OBSERVATIONS ON SPERM PENETRATION IN THE RAT

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ABSTRACT

The structural aspects of sperm penetration in the rat egg were investigated by electron microscopy. Eggs were recovered at intervals between 8 and 10:30 A.M. from females which had mated during the previous night. The oviducts were flushed with hyaluronidase and the eggs transferred into a 2 per cent osmium tetroxide solution, buffered at pH 7.8. After fixation, the eggs were mounted individually in agar, dehydrated in ethyl alcohol, and embedded in butyl-methyl methacrylate (3:1). The sperm penetrating the egg is covered by a plasma membrane which is present only on the side facing toward the zona pellucida; no membrane is visible on the side facing toward the vitellus. The sperm plasma membrane becomes continuous with the egg plasma membrane and forms a deep fold around the entering sperm. Cross-sections through the sperm midpiece in the perivitelline space show an intact plasma membrane. At the place of entrance, the plasma membrane of the sperm appears to fuse with the egg plasma membrane. After the sperm has penetrated the vitellus, it has no plasma membrane at all. The nuclear membrane is also absent. These observations suggest a new hypothesis for sperm penetration. After the sperm has come to lie on the plasma membrane of the egg, the egg and sperm plasma membranes rupture and then fuse with one another to form a continuous cell membrane over the egg and the outer surface of the sperm. As a result the sperm comes to lie inside the vitellus, leaving its own plasma membrane incorporated into the egg membrane at the surface of the egg.

There are only a few investigations that deal with the process of sperm penetration through the plasma membrane of the egg. In general, studies of fertilization are concerned with the approach of the gametes or the penetration of the extraneous layers of the ovum. Originally, penetration was thought to be accomplished by active movements of the sperm itself, but doubts were soon raised about this simple explanation. Wilson (24), for instance, concluded that "another and more important factor lies in some physical action that causes the sperm to be drawn passively into and through the membrane."

Allen, in his recent review (2), concludes that "there is no evidence that the movements themselves play any role in sperm entry," and ascribes the major role in sperm penetration to the acrosome filament, which may function as a "handle" by which the sperm is drawn into the egg. The acrosome filament itself, or the cytoplasm in which it is anchored, is thought to play the active role, while the plasma membrane proper is presumed to be broken mechanically.

Allen's view on sperm entry agrees with that previously expressed by Lillie (12) pertaining to sperm entrance in *Nereis*. After attachment of the spermatozoon to the egg membrane, the cortical cytoplasm of the egg is said to become denser at the point of attachment. This is followed by an active streaming of the surrounding cortical cytoplasm toward the sperm head, which is then carried into the egg by a centripetal movement of the condensed cytoplasmic mass.

From phase contrast microscope observations of sperm entrance into the rat egg, Austin (3) concluded that the sperm plays a passive role

and that penetration is due to some membrane activity through which the sperm head "sinks" into the vitellus. Ludwig (13) also believes that the egg cytoplasm is the active factor in sperm penetration. These observers, then, agree in principle in that they emphasize the role of the egg surface in sperm penetration.

Following the extensive studies on the reactive systems of fertilizin-antifertilizin and other surface agents, the idea developed that sperm attachment and activation of the ovum were due to specific surface reactions followed by a phagocytotic process which was responsible for the engulfment of the spermatozoon. Tyler (22) has recently proposed an elaborate pinocytotic scheme.[1] A similar process had been suggested by Bennett (5) in his "membrane flow" hypothesis for active transport of particles, even ions, into or out of cells.

While this hypothesis of sperm penetration is very attractive, there is no evidence for it. Indeed, in the electron micrograph of a recently penetrated sea urchin sperm published by Lord Rothschild (19), no membrane is seen surrounding the sperm. If the process of penetration involves phagocytosis or membrane flow, there should be at least one membrane around the sperm, namely that of the phagocytotic vesicle. None is visible, however.

The purpose of this paper is to examine with the help of electron micrographs the process of sperm penetration in the albino rat, *Rattus rattus*.

[1] Lewis (11) defined pinocytosis as "drinking by cells," and phagocytosis as "eating by cells," in describing the incorporation of fluids versus solids. Phagocytosis would be more accurate here than the term pinocytosis used by Tyler.

MATERIALS AND METHODS

Rats 70 to 100 days old were kept on daily 14-hour light and 10-hour dark periods in an artificially lighted, air-conditioned room. The estrus cycle of the female rats was followed by means of vaginal smears. Each female in proestrus was placed overnight with two vigorous males. The following morning the animals were examined for copulation plugs, and the presence of sperm was detected by vaginal smears. If there was evidence of mating, the females were sacrificed by cerebral dislocation. The eggs were collected between 8 and 10:30 A.M. by flushing the oviducts with hyaluronidase (Alidase, G. D. Searle and Co., Chicago, 150 USP/ml.) and were transferred into a buffered 2 per cent OsO_4 solution. This solution was prepared by dissolving 4 per cent OsO_4 in veronal acetate buffer at pH 7.8 and diluting it just before use with an equal volume of Tyrode's solution. Sucrose to yield a 0.25 M concentration was added.

After 30 minutes to 1 hour fixation, the eggs were rinsed with Tyrode's solution and embedded in 1 per cent bacto-agar. The agar block was dehydrated in increasing concentrations of ethanol and passed through *n*-butyl methacrylate. The eggs, surrounded with a small amount of agar, were embedded in a 3:1 mixture of *n*-butyl and methyl methacrylate. Three per cent benzoyl peroxide was added as a curing agent. Sections were prepared with a Porter-Blum microtome and a diamond knife, and were mounted on 200 mesh grids with carbon films. The specimens were examined in a Siemens Elmiskop II B. A 30 μ objective aperture was used. Some sections were stained with lead hydroxide (23).

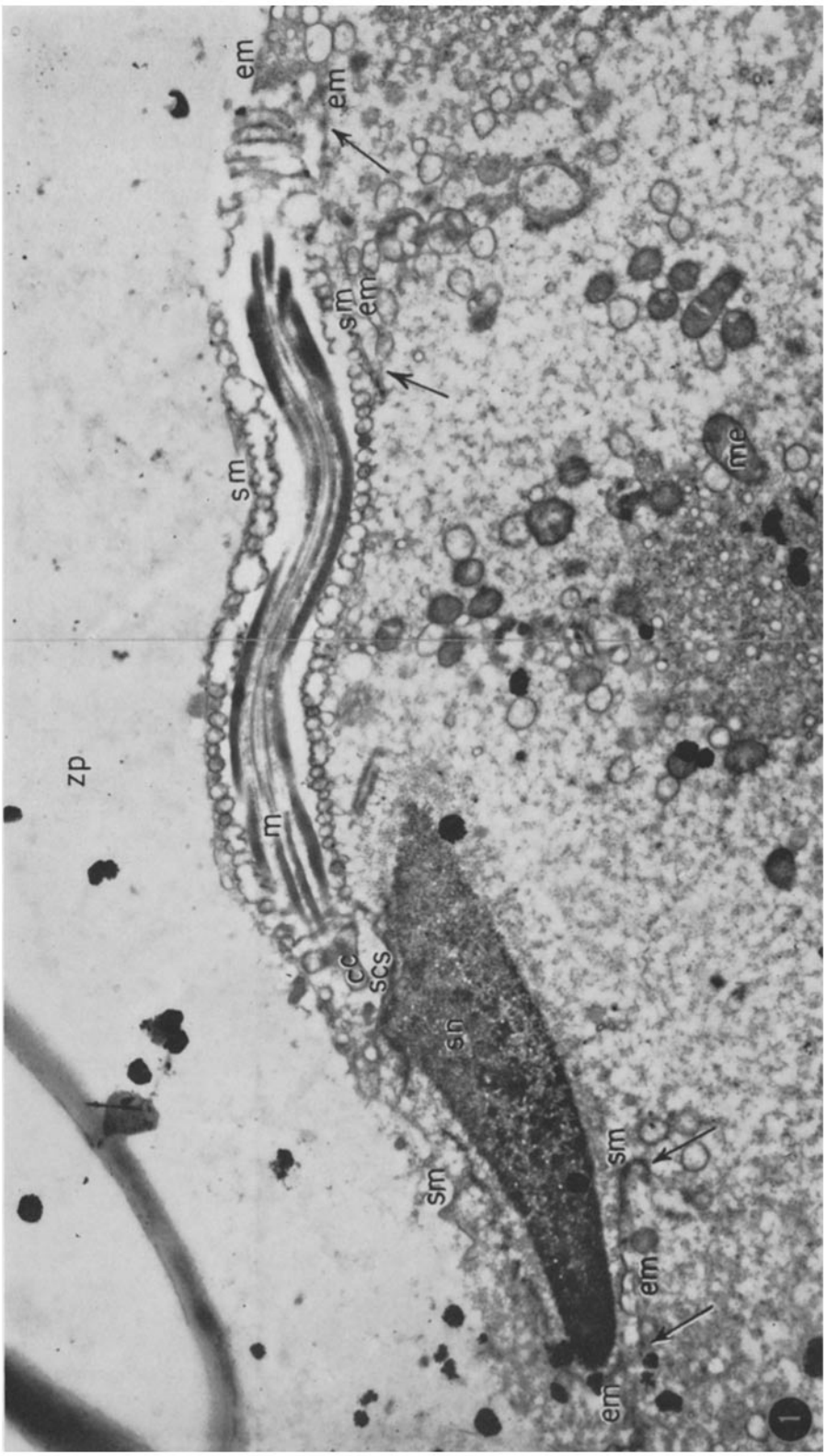
It was found that the time of ovulation, sperm penetration, and early pronuclear development in the Holzman strain rats used in this study agree well with the schedules published by Austin (4) and by Odor and Blandau (14).

Explanation of Figures

cc, centriolar complex
em, egg plasma membrane
m, midpiece
me, mitochondria of egg cytoplasm
ps, perivitelline space
scs, supracentriolar sheet
sm, sperm plasma membrane
sn, sperm nucleus
zp, zona pellucida

FIGURE 1

Rat sperm in the process of penetrating the egg. The arrows point to the folds where the plasma membranes of the egg and sperm are continuous. The section was stained with lead hydroxide for 5 minutes. The dark spots are deposits of lead carbonate. × 15,000.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RESULTS

Fig. 1 shows a longitudinal section of a recently penetrated sperm near the surface of a rat egg following normal mating conditions. The chromatin material is beginning to be dispersed and is much less electron opaque than the nucleus of a mature sperm (Fig. 3). The apparent granular structure of the sperm nucleus is reminiscent of a late spermatid (6). Near the caudal region of the nucleus the chromatin is further dispersed into finer components, showing fibrils about 40 A thick. These correspond to the smallest chromosomal fibrils that were observed in elongating spermatids of several species (18). At the apex of the nucleus no changes can be observed as yet in the electron opacity of the nuclear material. No acrosome is visible. Some dense fragments near the sperm nucleus may represent remnants of it. The centriolar complex and the midpiece are still attached at the base of the nucleus.

The most striking finding is that no plasma membrane appears to cover the sperm head on the side facing toward the vitellus. A plasma membrane is distinctly visible on the side facing toward the zona pellucida, however. The membrane makes a deep fold at the apex of the spermatozoon (arrows, left, Fig. 1) and another fold where the midpiece of the sperm moves out of the plane of section (arrows, right, Fig. 1). At both folds the plasma membrane of the egg is apparently continuous with the plasma membrane of the spermatozoon. This situation is represented diagrammatically in Fig. 2. The plasma membrane surrounding the egg is continuous with the membrane of the sperm along the axial filament which still protrudes into the perivitelline space. Another example of the continuity of the cell membranes of the two gametes is presented in Fig. 4.

Figs. 5 to 8 show cross-sections at different levels along the tail of a sperm penetrating an egg. These figures correspond to cross-sections at the places marked *A*, *B*, *C*, and *D* in Fig. 2. In Fig. 5, the midpiece lies in the perivitelline space and a continuous plasma membrane is clearly visible around the mitochondrial complex. The plasma membrane of the egg is still intact. Fig. 6 shows the attachment of the plasma membrane of the midpiece to that of the ovum. At the arrow, a continuity between the two membranes is suggested. Fig. 7 presents a view of the midpiece partially incorporated into the vitellus. The plasma membrane of the egg is continuous with the membrane enclosing the still protruding portion of the axial filament. In Fig. 8 the midpiece is completely within the vitellus. No plasma membrane surrounds the mitochondria of the midpiece in this case, but a plasma membrane is continuous above the incorporated portion of the spermatozoon.

Further sections of the same ovum (Fig. 9) show

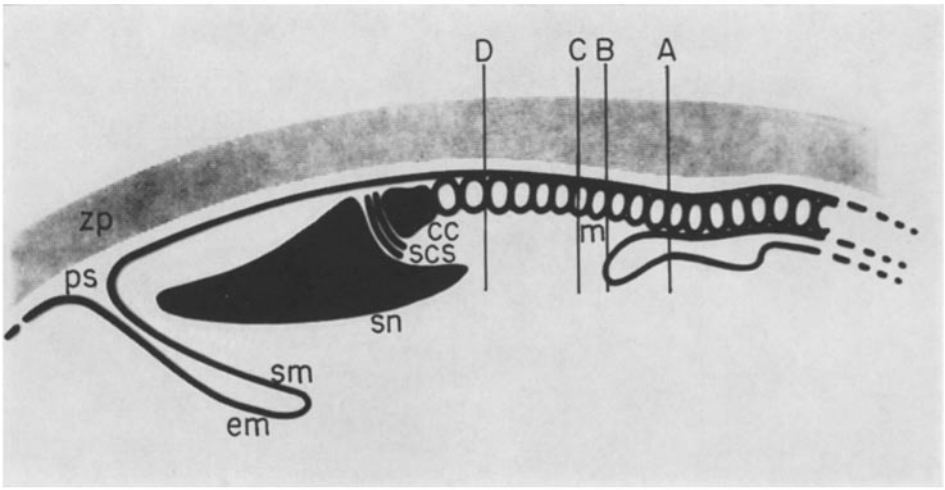


FIGURE 2

A diagrammatic representation of the sperm shown in Fig. 1, illustrating the behavior of egg and sperm plasma membranes during sperm entrance. The lines *A*, *B*, *C*, and *D* correspond to the planes of section through the midpiece shown in Figs. 5 to 8.

the sperm nucleus with the centriolar complex (or juxtanuclear body) and the "supracentriolar sheet" of Sotelo and Trujillo-Cenoz (21). This egg is the most advanced in its development of any described in this study. Neither a sperm plasma membrane nor a nuclear membrane is visible at any point. The nucleus of the spermatozoon has changed considerably. The swollen sperm nucleus now shows masses of 100 A fibers. Each fiber can be resolved into two parallel 40 A subfibrils (circles in Fig. 9). Three eggs were sectioned which showed the sperm in the process of penetration. In each case a fusion of the sperm and egg plasma membranes was visible. More than one hundred eggs were studied in which the sperm was already entirely inside the vitellus. No plasma membrane was ever seen to surround any part of these sperm. Parallel studies of eggs of the golden hamster indicate that the same process of sperm penetration is found here. The newly introduced paternal genome is thus exposed rapidly to the egg cytoplasm. This observation may lead to interesting theoretical considerations. Studies on further developments with regard to the sperm nucleus will be published elsewhere.

DISCUSSION

The electron micrographs presented here show that the sperm has no plasma membrane after it has penetrated the egg. During the process of penetration, however, a plasma membrane is still visible on the outer surface of the sperm. This membrane is continuous with the egg plasma membrane and forms a deep fold around the sperm. On the side facing toward the egg interior, the sperm has no visible plasma membrane. Serial cross-sections through the midpiece show all stages of penetration: in the region outside the vitellus, the midpiece is surrounded by a membrane; then comes the region where the egg and sperm plasma membranes fuse; finally, within the vitellus the midpiece is without a plasma membrane. These observations suggest the following hypothesis for sperm penetration.

The sperm head comes to lie against the plasma membrane of the egg. At a certain point the apposed plasma membranes rupture and the egg plasma membrane fuses with the sperm plasma membrane so that a continuous membrane is formed over the egg and around the outer surface of the sperm. In the regions where egg and sperm membranes have joined, the continuous membrane at first forms a deep fold around the sperm. This fusion of membrane proceeds from the point of attachment near the anterior end of the sperm toward the tail until the entire sperm lies inside the egg.

Fig. 10 illustrates schematically our hypothesis

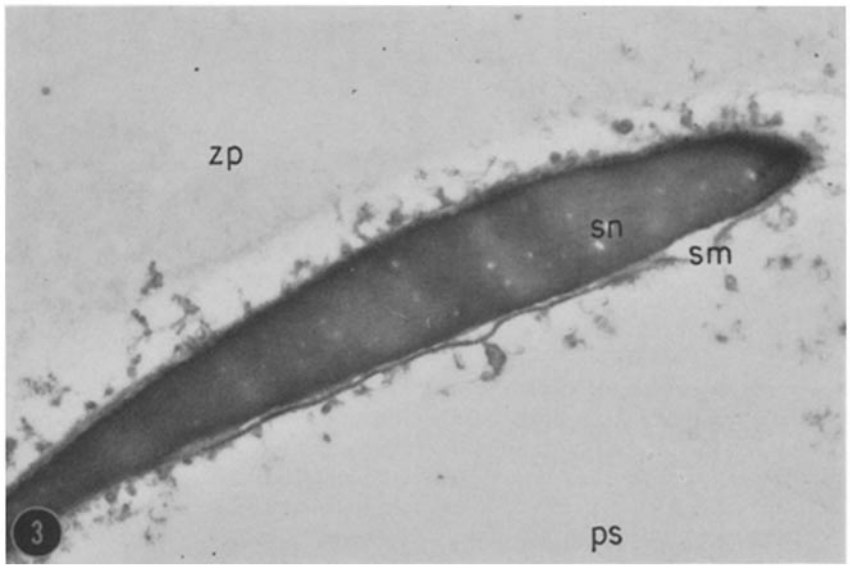


FIGURE 3

A spermatozoon in the perivitelline space. Observe the very dense nucleus and the cell membrane surrounding it. × 22,000.

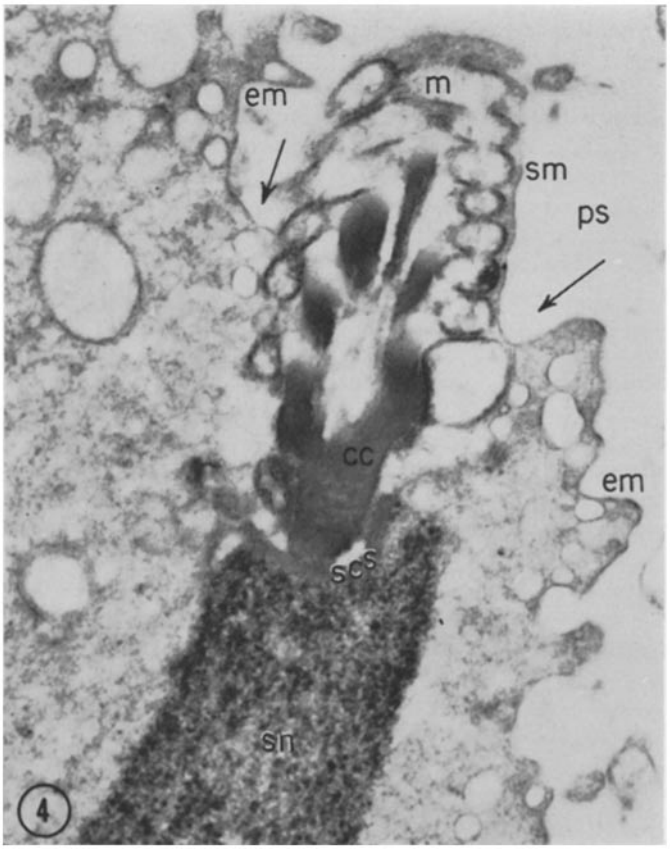


FIGURE 4

A sperm penetrating the rat egg. The plasma membrane of the egg is continuous with the membrane surrounding the sperm tail (arrows). × 37,000.

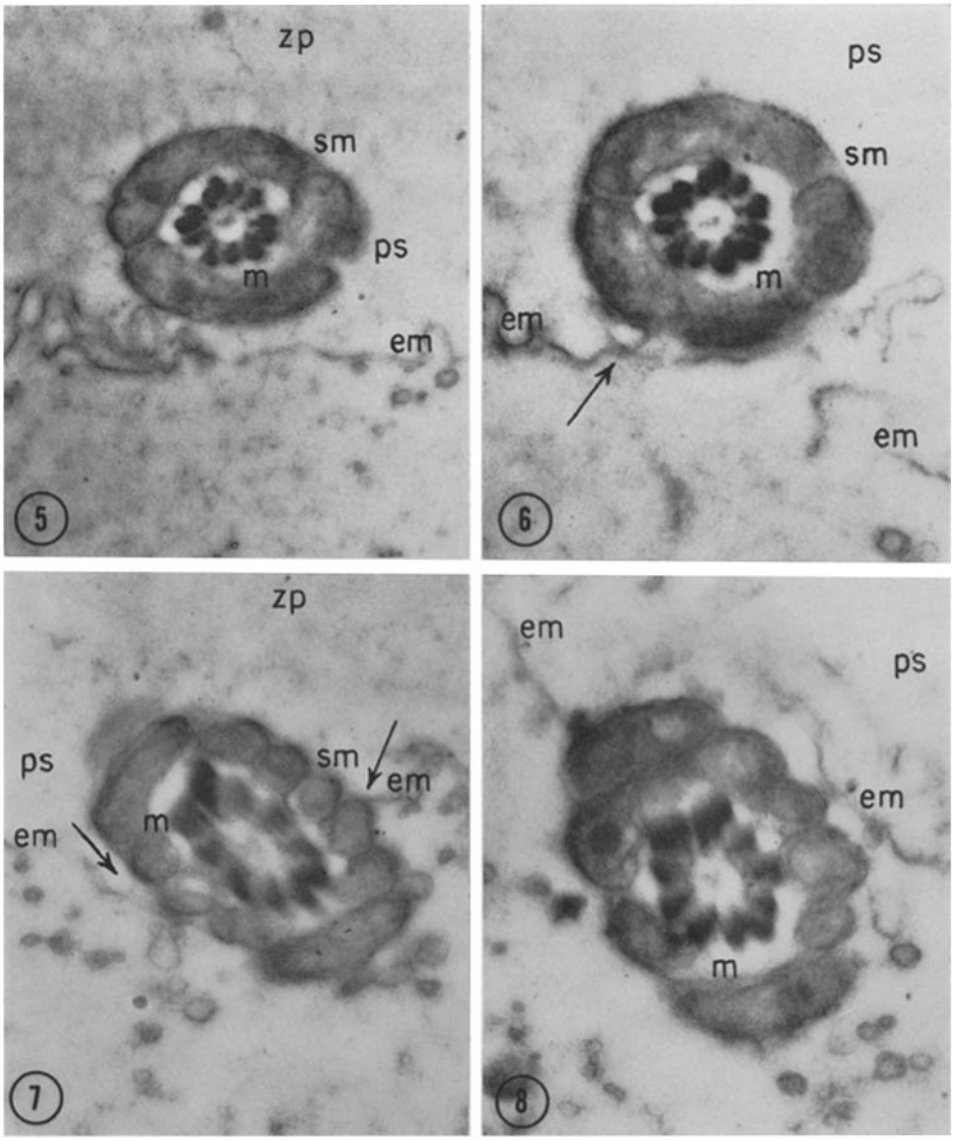
of sperm entry and contrasts it with the phagocytotic scheme suggested by Tyler (22). The solid black line corresponds to the plasma membrane of the spermatozoon, while the finely dotted line represents the plasma membrane of the egg.

It is clear that the phagocytotic scheme is not supported by our electron micrographs of sperm entry into the rat egg. If phagocytosis were the mechanism of sperm penetration, we would expect to find not only an intact sperm plasma membrane, but also a surrounding membrane of the phagocytotic vesicle. In fact, there are no membranes at all around the sperm after penetration.

A recent electron microscope study of conjugation in *Paramecium aurelia* (20) indicates that in the formation of the cytoplasmic bridge between conjugants a fusion of the cell membranes of the two cells occurs. This process resembles the fusion of egg and sperm plasma membranes, except for the restriction in space and time.

The process of sperm entrance by membrane fusion can be compared with the interesting membrane phenomenon described by Palade (16) for the discharge of the zymogen granules into the acinar lumen; he states that "the membrane of the zymogen granules becomes continuous with the cell membrane at the apical pole of the exocrine cell." The membrane phenomena in both of these processes would seem to be identical except for their direction.

Fusion of cellular membrane systems is a widely distributed phenomenon. Palade (15), in his studies on "The Endoplasmic Reticulum," reports that smooth surfaced vesicles of the endoplasmic reticulum establish contact with the cell membrane in many different cell types. He further suggests that, in the case of cells which are in-



FIGURES 5 TO 8

Cross-sections through the midpiece of an entering sperm at different levels.

FIGURE 5

The posterior region still in the perivitelline space. The intact plasma membranes of the egg and sperm are clearly visible. × 28,000.

FIGURE 6

The egg and sperm plasma membranes touch and apparently fuse at the arrow. × 34,000.

FIGURE 7

The midpiece is partially incorporated into the vitellus. The continuity of the plasma membrane of the egg with the sperm plasma membrane is visible at the arrows. × 31,000.

FIGURE 8

The midpiece is now entirely within the vitellus. The plasma membrane is continuous outside the incorporated spermatozoon. × 35,000.

volved actively in pinocytosis or phagocytosis, large quantities of membrane material will be incorporated into the cytoplasm and that a unidirectional flow of membrane material is unlikely. Palade suggests also that the membrane is repeatedly circulated between the cell surface and the interior of the cell.

Hodge *et al.* (9) propose that the lamellar systems of plant cells are generated by fusion or coalescence of vesicular elements. The most important example they give is the formation of the internal membrane systems in developing chloroplasts.

Other examples of membrane fusion during fat absorption in the intestinal epithelium have been described by Palay (17).

Close contact of egg and sperm plasma membranes can be seen in electron micrographs published by Afzelius and Murray (1) in the case of the sea urchin, and by Colwin *et al.* (7) and Colwin and Colwin (8) for annelids. These investigators, however, did not show later stages of sperm penetration.

The hypothesis we have presented above allows us to examine in a new light other questions related to sperm penetration. The formation of the fertilization cone and its creeping up along the acrosome filament (*cf.* 8, 10) may be interpreted as cytoplasmic streaming or redistribution of the egg cytoplasm after membrane fusion has taken place. This allows the sperm head to "sink" gradually into the egg. Kille's (10) recent description of the penetration of the lamprey sperm provides a good example of such a process. Fertilization in *Nereis*, as described by Lillie (12), can be interpreted in the same fashion.

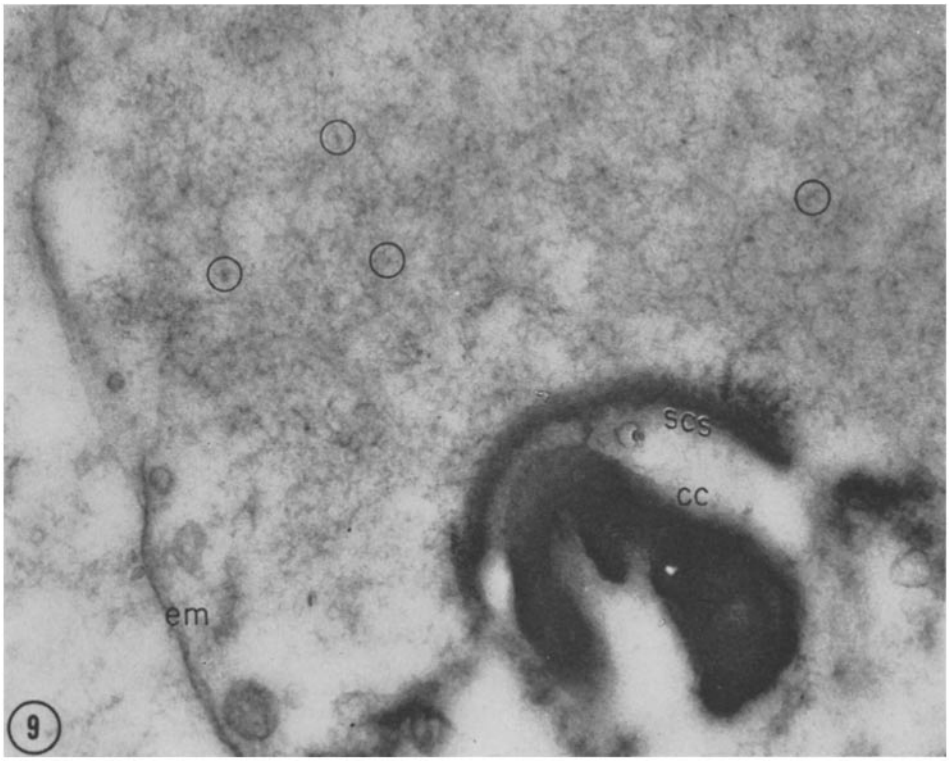


FIGURE 9

The sperm nucleus with the centriolar complex is shown in further sections of the same egg that is illustrated in Figs. 5 to 8. On the left the egg plasma membrane is visible. Inside, no sperm plasma membrane or nuclear membrane around the sperm chromatin mass can be observed. At the base of the nucleus is the "supracentriolar sheet," which is the only membrane associated with the nucleus. The sperm nucleus shows masses of 100 A fibers, each of which is composed of two 40 A subfibrils (circles). The section was stained with lead hydroxide for 5 minutes. × 65,000.

Such membrane fusion may take place only between membranes of similar molecular organization. The reasons for species specificity in fertilization may be sought in part in a specificity of membrane structure. Similarly, the block to polyspermy could be the reflection of a reorientation of the reactive sites within the membrane following the fusion of egg and sperm membranes.

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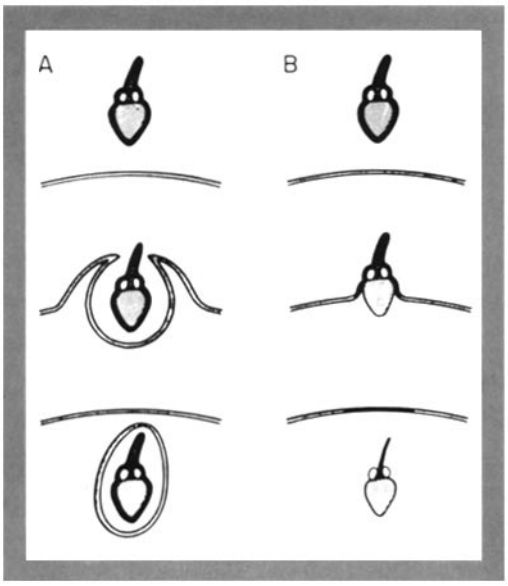

FIGURE 10

A diagrammatic representation of sperm penetration by phagocytosis (A) and by membrane fusion (B).

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