. × 54,000.

FIGURE 21

Eversion of the outer zone is nearly completed. The acrosomal orifice is wider than previously. The acrosomal granule has disappeared. Some of the acrosomal tubules of the inner zone are a little convoluted. × 54,000.

FIGURE 22

The intermediate zone has started to evert. The acrosomal orifice is larger than in the previous stage. This unusual specimen was progressing through the vitelline membrane along a somewhat unusual non-radial path. Everting intermediate zone granules imperceptibly mingle with previously everted outer zone granules, and both sets of granules mingle with vitelline membrane material in widening streak that trails from advancing orifice of acrosome to periphery of vitelline membrane. Sperm plasma membrane and acrosomal membrane appear as a continuum. × 51,000.

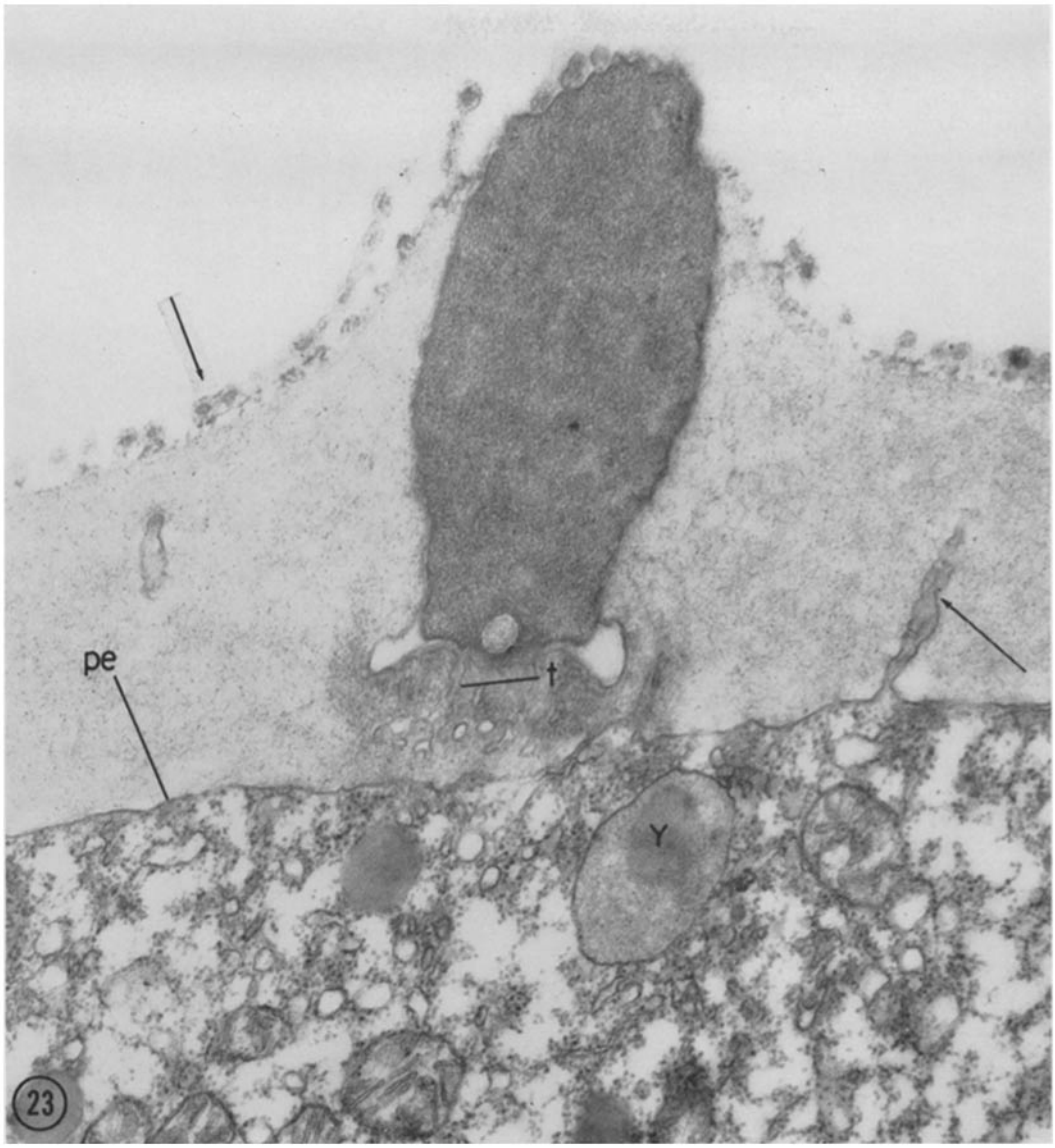

FIGURE 23

Late stage of eversion of intermediate zone of acrosome. Spermatozoon has traversed much of vitelline membrane. Trailing diffuse areas stretch from periphery of vitelline membrane to area where granular material is everting. Beneath arrow at left, granules and particles of OBL of vitelline membrane show typical unaffected appearance of these elements in areas somewhat removed from site of sperm penetration. Arrow at right points to proximal part of microvillus of egg. (Hole in Formvar causes artificial circle within apex of sperm nucleus.) × 54,000.

elongations (not uncoilings) of already existing structures. It appears, then, that the acrosome filament of the starfish and the acrosomal tubules of *Hydroides* are formed in essentially different ways. Whether these differences reflect fundamental differences in the manner of sperm association and egg penetration in these species, or whether further studies on these and other species will show that the differences are only apparent, remains to be seen.

In the attached spermatozoon the mechanism by which the tubules become enlarged is unknown. There appear to be granular constituents within the tubular invaginations of the unreacted spermatozoon. If this material consisted of polymerized molecules, then its breakdown to smaller units would increase the osmotic pressure within the tubules. But the tubules lengthen more than they increase in diameter. Moreover, their openings seem to permit communication with the material (sperm cytoplasm) which lies in the area between the acrosome and the nucleus. The solution of the entire problem of tubule enlargement must await further investigation.

CONCLUSION

Whatever the exact role of each component may be, it is clear that in *Hydroides* the acrosomal region provides the machinery necessary to deliver the nucleus to the egg proper.

This investigation was supported by Research Grant RG 4948 from the National Institutes of Health, United States Public Health Service.

We are very grateful to Mr. Lawrence Melia for his technical assistance during these studies.

This is one of a series of three papers the substance of which was presented as a lecture at the meetings of the International Institute of Embryology held at Pallanza, Italy, in September, 1960.

Received for publication, September 12, 1960.

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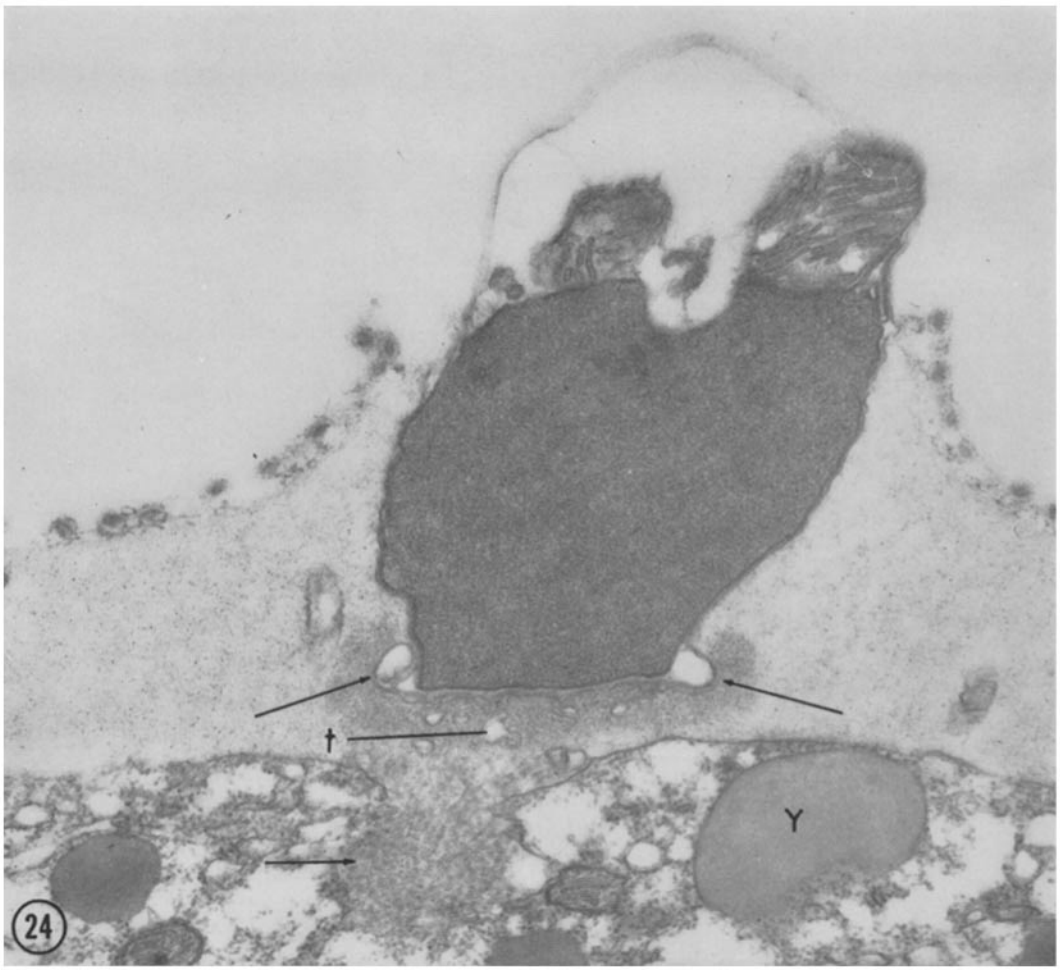
FIGURE 24

Spermatozoon with completely everted outer and intermediate zone of acrosome. Branching invaginated tubule of acrosomal membrane (*t*) is apical element as spermatozoon nears egg plasma membrane. Coincidentally, a cortical granule (lower left arrow) of egg is empyting into perivitelline space in this vicinity. At right arrow, plasma-acrosomal membrane has shortened to mere bulge (shown in enlarged view in Fig. 26). At upper left arrow, plasma-acrosomal membrane has begun to fold back beside nucleus. × 54,000.

FIGURE 25

Apical region of spermatozoon in slightly later stage and at higher magnification than that shown in Fig. 24. Note further folding of plasma-acrosomal membrane. At right, fold clearly shows continuity of acrosomal with plasma membrane. Elongated branching invaginated tubules (*t*) of acrosomal membrane lie among inner zone granular material, which is now mingling with vitelline membrane material. Right arrow: apparent small vesicles within a tubule. Left arrow: small vesicle at inner end of fold in membrane. Line *A* lies in approximate plane of section shown in Fig. 28; line *B* in that of Fig. 29; line *C* in that of Fig. 30. × 118,000.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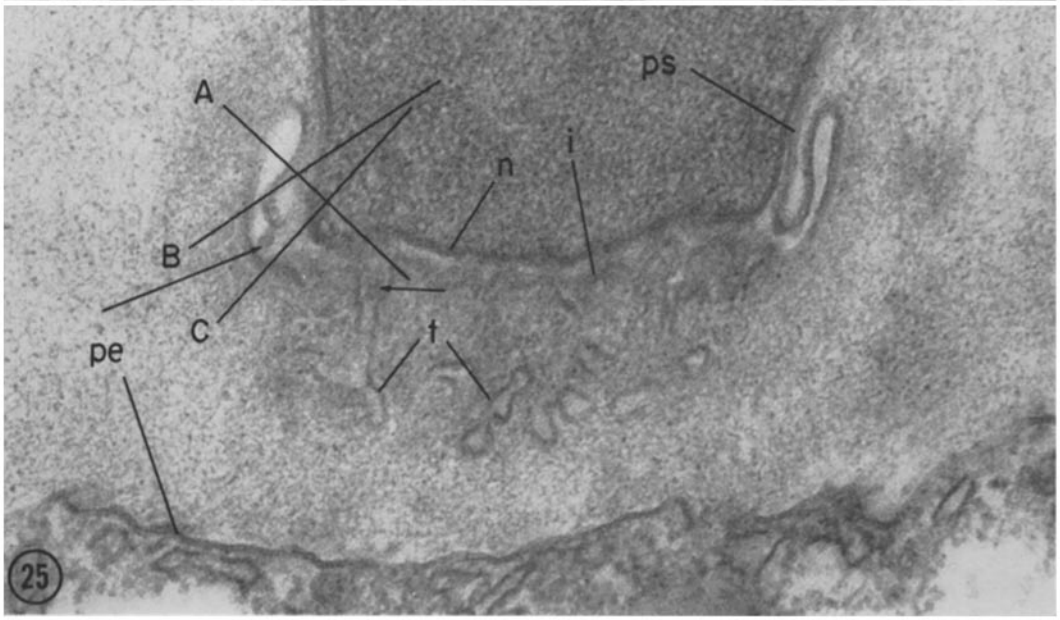
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FIGURE 26

Enlarged view of right side of apical region shown in Fig. 24. Much shortened but not yet folded plasma-acrosomal membrane. Right arrow: part of intermediate zone granular material which persists as acrosomal remnant in later stages. Left arrow: note that acrosomal membrane runs continuously from bulged area to invaginated tubule. × 127,000.

FIGURE 27

Left half of nearly mid-section of apical region. Shows folded plasma-acrosomal membrane. Granular material of inner zone surrounds branching invaginated tubules (*t*). Only a small remnant of everted intermediate granular material is still visible. Arrow: presumed vesicle of type often found at inner end of fold. × 139,000.

FIGURE 28

Section cut through approximate plane indicated by line *A* in Fig. 25. Invaginated tubules of acrosomal membrane form a group or tuft which is most apical element of spermatozoon as it approaches egg plasma membrane. Everted acrosomal material meets ML material of vitelline membrane. Arrow: apical part of fold of plasma-acrosomal membrane. × 54,000.

FIGURES 29 AND 30

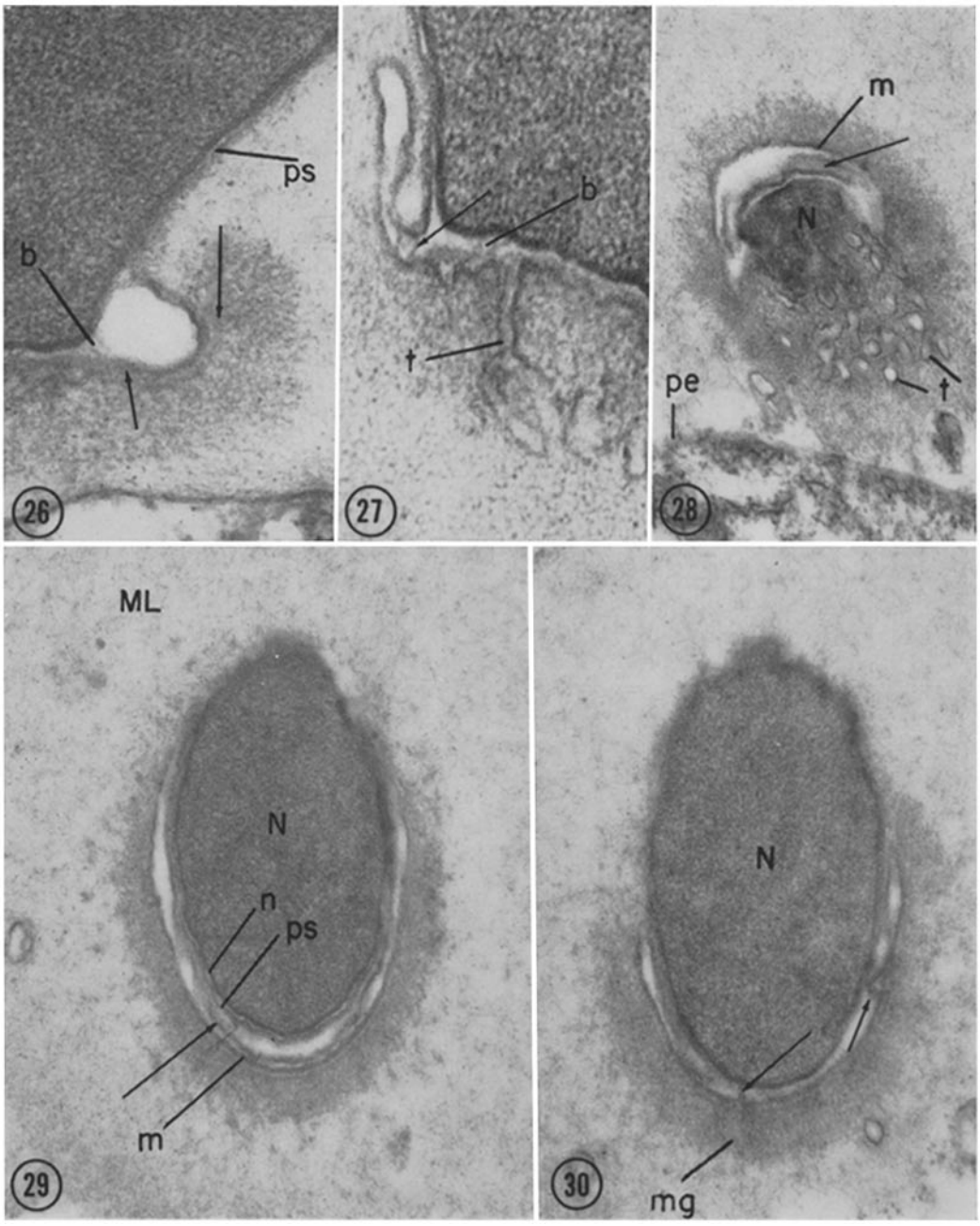
Somewhat oblique cross-sections of spermatozoa in stages close to that shown in Fig. 25. Each spermatozoon is surrounded by material of the ML of the vitelline membrane.

FIGURE 29

Section in plane indicated by line *B* in Fig. 25. Everted acrosomal material lying just outside acrosomal membrane is more sharply defined than that at greater distance from membrane. Arrow: plasma-acrosomal membrane. × 51,000.

FIGURE 30

Section in plane indicated by line *C* in Fig. 25. Arrow at right: small vesicle of type frequently found at inner end of folded plasma-acrosomal membrane. Arrow at left: small vesicle apparently not attached to inner end of a fold. × 54,000.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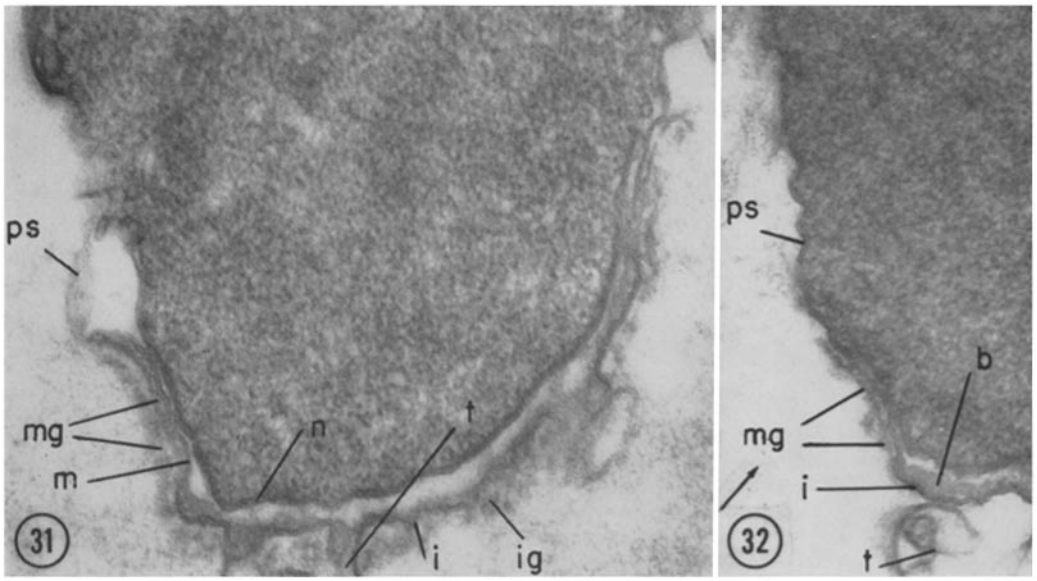

FIGURE 31

Fold has disappeared. Site of junction of acrosomal and plasma membranes now marked only by remnant of intermediate zone granules (*mg*) which lies just outside membrane. A few inner zone granules also persist. Specimen fixed 3 minutes 5 seconds after insemination. × 109,000.

FIGURE 32

Small remnant of everted intermediate zone granular material (*mg*) still lies close to sperm plasma membrane even though this part of sperm head now lies in space (arrow) from which ML of vitelline membrane has been dissolved. This remnant is sole reminder of acrosomal origin of apical part (*i*) of now continuous sperm plasma membrane. Specimen fixed 3 minutes 5 seconds after insemination. × 109,000.

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