ZYGOTE FORMATION IN *ASCARIS LUMBRICOIDES* (NEMATODA)

W. EUGENE FOOR

From the Department of Parasitology, Tulane Medical School, New Orleans, Louisiana 70112

ABSTRACT

Ultrastructural observations of the *in utero* sperm of *Ascaris lumbricoides* reveal that it consists of a relatively clear, ameboid anterior region and a conical posterior region containing numerous surface membrane specializations, dense mitochondria, a lipid-like refringent body of variable size, and a dense nucleus which lacks an apparent nuclear envelope. No acrosomal complex was observed. Pseudopods emanating from the anterior cytoplasm make first contact with the primary oocytes and appear to be responsible for the localized removal of the extraneous coat covering the oolemma. Subsequently the gamete membranes interdigitate and finally fuse. Because this pseudopodial action appears similar to that reported for the acrosomal filaments in flagellated sperm, the anterior region of the *Ascaris* sperm is thought to serve an acrosomal function. Following gamete-membrane fusion, the sperm nucleus acquires a particulate appearance and becomes disorganized. Once inside the oocyte, the sperm cytoplasm consists of dense mitochondria, ribosomes, and vesicles derived from the surface membrane specializations. The refringent body, whose contents possibly contribute to the synthesis of ribosomes, is usually absent by the time the sperm cytoplasm attains a central position in the egg.

INTRODUCTION

Early cytologists depicted the ameboid, nonflagellated sperm of *Ascaris* as having a broad anterior region containing no organelles and an angular posterior region possessing a dense nucleus, numerous mitochondria, and a large, refractile, triangular inclusion called the refringent body. Because certain studies (16) indicated that the refringent body was a product of the Golgi apparatus, it was considered homologous to the acrosome of flagellate sperm by many authors (1, 28, 42, 47). Others have contended that the body consists of a highly specialized protein (ascaridine) (10, 11, 13) which contributes to the synthesis of RNA immediately following its entry into the oocyte (29, 30).

In an ultrastructural study, Favard (12) confirmed earlier (10, 42) observations that the refringent body arises from the coalescence of ascaridine granules and noted that the ascaridine originates from rough endoplasmic reticulum. In addition, he described vesicles adjacent to the sperm surface membrane which he called proacrosomal granules. Recent reports tend to support the assumption that the vesicles are acrosomal (3).

Jamuar (17) has reported membranous elements in *Nippostrongylus* sperm which appear similar to the proacrosomal granules in *Ascaris*. However, he has called them "mitochondrion-like inclusions" since they stain with Janus Green. In a comparable study Lee and Anya (21) failed to observe these mitochondrion-like inclusions in the sperm of *Aspiculuris*.

In view of the questionable presence of an acrosome in nematode sperm, the present study was undertaken in an effort to determine the mode

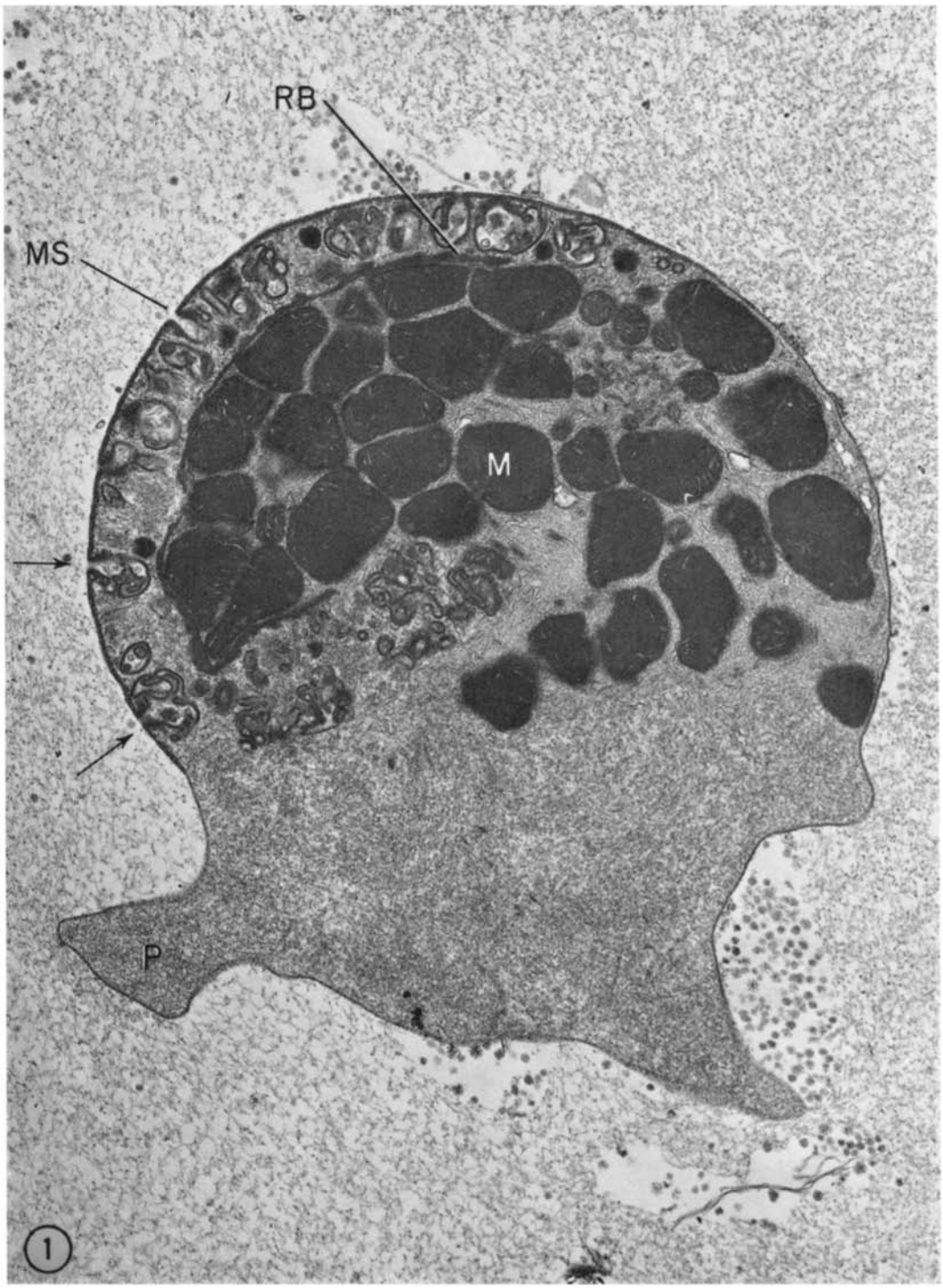

FIGURE 1 Sperm free in the uterus lumen at the level of the oviduct-uterine junction. The broad anterior cytoplasm, modified by the presence of pseudopods (*P*), contains only filamentous elements. The refringent body (*RB*), dense mitochondria (*M*), and plasma membrane specializations (*MS*), with pore-like openings to the exterior (arrows), are confined to the conoid posterior region. × 15,000.

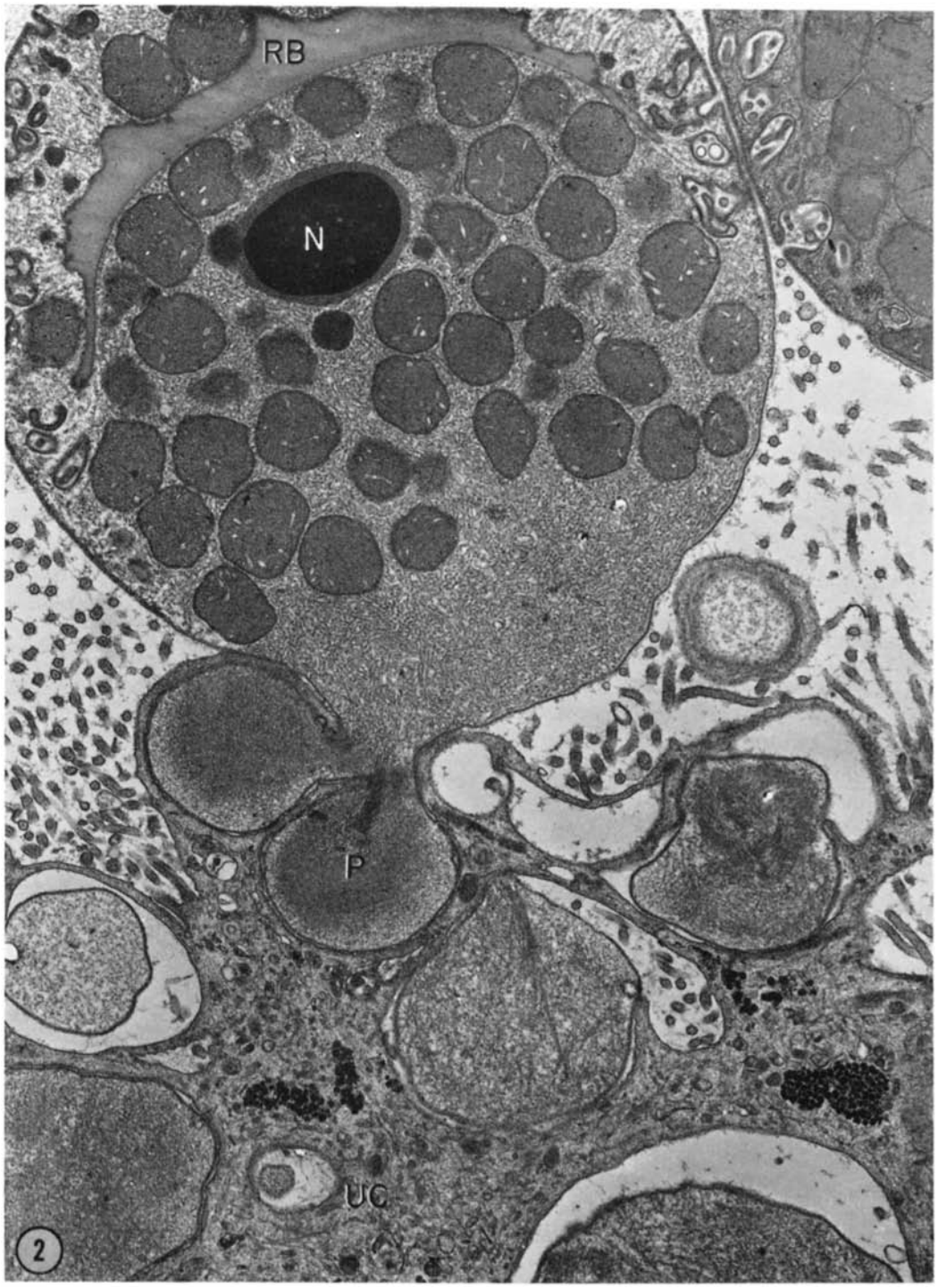

FIGURE 2 Sperm attached to the uterine wall near the oviduct-uterine junction. This micrograph illustrates the profuse interdigitations of the pseudopods (*P*) and the uterine cell (*UC*) plasma membrane. A lipid-like refringent body (*RB*) and dense nucleus (*N*) are apparent in the posterior cytoplasm. × 16,200.

of oocyte penetration in *Ascaris*. Special attention is directed to the appearance of the nucleus and refringent body prior to and following gamete-membrane fusion.

MATERIALS AND METHODS

Adult female *Ascaris lumbricoides* were obtained from patients after treatment with Antepar. The upper portions of the uteri were removed from freshly recovered worms, cut into short lengths, and immersed in cold 3% glutaraldehyde buffered to pH 7.4 with 0.1 M Sorenson's phosphate, for 1½ hr (37). After the initial fixation, the tissues were washed overnight in buffer and subsequently fixed in cold 1% osmium tetroxide for 1 hr. The fixed tissues were dehydrated at 4°C in graded dilutions of ethanol and embedded in Epon 812 (22). Thin sections were cut with a Sorvall MT-2 microtome (Ivan Sorvall Inc., Norwalk, Conn.) and mounted on uncoated or Formvar-coated grids. The mounted sections were stained with uranyl acetate (48) and then with lead citrate (34) and were examined with a Siemens Elmiskop IA.

RESULTS

The distal portion of the uterus is laden with sperm and newly fertilized eggs. The sperm are

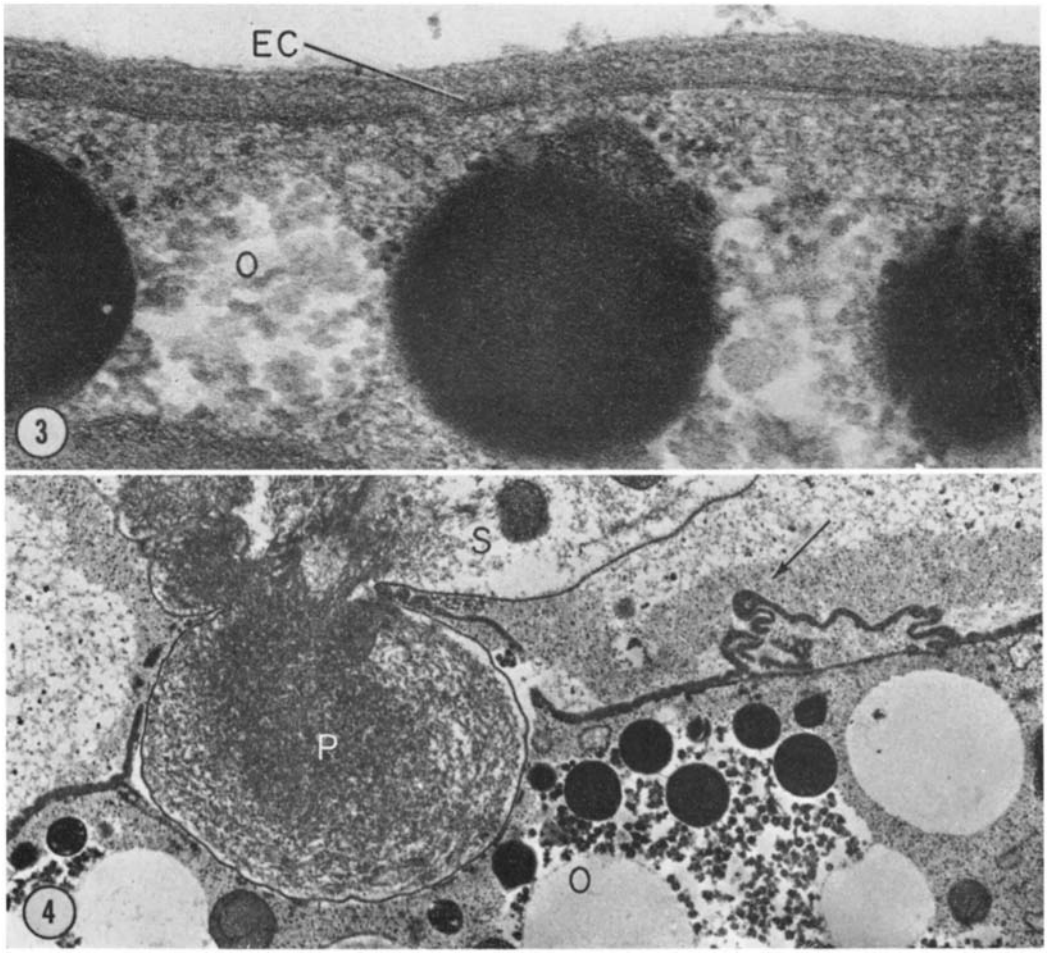

FIGURE 3 High magnification showing the extraneous coat (*EC*) about the primary oocyte (*O*) as the oocyte enters the uterus from the oviduct. × 28,500.

FIGURE 4 Initial contact between sperm (*S*) and oocyte (*O*). Folds (arrow) in the extraneous coat are indicative that it has been pushed aside by physical action of the pseudopod (*P*). × 10,500.

either free in the lumen or aggregated adjacent to the luminal surface of the uterine cells.

Electron microscopic images (Figs. 1 and 2) of the *in utero* spermatozoan are in agreement with earlier reports that it consists of a broad, clear, anterior region and a conical, posterior region containing numerous organelles. The clear anterior cytoplasm contains some fibrillar components, and the cell surface is frequently projected as pseudopodial processes (Figs. 1 and 2). In those sperm adjacent to the uterine wall (Fig. 2), the pseudopodial processes interdigitate with the plasma membrane of the uterine cells and seemingly serve as holdfast structures to maintain the sperm's position in the uterus.

In contrast, the posterior cytoplasm contains

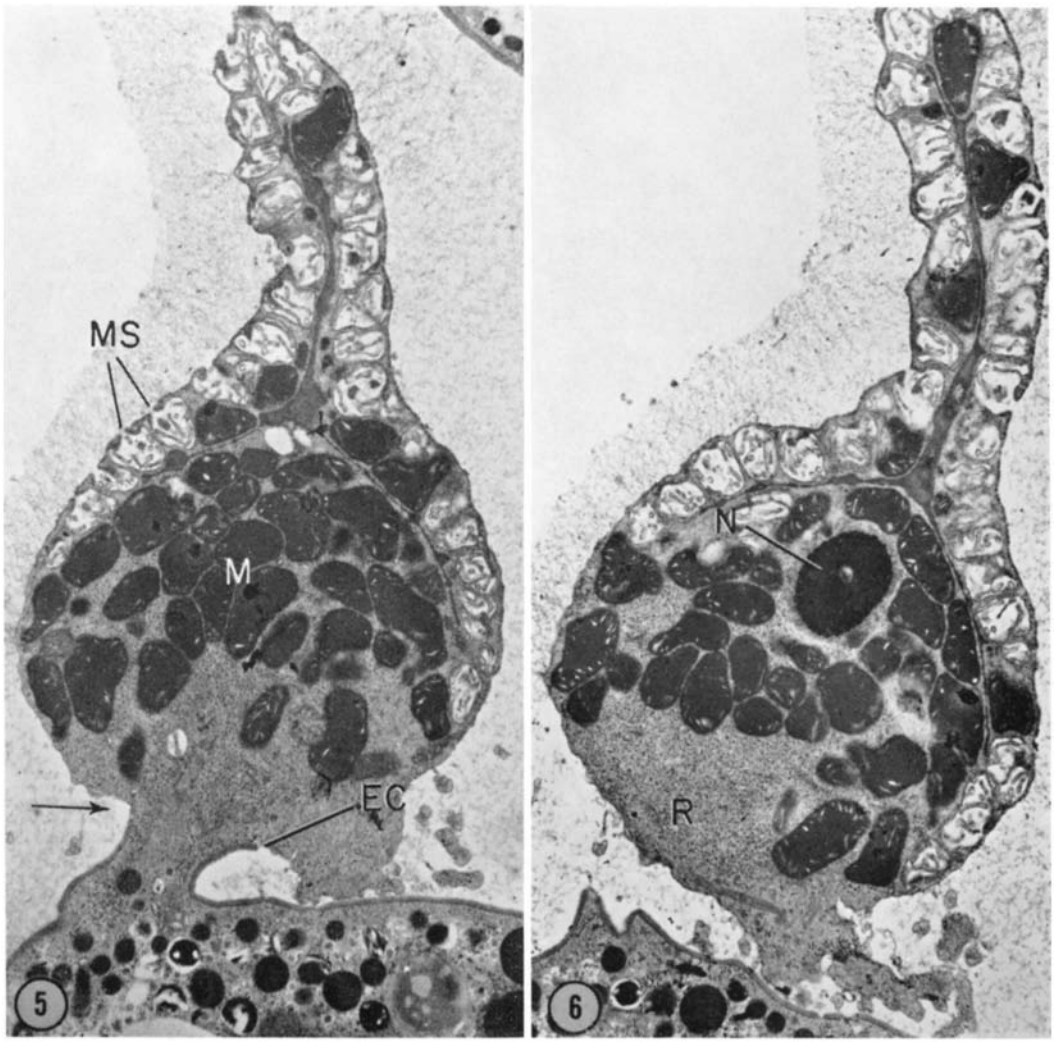

FIGURE 5 Illustrates initial continuity between the uniting gamete cytoplasms. The extraneous coat (*EC*) remains associated with the oolemma at the site of fusion (arrow) of oolemma with the sperm plasma membrane. *MS*, membrane specializations; *M*, mitochondria. × 8,400.

FIGURE 6 Corresponding section of the uniting gametes shown in Fig. 5 illustrating the dense sperm nucleus (*N*) and the beginning formation of ribosomes (*R*) in the sperm cytoplasm. × 8,400.

numerous dense mitochondria, vesicular components, the refringent body, and a dense nucleus which lacks an apparent nuclear envelope (Figs. 1 and 2). The vesicular components (the proacrosomal granules described by Favard) located along the peripheral margin (Fig. 1, *MS*) contain numerous microvillus-like processes and possess a porelike opening to the exterior. The sperm plasma membrane appears continuous with the membrane of the vesicular structures, and the latter are thought to be specializations of the surface membrane.

The primary oocytes, characterized by an extraneous coat 50–100 mμ in thickness (Fig. 3, *EC*), are fertilized shortly after they enter the uterus. Pseudopodial processes, emanating from the clear anterior region of the sperm, apparently make first contact with the oocyte.

From certain images (Fig. 4) depicting this initial contact, one gains the impression that the extraneous coat is easily separated from the oolemma and is merely pushed aside by physical action of the pseudopods. There is no evidence that the coat is lysed by products released from the sperm. As seen in Figs. 4 and 5, the coat remains continuous about the oocytes even in that area immediately adjacent to the sperm plasma membrane.

Once the sperm membrane comes into direct contact with the oolemma, gamete membrane fusion can occur. In certain uniting gametes (Figs. 5 and 6), the sperm membrane fuses with the oolemma almost immediately. In others (Fig. 8), membrane fusion occurs less rapidly, and the pseudopodial processes, often containing aggregates of dense nonfibrillar material (Fig. 8 *DM*), interdigitate with the oolemma. Subsequently, the membrane about the lateral margin of the sperm also fuses with the oolemma, and the interdigitating membranes disappear (Fig. 9). The gamete cytoplasms are now continuous and the sperm contents (the refringent body and surface membrane specializations being last) enter the oocyte (Figs. 9, 11, 12).

In areas where it is continuous with the sperm membrane, the elevations of the oocyte membrane are not regarded as originating from a fertilization cone, since these elevations do not appear prior to actual membrane fusion. Possibly the elevations are due to a gradual expansion of the area where the gamete cytoplasms initially become continuous. Once the sperm cytoplasm has entered the oocyte, the elevations recede but, as in other zygotes, a portion of the sperm membrane is retained at the site of entry (Fig. 11, arrow). This mosaic character of the zygote membrane is apparent for only a short period of time, since formation of the primary envelopes usually begins immediately following sperm penetration (14).

Immediately following gamete - membrane fusion, the dense sperm nucleus acquires a particulate appearance (Fig. 7), and numerous ribosomes

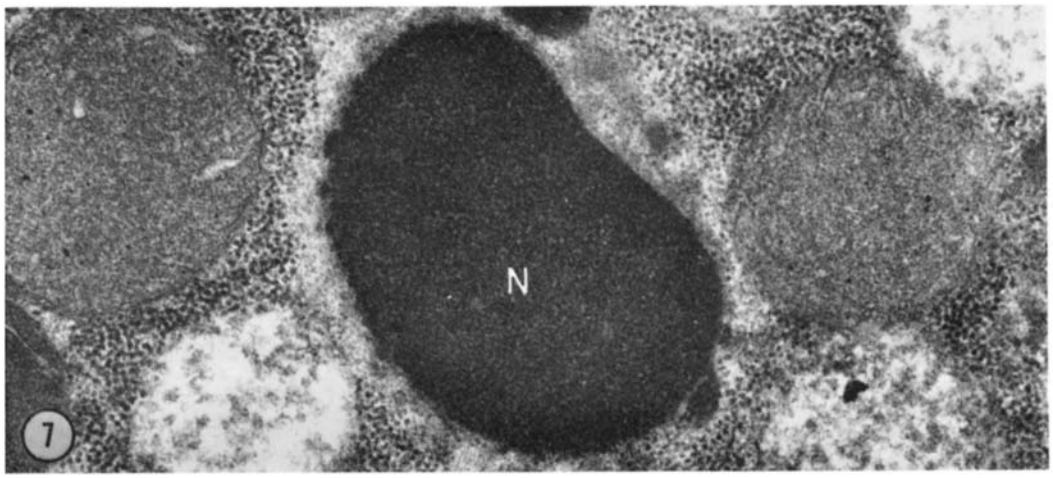

FIGURE 7 High magnification showing the particulate appearance of a sperm nucleus (*N*) corresponding to the nucleus shown in Fig. 6. × 38,500.

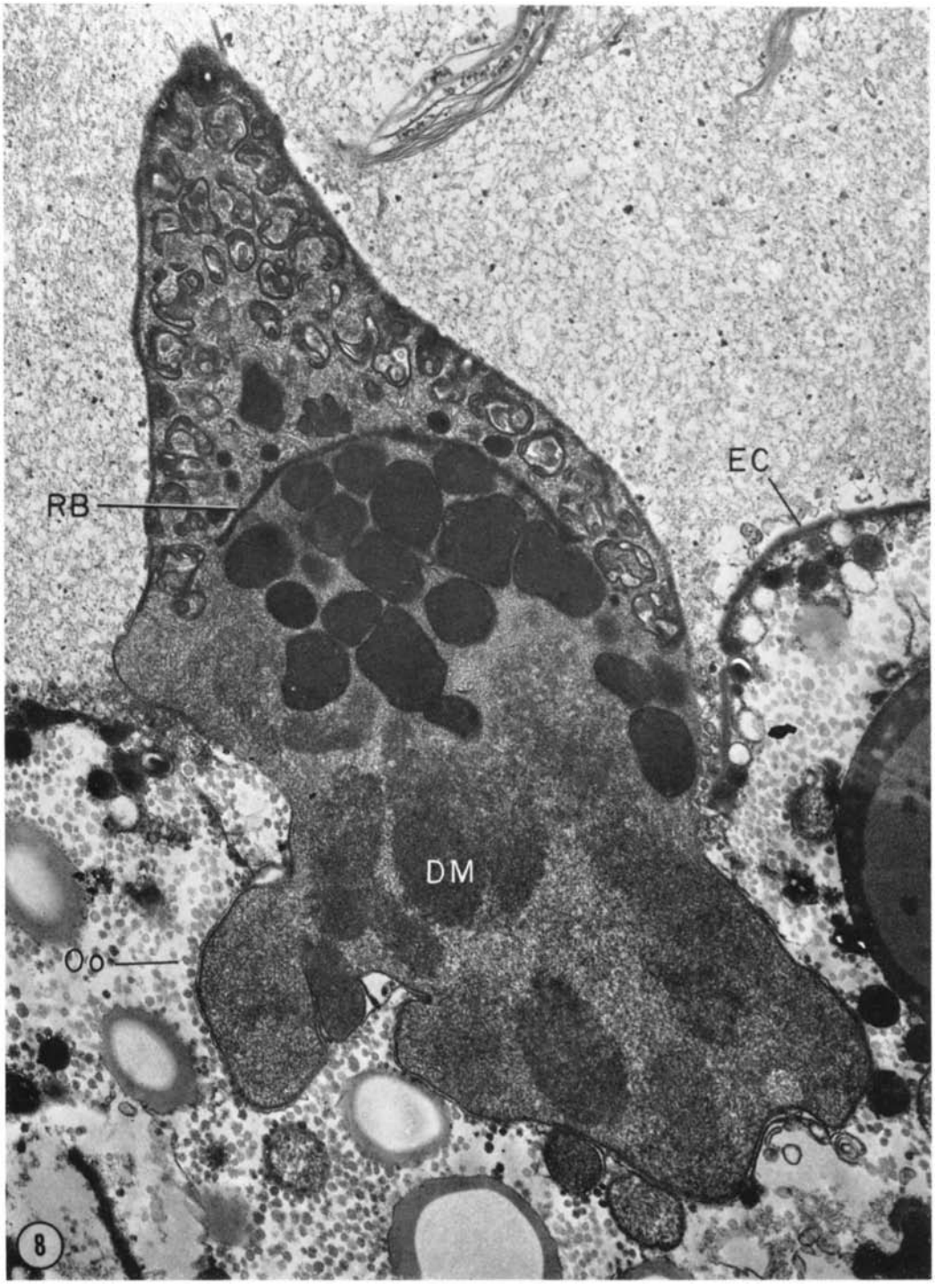

FIGURE 8 Micrograph showing interdigitations between the oocyte oolemma (*Oo*) and the plasma membrane of a penetrating sperm. Aggregations of dense material (*DM*) are apparent in the anterior sperm cytoplasm. *RB*, refringent body; *EC*, extraneous coat. × 15,600.

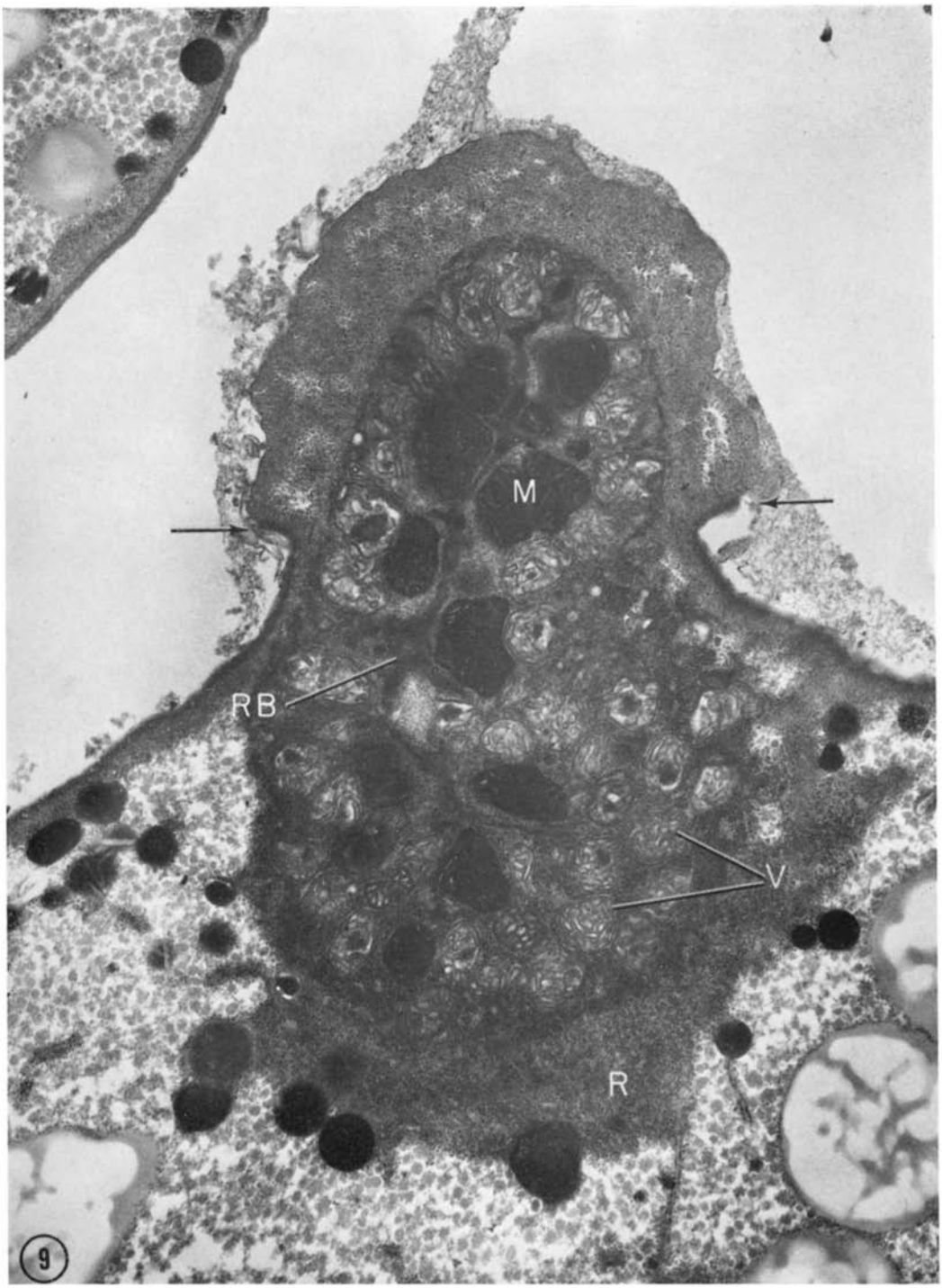

FIGURE 9 Sperm cytoplasm entering the primary oocyte. Fusion of the gamete membranes is seen at arrows. Note the rapid proliferation of ribosomes (*R*) in the newly penetrated sperm. *RB*, refringent body; *M*, mitochondria; *V*, vesicles derived from specializatives of the sperm membrane. × 16,000.

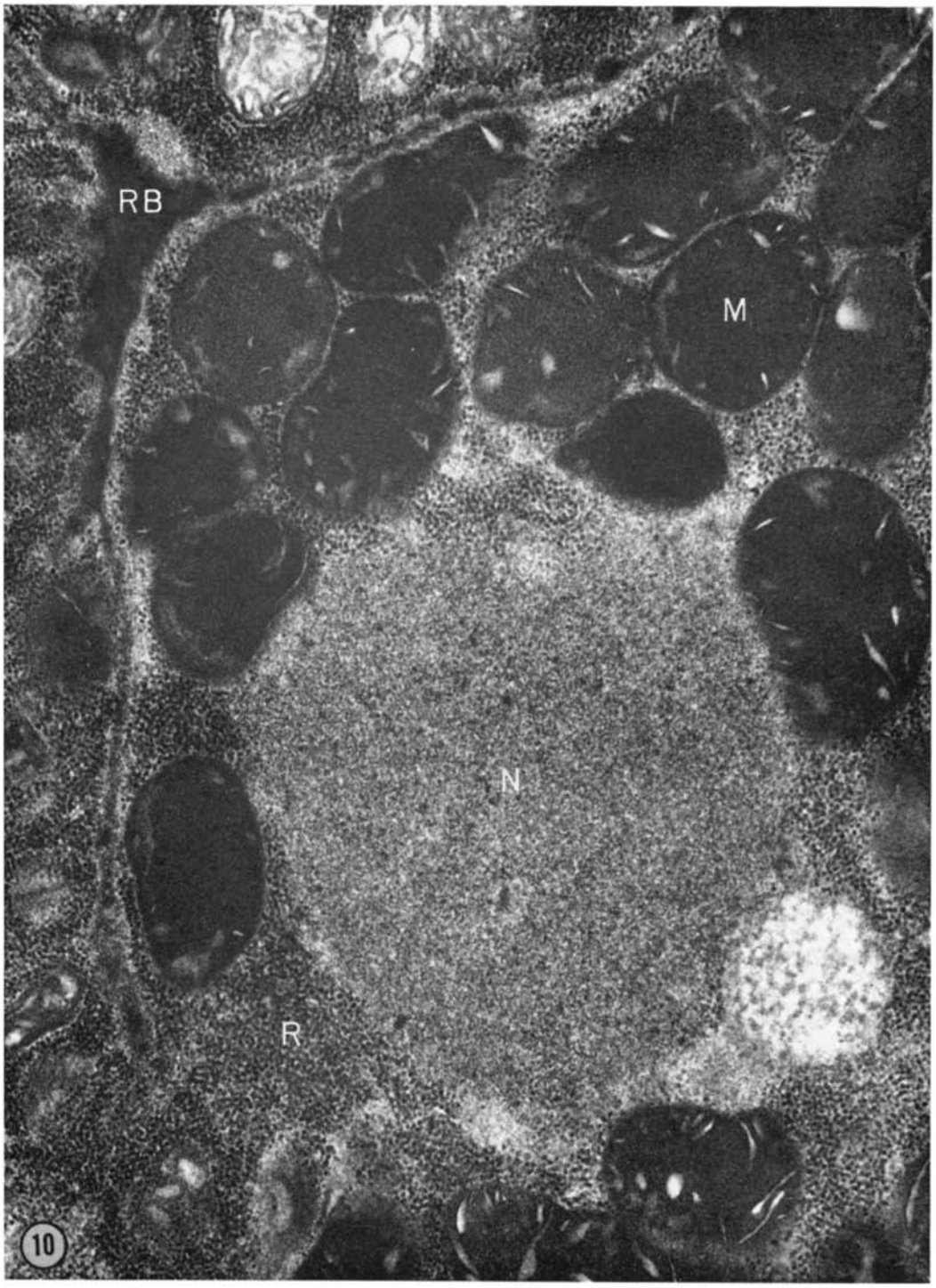

FIGURE 10 Micrograph illustrating the sperm nucleus (*N*) immediately after its entry into the oocyte. Note the decrease in density and the apparent absence of a nuclear envelope. *RB*, refringent body; *M*, mitochondria; *R*, ribosomes. × 35,750.

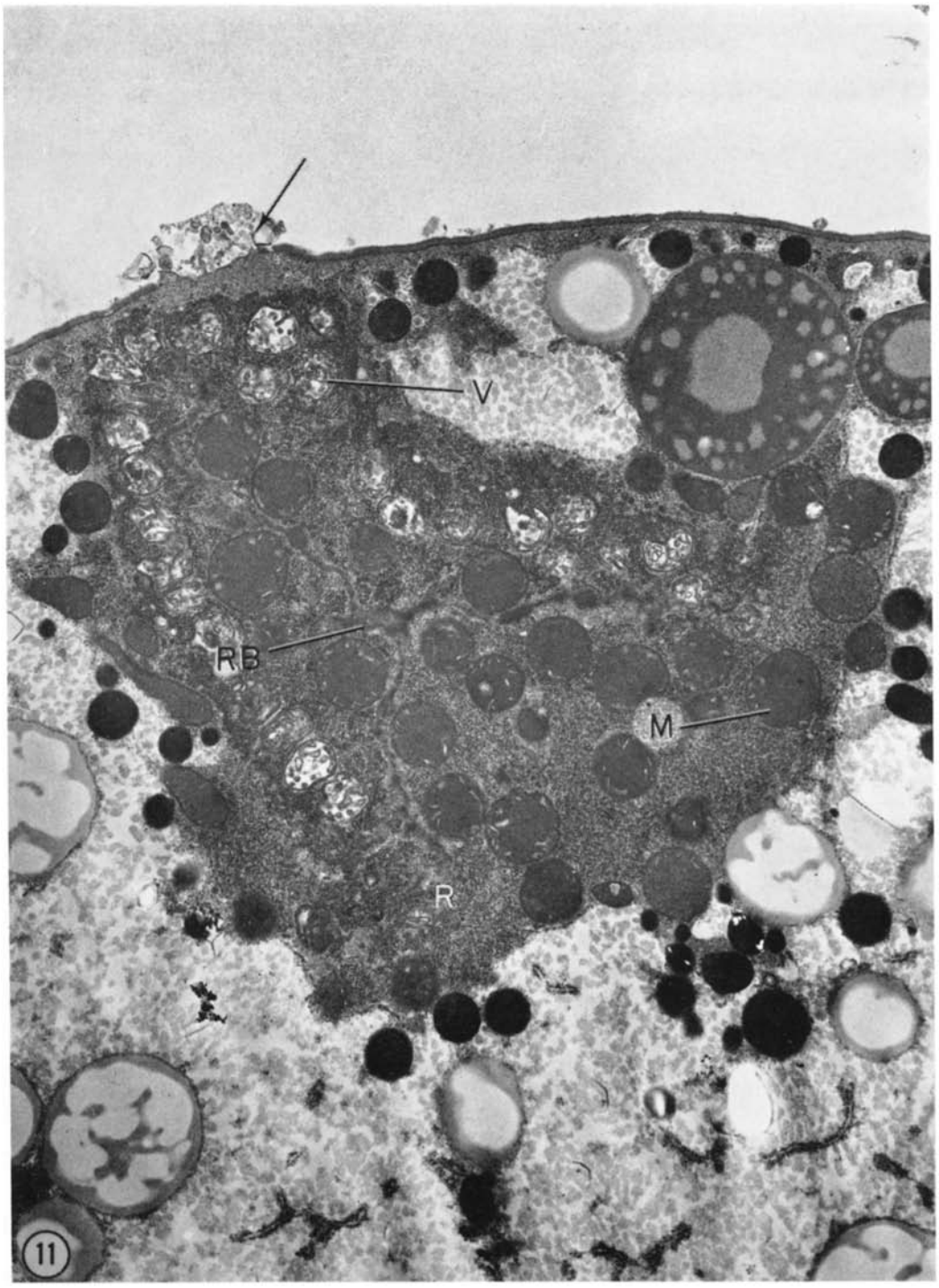

FIGURE 11 Complete fusion of the sperm and oocyte cytoplasms. The refringent body (*RB*), mitochondria (*M*), and vesicles (*V*) are still apparent in the sperm cytoplasm. The sperm nucleus is no longer present. Note the mosaic character of the egg membrane (arrow) at the site of sperm entry. *R*, ribosomes. × 12,900.

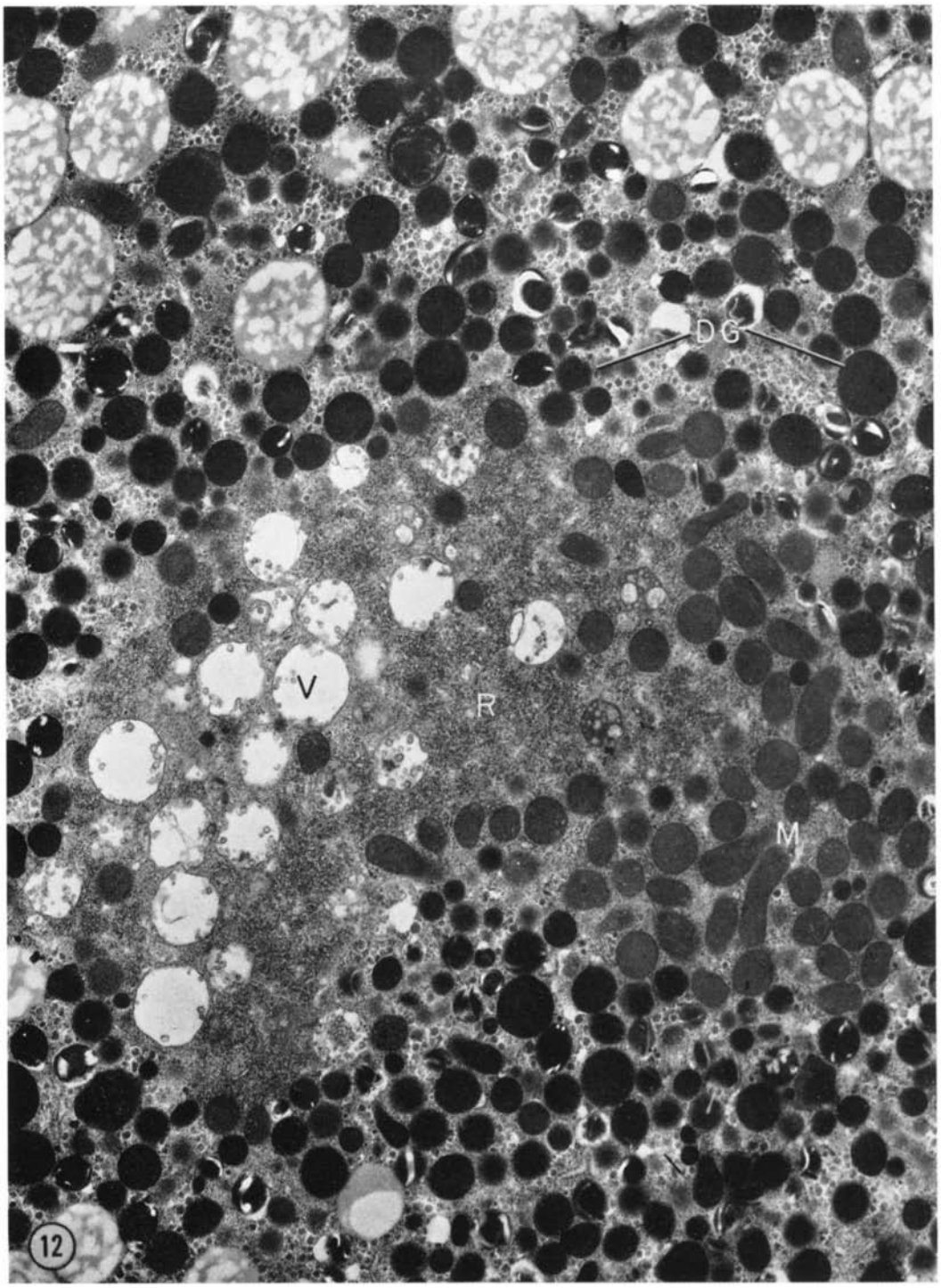

FIGURE 12 The sperm cytoplasm after it migrates to the center of the egg. The refringent body has disappeared. The vesicles (*V*), ribosomes (*R*), and mitochondria (*M*) are surrounded by accumulations of dense granules (*DG*). × 13,000.

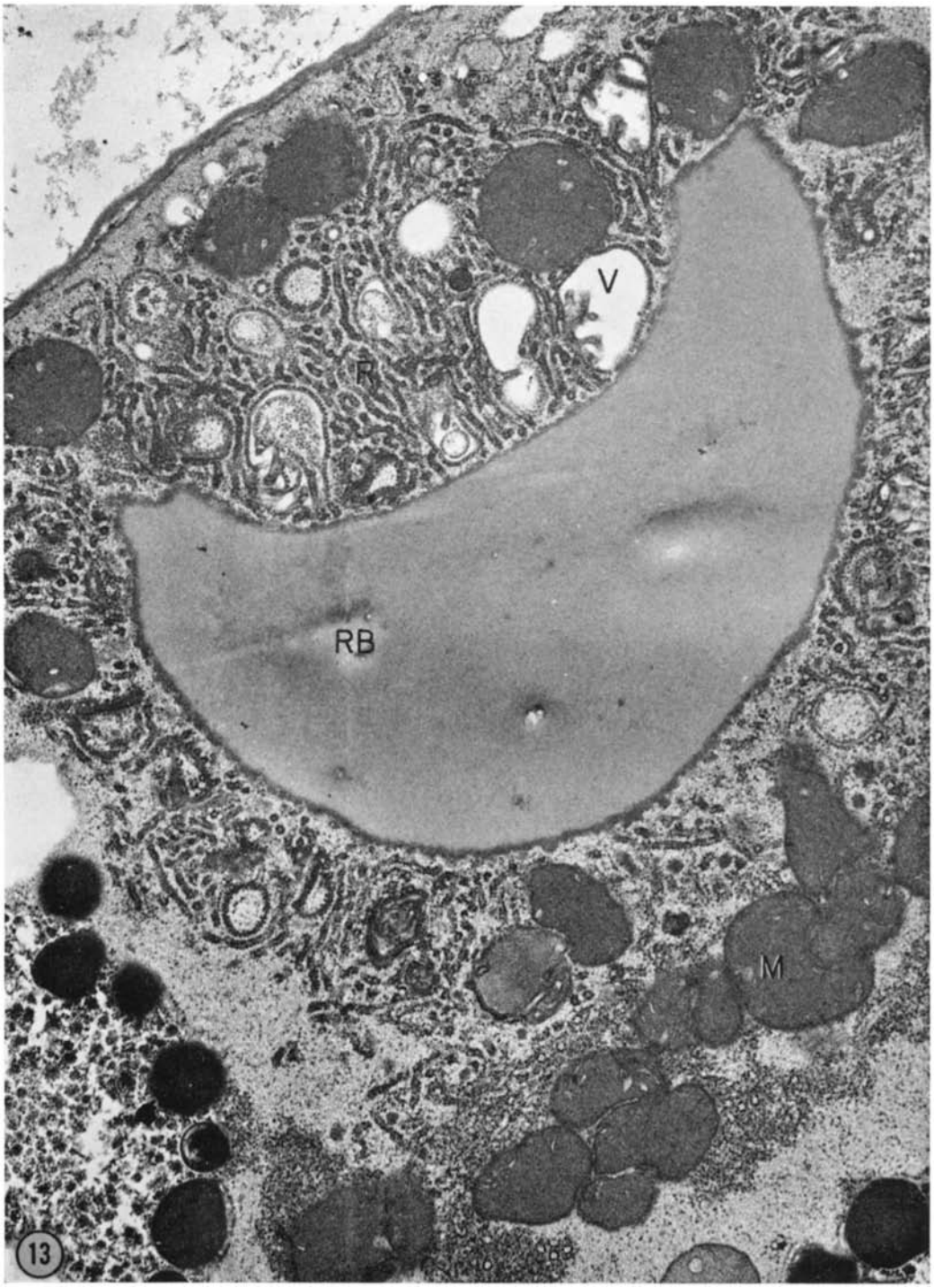

FIGURE 13 Newly penetrated sperm with linear strands of ribosomes (*R*) radiating from a large, lipid-like refringent body (*RB*). *V*, vesicles; *M*, mitochondria. × 17,000.

are observed in the area of the sperm cytoplasm (Figs. 9 and 11). During further penetration into the egg, the sperm nucleus gradually decreases in density and undergoes dissolution (Fig. 10), which is complete by the time the sperm cytoplasm attains a central position in the egg (Fig. 12). Whether this apparent change in the sperm nucleus is associated with the sudden appearance of ribosomes is unknown.

After entry into the oocyte, the sperm cytoplasm is characterized by large dense mitochondria, ribosomes, vesicles derived from the surface membrane specializations, and the refringent body (Fig. 11). Although the refringent body gradually disappears, the remaining components migrate to the center of the egg where they are surrounded by an aggregation of dense granules (Fig. 12). Subsequently, the vesicles disappear, but the mitochondria (much reduced in size) and many of the ribosomal elements are dispersed in the egg cytoplasm. The reappearance of a well-defined sperm nucleus was not observed in this study.

Although most of the sperm penetrating mature primary oocytes had a comparatively small refringent body, it was observed that sperm having a large (4–5 μ), lipid-like, refringent body are also capable of penetration (Fig. 13). There is no major difference in the appearance of the nucleus, vesicles, and mitochondria in the newly penetrated sperm having a large refringent body; however, the ribosomes are initially less numerous. In addition, the ribosomes are not randomly distributed but appear as linear strands radiating from the refringent body. As the sperm cytoplasm migrates to the center of the egg, the ribosomes become more numerous and less organized. The refringent body decreases in size and is usually absent by the time the sperm cytoplasm attains its central position. Following the disappearance of the refringent body, there is no appreciable morphological difference between the two types of penetrated sperm.

DISCUSSION

A well-defined acrosome has not been demonstrated in the anterior cytoplasm of nematode sperm. Nevertheless, it is generally agreed that during zygote formation the anterior portion of the sperm is first to enter the egg (25). Examinations of the anterior cytoplasm in most nematode sperm, including those having a flagellate form (21), have shown that it is modified by the presence of pseudopods. Although reported to contain periodic acid–Schiff–positive materials (27), the pseudopods have not been considered seriously as an acrosome; rather, they have been thought to be associated with sperm movement in the uterus.

The results of this study are in agreement with earlier reports that organelles are lacking in the anterior cytoplasm of *Ascaris* sperm. The filamentous elements within the pseudopods, although similar to those reported in the acrosome of certain crayfish (26, 50), are not considered to be acrosomal since they were not confined to vesicles. Presumably these filaments, like the microtubules in the ectoplasm of certain sarcodinians (32, 45), assist in directing the flow of the ameboid cytoplasm.

There can be little doubt that the pseudopods in *Ascaris* sperm are motile. They appear capable of applying considerable force against adjacent cell surfaces since they produce and maintain indentations in the plasma membrane of uterine cells. There is no indication that the indentations are formed by the action of lytic substances released by the sperm, nor is there evidence to support previous assumptions that sperm in this position are degenerate and are being engulfed by the uterine cells (19, 35, 36). It appears, rather, that this relationship between the sperm and uterine cells is associated with the retention of sperm in the female reproductive tract. Possibly these areas of sperm "storage" are comparable to the previously described seminal receptacle.

Colwin and Colwin (4) have shown that in *Saccoglossus* and *Hydroides* the acrosome dehisces as the sperm approaches the egg. The contents of the acrosome are released; the remaining membrane, in addition to becoming continuous with the sperm plasma membrane, is rapidly modified by the appearance of acrosomal filaments. Presumably, the released contents cause a localized erosion of the extraneous coat about the egg (5) and thereby enables the filaments to contact and interdigitate with the egg plasma membrane. This activity of the acrosome, called the "acrosomal preliminaries," is thought to represent a fundamental pattern in zygote formation since there is some evidence that it occurs in a wide variety of species (4, 7–9).

Electron micrographs of the initial contact and interdigitation of the gamete membranes in *Ascaris* reveal major differences compared with electron micrographs of these events in species possessing a well-defined acrosome. In addition to inherent

differences related to the presence of pseudopods, it is notable that the anterior membrane remains intact as the sperm approaches the oocyte. There is no evidence that substances are released from the sperm cytoplasm, and it appears that the extraneous coat about the oocyte is removed by physical action of the pseudopods. If the extraneous coat were removed by lytic substances, one would not expect it to be present on the oolemma at the exact site of the fusion of the oolemma with the sperm membrane as shown in Figs. 5 and 9. Once the extraneous coat is removed, however, the pseudopods interdigitate with the oolemma in a manner not unlike that reported between the acrosomal filaments and egg membrane in *Hydroides* (5). Apparently, the activity of the pseudopods in *Ascaris* sperm is comparable to the "acrosomal preliminaries" in flagellated sperm as described above.

At this particular stage of syngamy in both *Ascaris* and *Hydroides*, the gametes' cytoplasms are separated by two interdigitating plasma membranes. Whether the sperm membrane at this stage is derived from the original plasma membrane as in *Ascaris* or from the membrane of the acrosomal vesicle as in *Hydroides* seemingly has little effect on subsequent fusion of the respective gametes. In both species, the separating membranes disappear, and the gamete cytoplasms become continuous.

The initial contact between the gametes of both *Ascaris* and *Hydroides* supports Pethica's (31) suggestion that cells make their first adhesive contact at points having a low radius of curvature. It is also notable that once the extraneous coat is removed from the *Ascaris* oocyte the activity of the pseudopods appears comparable to that reported for the acrosomal filaments of flagellated sperm. Seemingly, the pseudopods of *Ascaris* sperm represent a modification of the fundamental structural plan of the acrosome present in flagellated sperm.

Contrary to previous reports (1, 3, 12, 27), neither the refringent body nor the surface membrane specializations (proacrosomal granules) are considered either homologous or analogous to the acrosome of flagellate sperm. In addition to occupying a posterior position in the sperm, these structures undergo no apparent morphological change immediately prior to sperm contact with the oocyte Furthermore, it has been observed that subsequent to gamete membrane fusion they enter the ooeyte intact and remain apparent for some time in the zygote cytoplasm. No comparable activity has yet been ascribed to the acrosome of flagellated sperm.

Nucleus

From information now available, it appears that there is little uniformity in the location and appearance of the nucleus in mature nematode sperm. In *Aspiculuris*, the chromatin is reportedly associated with microtubules in the cytoplasm (21); in *Nippostrongylus* it is arranged as a taillike structure (17). Although located in the posterior cytoplasm in *Ascaris*, it occupies a position anterior to other organelles. In all three species a nuclear envelope has not been observed.

As long ago as 1883 Schneider (39), working with *Ascaris megalocephala* (*Parascaris equorum*), denied the existence of a male pronucleus in newly fertilized eggs. He surmised that the two pronuclei are formed from a division of the female nucleus. Although that early report has been neither confirmed nor refuted in the present study, the observed dissolution of the nucleus in *Ascaris lumbricoides* sperm following gamete fusion is of particular interest since it coincides with the rapid appearance of ribosomes in the newly penetrated sperm cytoplasm. It remains to be determined whether the nucleus is involved in the production of ribosomes from materials contained within the refringent body as suggested by Panijel and Pasteels (29).

The paucity of information concerning the appearance of newly penetrated sperm nuclei in other organisms makes it impossible to interpret conclusively the observed dissolution of the sperm nucleus in *Ascaris*. One might suggest, however, that this phenomenon is comparable to the vesiculation and subsequent disappearance of the nuclear envelope in the newly penetrated sperm of *Hydroides* and *Saccoglossus* (4) and thereby fits into a fundamental behavioral pattern of all newly penetrated sperm nuclei. The dissolution of the sperm nucleus is *Aspiculuris* prior to oocyte penetration possibly represents a further modification of this behavioral pattern.

Refringent Body

There has been much speculation as to the origin and function of the refringent body present in ascarid sperm. This body has been described

as yolk (19, 23), chromatic (36) or achromatic material (38), a chromatoid body (24), or a remnant of the Golgi apparatus (16). There is now strong evidence that it consists largely of a highly specialized protein (ascaridine) (10, 13, 30) synthesized in the testes. It reportedly originates from ribosomes (12) but acquires a lipid moiety during subsequent development (27).

Histologically, the material comprising the refringent body is first observed as numerous, small, Gram-positive granules in the spermatogonia. Although the granules enlarge during subsequent developmental stages, they do not coalesce to form the large refringent body until the sperm are deposited in the female reproductive tract (10, 12, 27). Presumably the coalescence of granules, as well as other morphological changes, are associated with the final maturation of the sperm (46). Cunningham (6), however, noted that the sperm can effect fertilization prior to achieving its final conoid form.

The present studies show that sperm having either large or small refringent bodies are capable of penetrating primary oocytes. In addition, they tend to support Panijel and Pasteel's conclusion that immediately following penetration the materials contained within the refringent body participate in the synthesis of ribosomes. The marked difference in the arrangement of ribosomes and the seemingly slower rate of synthesis about the large refringent body give some indication that certain materials contained within the body must be unmasked (possibly separated from lipid) before synthesis can occur. The sudden appearance of ribosomes about penetrated sperm having a small refringent body suggests that the unmasking of the materials has occurred prior to penetration. Whether the dense material observed in the anterior cytoplasm of the sperm depicted in Fig. 8 is indeed the unmasked product remains unknown. There is no evidence to support previous assumptions that sperm having a small refringent body are undergoing degeneration (10, 12). It appears that the size of the refringent body is merely a reflection of the time period the sperm has been in the uterus.

In any discussion of the refringent body, the question arises whether it is comparable to any structure in flagellated sperm. Histochemically it appears somewhat similar to the RNA-containing chromatoid body reported in the male germ cells of certain vertebrates and invertebrates (see Sud reference 43, for review). The chromatoid body, considered isomorphic to the residual body of Regaud (33), is usually described as an aggregation of osmiophilic granules (41, 2) or an irregular, homogeneous, electron-opaque mass (15, 49). It reportedly originates from the ground substance. In the hagfish, it is thought to form from the fusion of smaller granules (40), but, in the rat, it is apparently associated with annulate lamellae (44). The body disappears during meiosis but is later reconstituted in the daughter cells. With few exceptions, it disappears during subsequent development and makes no visible contribution to the mature spermatozoan. Smith and Lacy (41) have confirmed Regaud's (33) observation that the residual body is phagocytosed by the Sertoli cells. In addition, they postulated that the bodies may be indirectly responsible for the local control of spermatogenesis.

The chemical processes responsible for the disappearance of the chromatoid body in the sperm cytoplasm in certain species possibly correspond to those responsible for the gradual decrease in size of the refringent body in *in utero* sperm of *Ascaris*. It is interesting to recall Van Beneden and Julin's (46) contention that *Ascaris* sperm undergo their final maturation in the female uterus. Mayer's (24) supposition that the refringent body is homologous to the chromatoid body may yet prove to be valid.

Contrary to previous reports that little or no new ribosomal RNA is synthesized in most newly fertilized eggs (20), the present report is in agreement with recent evidence which indicates that a rapid synthesis of RNA occurs in *Ascaris* (18) immediately following entrance of the sperm. The initial mingling of the sperm nucleus and refringent body with the oocyte cytoplasm of *Ascaris* deserves more attention than it has attracted thus far.

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