MEMBRANE PARTICLE CHANGES ATTENDING THE ACROSOME REACTION IN GUINEA PIG SPERMATOZOA

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ABSTRACT
To examine the freeze-fracture appearance of membrane alterations accompanying the preparation of sperm membranes for fusions—the first preparatory stage occurring before physiological release of the acrosomal content, the second afterward—we induced the acrosome reaction in capacitated guinea pig spermatozoa by adding calcium to the mixture. The most common features observed before fusion of the acrosomal and plasma membranes were the deletion of fibrillar intramembranous particles from the E-fracture faces of both membranes, and the clearance of globular particles from the P face of the plasma membrane—events taking place near the terminus of the equatorial segment. Large particles, >12 nm, remained not far from the cleared E-face patches. The P face of the outer acrosomal membrane is virtually clear from the outset. In addition, when fusion was completed, occasional double lines of large particles transiently embossed the P face of the plasma membrane (postacrosomal) side of the fusion zone. Behind the line of fusion, another series of particle-cleared foci emerged. We interpreted these postfusion membrane clearances as a second adaptation for sperm-egg interaction. Induction of the acrosome reaction in media containing phosphatidylcholine liposomes resulted in their apparent attachment, incorporation, or exchange in both the originally and secondarily cleared regions. Our observations support the concepts that membranes become receptive to union at particle-deficient interfaces, and that the physiologically created barren areas in freeze-fracture replicas may herald incipient membrane fusion.

After epididymal maturation, four stages of the spermatozoon's life are critical for successful interaction with the egg (4, 6, 8, 38). One step is activation, the change in the rate and form of the tail movement from that of a slow-driven windshield wiper to a faster, undulating whip. Another is capacitation, an ill-defined phenomenon normally occurring in the female genital tract while the sperm membranes are undergoing modification to facilitate fusion between the acrosomal and plasma membranes. The third phase is this fusion itself, the acrosome reaction. The last crucial episode is the preparation of the plasma membrane for fusion with the egg.

The intramembranosus events associated with these important stages have barely been examined.
enzymes to help the sperm permeate the cells of the oocyte cumulus oophorus and the Zona pel-
region, dense ridges span the slender space be-
tween the outer acrosomal membrane and the plasma membrane. In order for the acrosomal enzymes to help the sperm permeate the cells of the oocyte cumulus oophorus and the Zona pel-
Lucida, the acrosomal content must first be released. It is here in the equatorial segment, in front of the compact ridges, where the ultimate fusion between acrosomal and plasma membranes usually occurs, relinquishing the contents of the granule while still encasing the sperm with a continuous, hybrid membrane now comprised of the acrosomal and plasma membranes. The interior of the acrosomal membrane is then exposed over the anterior two-thirds of the nucleus. This massive exocytotic event is naturally triggered by unknown factors in the vicinity of the egg in vivo, and in vitro by the addition of 1.5-5 mM CaCl2 to spermatozoa incubated in a capacitating medium (39).

As well as providing a good model to explore the intramembranous events preceding fertilization, the spermatozoan acrosome we have selected here is of particular advantage for studying exocytosis. There is a well-defined stimulus for inducing the acrosome reaction in vitro, and the responses to calcium stimulation are moderately consistent, within 5-15 min in 30-40% of the cells. Moreover, the exocytotic reaction can be quantitated by light microscopic determination of the percentage of reactive sperm, and its success tested by in vitro fertilization. The final fusion takes place in a 1 x 6-μm band across the girth of the flattened equatorial segment rather than at random foci. Unlike the situation in other exocytotic model systems, once this secretory hemi-
orifice is formed, it persists for the rest of the cell’s life. The patterns of particle admixture within a hybrid membrane may then be observed. Further-
more, we can be assured that no new proteins are being synthesized; the head lacks nuclear pores and rough-surfaced endoplasmic reticulum. Last (and significantly), freeze-fracture-thin-section correlates of sperm cell membranes have been fully documented (8-11, 14).

With this well-defined system, we can now ask what intramembranous changes can be viewed in freeze-fracture, and also whether the membrane alterations related to exocytosis in this mammalian cell parallel those described during exocytosis and fusions in other diverse systems (2, 7, 15, 16, 18, 25-27, 32, 33, 37).

MATERIALS AND METHODS

Spermatozoa

Spermatozoa were removed from transected tubules of the epididymal tails of adult guinea pigs (>500 g) in 31 separate experiments. The sperm were prepared in three fashions: (a) Freshly-removed spermatozoa were mixed in calcium-free Tyrode’s solution (39) at room temperature and fixed for electron microscopy within several minutes (b) other samples were incubated at 37°C in calcium-free Tyrode’s solution containing 6 mM glucose and/or 0.25 mM pyruvate (30) under sterile mineral oil for 12-16 h before fixation; and (c) to half these latter preparations, 1.5-5 mM CaCl2 was added and the incubation was continued for another 10-30 min before fixation. Samples of the sperm being incubated were removed at various times for examination by phase-
contrast microscopy.

For cation localization, we explicitly followed the K-
pyroantimonate method of Ravazolla et al. (29) and Spicer et al. (36) (K-pyroantimonate, 262.90 mol wt, Merck A.G., Inc., Darmstadt, West Germany).

In four experiments, portions of the sperm samples were incubated with artificial lipid vesicles in the capacita-
ting medium. Unilamellar, egg lecithin, phosphatidyl-
choline liposomes (average diameter, 25 nm) were kindly provided by Doctors R. Hamilton, J. Goerke, M. Williams, and R. Havel of the University of California at San Francisco (processing procedure in preparation). Large, negatively charged multilamellar liposomes were devised, using standard procedures (5). 63 μmol egg lecithin, 18 μmol dicetyl phosphate, and 9 μmol cholesterol, were combined in 4:1 chloroform-methanol. The solvent was removed under reduced pressure, and the dry lipid was dispersed into calcium-free Tyrode’s solution by hand agitation. We purchased materials for the liposomes as kits from Avanti Biochemicals, Birmingham, Ala.

Fixation was accomplished by immersing droplets of spermatozoa in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4, room temperature). Some preparations for thin-sectioning were fixed in tannic acid as well, either by the method of Mizuhira and Futaiwak
(22) or by that of Simionescu and Simionescu (34). Both methods displayed the glycoalyx to advantage. Primary fixation with tannic acid, however, was more propitious for demonstrating microtubules. Tannic acid fixation as achieved by the Simionescus was excellent for staining the bilayers of liposomes and helping to distinguish them from native membranes.

Fixation of the material processed for thin sectioning lasted for 1 h, and that for freeze-fracture processing, for 15-30 min. The procedures used for subsequent processing for electron microscopy have been reported previously (10, 11). Sections and replicas were examined in a Siemens Elmskop 101 electron microscope.

RESULTS

Prelude

Published descriptions of the guinea pig spermatozoon as it appears in thin-section, freeze-fracture, surface replica, and scanning electron microscope preparations are abundant (8, 9, 14). As indicated in Fig. 1, sperm removed from the cauda epididymidis are arranged in stacks. K-Pyroantimonate precipitates cations heavily in two regions of the acrosomal cap, where initial vesiculation and fusions between the acrosomal and plasma membranes will occur (Fig. 2). The final union of the two structures will be far more posterior, in the acrosome's equatorial segment, the region most relevant to the observations discussed in this paper.

Spermatozoa Incubated in Calcium-Free Tyrode's Solution

EARLY CHANGES: During incubation in calcium-free Tyrode's solution, a capacitating medium (24, 39), the sperm separate (Fig. 3a). But as observed in spermatozoa just removed from the tail of the epididymidis or the initial portion of the vas deferens, the plasma membrane overlying the equatorial segment of the acrosome at first (1 h) retains a prominent tannic acid-stainable glycoalyx (Fig. 3b). The outer acrosomal membrane is ~8 nm thick and keeps this width as it curves forward to form the inner acrosomal membrane, entrapping the uniformly dense acrosomal content within the fold thus created (Figs. 2, 3b). The outer acrosomal membrane is ~8 nm thick and keeps this width as it curves forward to form the inner acrosomal membrane, entrapping the uniformly dense acrosomal content within the fold thus created (Figs. 2, 3b). As in freshly removed spermatozoa, when the spermatozoon is sectioned lengthwise, several ellipsoid masses bridge the space between the plasma membrane and the outer acrosomal membrane (Figs. 3b, 4a). Grazing sections parallel to the plane of the flattened head reveal the correspondence in contour and location between these dense structures (Fig. 3d) and the palisade of elevations evident in fractures (Figs. 3c, 4b, c). Immediately fronting these landmarks, the ultimate fusion between the acrosomal and plasma membranes will eventually transpire. In front of the plasma membrane palisade, intramembranous particles on the P face are fewer than behind it. As will be observed later, elongated fragments can be seen especially well on the E-fracture face in the forefront of the palisade (Fig. 5a, b).

Fractures exposing the E face of the outer acrosomal membrane reveal an orderly, uniform, globular particle population (8-10). The P face is particle-poor. Fractures through the inner acrosomal membrane, however, have not been positively identified.

LATER CHANGES: Several membrane modifications become visible in spermatozoa incubated in Tyrode's solution for 12-16 h before the addition of CaCl₂ to trigger membrane fusion and acrosomal content release. As previously reported (or illustrated later), the quilt pattern noted in freeze-fracture over the acrosomal cap and equatorial segment fades (9, 12); geometric clusters of particles assume prominence in front of the striated ring (9, 12); and among this population which contains approximately 30% of the sperm swimming with an activated movement, the small particles in the plasma membrane midpiece have lost their orderliness as discrete strands and scatter the terrain at random (9, 11, 12). At this time, the majority do not manifest any activated motion or reveal this change in midpiece particle distribution. Also, as illustrated, tannic acid staining of the plasma membrane glycoalyx decreases (Fig. 4a), and the palisading impressions convert to obscurity as the plasma membrane partially lifts away (Figs. 4a, 5c, d).

The major morphological features which precede membrane fusion in the equatorial region are observed to best advantage on the E-fracture faces of the outer acrosomal and plasma membranes. In the former membrane, fibrillar profiles become prominent in front of the ridges (Fig. 4b, c). Random, large, globular particles adorn the membrane as well, and patches completely devoid of particles are also commonly encountered in this E face (Fig. 4d, e); while the P face (Fig. 5b) remains visually unchanged.

The E face of the overlying plasma membrane fully mirrors this same constellation of fibrillar profiles, scattered large particles (>12 nm), and clearings (Fig. 4a-d). In fact, in the absence of...
Figure 1  A stack of guinea pig spermatozoa before their incubation in a capacitating medium. Dense pyroantimonate-cation deposits fill two areas of the acrosomal cap (arrows). During the acrosome reaction, vesiculation commences in these regions and terminates in the equatorial segment (asterisk). A, acrosome. × 18,000.

other landmarks, the greater degree of plasma membrane puckering (reflected in thin section in Fig. 4a and in fracture in Fig. 5 b-d) may be the only peculiarity by which the E faces of the two different membranes may be tentatively distinguished. However, in the P face of the plasma
membrane, particles are likewise dispelled, with the clearances forming a series of patches (Fig. 5e) or sometimes short confluent bands (Fig. 5f).

Spermatozoa after the Addition of Calcium and Induction of the Acrosome Reaction

After the addition of 1.5-5 mM Ca++, as many as 40% of the spermatozoa (at an approximate concentration of 1-3 × 10⁸) undergo the acrosome reaction, most precisely confirmed in thin sections as fusion between the plasma membrane and outer acrosomal membrane (Figs. 6a, b, 7a). While vesiculation happens anterior to this band and may also take place in disrupted sperm (11), the reestablishment of membrane continuity in the equatorial segment, and consequently the maintenance of the cell's membrane potential, constitute the essential aspects of the acrosome reaction. Spermatozoa remain viable for several hours afterward. Due to the acute curvature of the area of final fusion, it is usually extremely difficult to obtain replicas which demonstrate the full contour of the hybrid plasma-acrosomal membrane union at its inception. When perceived, however, the plasma membrane portion of this zone is usually particle-poor (Figs. 6c, 7b-d). What we interpret as images directly following fusion comprise single or double strands of large particles (Fig. 7b-d), the same in diameter as the newly uncovered, inner acrosomal membrane particles at the juncture of the two membranes. The strands may be transient. This arrangement is best seen when the
FIGURE 3  (a) Scanning electron micrograph of the head and part of the tail of a guinea pig spermatozoon. The horizontal lines indicate the equatorial segment depicted in thin section in Fig. 3b. (b) A sagittal section through the equatorial segment of a tannic acid-fixed guinea pig spermatozoon, with the lines in the same position as in Fig. 3a. The cell membrane, with its prominent glycocalyx, is apparently bridged to the outer acrosomal membrane by an interrupted, dense matrix. The two membranes usually will fuse in front of this compact area (arrow). (c) and (d) In freeze-fracture (c), the P face of the plasma membrane reflects the palisade of projecting "fingers" seen in tangential section through the rear margin of the equatorial segment (d). (c) Here, the angle of shadowing originates from the top. Particles are scarcer in front of the projections than behind. (a) × 5,000; (b) × 98,000; (c) × 66,000; (d) × 120,000.

FIGURE 4  Portions of sperm membranes after a 12–16-h incubation in calcium-free Tyrode’s solution. The micrographs in this plate are mounted with the tips of the sperm heads pointing downward. (a) The glycocalyx of the plasma membrane stains more faintly as the membrane pulls away from the body of the acrosome. The plasma membrane stays relatively tight in the equatorial segment (above the arrow). AM, acrosomal membrane. (b) P face of the plasma membrane over the ridges and E face of the outer acrosomal membrane. Fibrillar profiles now dominate the outer acrosomal membrane in the foreground of the ridges, while more typical globular particles persist frontally (in the lower part of the micrograph). (c) Fibrillar E face of the outer acrosomal membrane underlying a small clearing (arrow) in the plasma membrane P face (cf. area designated by the arrow in Fig. 4a). (d) During particle clearance on the E face of the outer acrosomal membrane, elongated profiles balustrade the edges of the smooth region. (e) In addition to fibrillar ones, large particles scatter the E face of the outer acrosomal membrane. They have no discernible orientation in respect to the two clearings (upper part of micrograph) fronting the parallel elevations. (a) × 90,000; (b) × 54,000; (c) × 70,000; (d) × 100,000; (e) × 88,000.
The orientation of these micrographs presents the tips of the sperm heads pointing to the left.

(a) Before incubation, the E face of the plasma membrane reflects the imprints of the parallel projections, with some fibrillar images admixed with the more globular intramembranous particles in the foreground.

(b-f) Portions of sperm membranes after a 12-16-h incubation in calcium-free Tyrode’s solution. (b) The P face of the outer acrosomal membrane (OAM) retains its finely granular character as it encroaches upon the E face of the plasma membrane (PM). E, E (extracellular) fracture-face. P, P (cytoplasmic) fracture-face. (c) and (d) E faces of the plasma membrane. The parallel ridges, directed to the left, become obscured as the membrane undulates; fibrillar elements now more fully characterize the fracture-face, and particle-free areas (arrows) emerge. (e) Small clearings materialize in the plasma membrane (PM) P face, while persisting fibrils can be observed in the outer acrosomal membrane E face. (f) Eventually, some smooth zones converge. (a) × 88,000; (b) × 80,000; (c and d) × 100,000; (e) × 68,000; (f) × 60,000.
FIGURE 6 Heads of spermatozoa induced to undergo the acrosome reaction by the addition of 0.5 mM Ca\(^{++}\) to Tyrode’s solution. Here, the tip of the sperm head is upward. (a) Vesiculation is a common occurrence over what was formerly the acrosomal-cap region. Note the looping, continuous hybrid membrane which has formed between the acrosomal membrane of the equatorial segment and the plasma membrane. (b) The continuity (arrow) between the cell membrane (PM) and the outer acrosomal membrane (AM) is more distinct in this higher magnification view of another spermatozoon reacted as indicated above. Observe the length and content of the remaining equatorial segment of the acrosome. (c) Freeze-fracture images also disclose the considerable degree of curvature in the fused (arrow) hybrid membrane. Such a configuration makes it difficult to obtain replicas showing large areas of the same fracture-face in the conjoined membranes. When visible, the juncture usually exhibits but few particles. (Figs. 7 and 8 better illustrate subsequent changes in particle density at this point.) (a) × 24,000; (b) × 165,000; (c) × 30,000.
final line of re-established membrane continuity occurs at the posterior terminus of the equatorial segment, thus permitting a flatter fracture plane (Figs. 7, 8a, b).

Succeeding membrane fusion and consecutive loss of the sharp demarcation between the P faces of the plasma membrane and what must now be the inner acrosomal membrane (Fig. 8a, b), a second series of particle clearings emerges, arising as a string of clear sites (Fig. 8a, b), or as a smooth band across the plasma membrane of the postacrosomal segment (Fig. 9a).

Other plasma membrane modifications induced earlier, such as the increased clusters of particles fronting the striated ring (Fig. 8c) and the random dispersion of midpiece particles (Fig. 8d), remain unchanged.

**Phosphatidylycholine Liposomes**

Figs. 9 and 10 illustrate various images of the spermatozoal head after incubation and the acrosome reaction in liposome-containing media (−100−1,000 vesicles per cell). (We refer to multilamellar liposomes simply as large ones; unilamellar liposomes are so designated.) Before the addition of calcium, large liposomes are associated with the membrane in the equatorial region (Fig. 9b). Small unilamellar ones (not shown) cluster there as well. Clear circles in the plasma membrane over the acrosomal cap (Fig. 10a) and even some bulging ones (Fig. 10c) are readily demonstrated. With the addition of calcium, unilamellar liposomes appear to be attached to (Fig. 9c), or continuous with (inset, Fig. 9c) the hybridized plasma membrane in the immediate postequatorial zone. Large, smooth protuberances can also be observed in fractures of the plasma membrane equatorial regions (Fig. 10b).

Larger (>150 nm) unilamellar vesicles become abundant after the calcium is added (inset, Fig. 10a); nearly all were ~25 nm in diameter before this—vesicles identical to the smaller ones depicted in Fig. 9c and in the left side of the inset, Fig. 10a.

**DISCUSSION**

The study of changes in particle distributions attending membrane fusions in the spermatozoan system is plagued by a single discouraging feature and facilitated by several strongly beneficial ones. The pitfall in interpreting freeze-fractures of acrosome-reacted sperm lies in the similarity of the focal membrane particle deficiencies in reacted sperm to areas also present in injured sperm, the damage produced by pathological or mechanical rupture of the acrosomal and plasma membranes before fixation (11, 13, 14, 31). Even in the most successful of experiments when 40% of the sperm population have experienced acrosomal reactions, some acrosome-disrupted (11) spermatozoa remain, and they too may disclose barren areas.

Also, the effects of cryoprotectants on unfixed sperm are comparable to those on lymphocytes and *Amoebae* (19, 20), namely, the induction of particle-deficient domains. Thus, we work solely with fixed preparations. Observing such “wastelands” alone, then, is inadequate grounds for identifying sites of presumptive membrane fusion. Some vacant areas merely represent reversible or irreversible membrane injury (13).

Fortunately, however, in the guinea pig spermatozoon, a constellation of membrane changes correlates with the preparedness of sperm (capacitation) to undergo fusion of the acrosomal and plasma membranes. The combination of recognizing these corollary alterations and knowing the...
Freeze-fractures of the P face of the hybridized plasma membrane after the acrosome reaction. The tips of the sperm heads now point to the right. (a) and (b) Postacrosomal segment. At the far right, the juncture of the former acrosomal and plasma membranes is still visible. Behind it, a second series of clearings crop out (arrows, b). We speculate that this postacrosomal reaction clearing may be preparatory to sperm-egg fusion. (c) and (d) Displays of two features characteristic of capacitated and activated sperm populations, respectively; distinctions which persist after the acrosome reaction. (c) Particle clusters in front of the striated ring become more pronounced. (d) Small particles assume a disorderly arrangement in the midpiece, replacing the typical organized strands commonly found in spermatozoa before incubation (9, 11, 12). (a) × 42,000; (b) × 112,000; (c) × 50,000; (d) × 44,000.
FIGURE 9 (a) Postacrosomal segment after the acrosome reaction. On the right, the P face of the plasma membrane sometimes retains a slick-surfaced band behind the line of fusion (arrows). The remaining micrographs (Fig. 9, 10) portray varied appearances of cells incubated with liposomes in the Tyrode's solution. (b) and (c) Observed in fracture, large (multilamellar) liposomes hover in the region of the equatorial segment before the acrosome reaction (b). During and after the reaction, small unilamellar ones as well appear to attach (c) and fuse with the native membrane (inset) in loci corresponding to the zones of particle-cleared patches. V, small unilamellar liposomes. (a) × 60,000; (b) × 48,000; (c) × 110,000; inset, × 120,000.
FIGURE 10 The inset reflects the utility of tannic acid-mordant action for discriminating between vesicular unilamellar liposomes and the heavily stained native acrosomal (AM) and plasma (PM) membranes. The larger liposomes on the right are more common after the addition of calcium. × 100,000. (a) and (c) During incubation in capacitating media, in this instance with liposomes, clear circles (arrows) develop in the plasma membrane over the acrosomal cap where vesiculation will later commence. Occasionally, the sperm exhibit smooth bulges in these areas (c). (b) After the addition of calcium, more notable bulges are seen in the equatorial segment. We have not yet established whether the protuberances represent a stage in the incorporation of liposomes or the deletion of a lipid-rich region of the native membrane (as occurs in mast cells [16]). (a) × 44,000; inset, × 96,000; (b) × 50,000; (c) × 60,000.
location of the 1 × 6-μm zone of fusing membrane eases interpretation. As mentioned, corroborative modifications in the plasma membrane of the head include the growth of particle clusters in front of the striated ring (some are there at the outset), loss of the quilted pattern in the plasma membrane over the acrosome, and eventually, vesiculation of the acrosomal and plasma membranes anterior to the equatorial segment. In the midpiece, the herald of the acrosome reaction is the disaggregation of small particle strands overlying mitochondria, while in the principal piece, the population of 6-nm particles increases. The site of the broad zone where final acrosome membrane-plasma membrane fusion takes place is consistently within 1 μm of the parallel elevations which mark the end of the equatorial segment. And it is only this area, cleared of particles and morphologically coinciding with the other membrane changes, which we can confidently consider prepared for fusion between the plasma and acrosomal membranes.

Keeping in mind these confirmative points, and dismissing membrane clearances which are but preparative procedure−, or injury-induced artifacts, we may well ponder what the pervasive features which antecede fusion at this site are and ask what those changes actually mean. In the equatorial region, consistent alterations include a clearing of particles on the P face of the plasma membrane, accentuation of fibrillar profiles followed by their disappearance from the E faces of the outer acrosomal membrane and overlying plasma membrane, and, on both of these E faces, the scattering of large (>12 nm) particles. Therefore, incubation in a capacitating medium stimulates the development of particle-free areas in three of the four membrane halves which will be involved in the fusion. The fourth layer concerned, the outer half of the outer acrosomal membrane, is virtually devoid of particles from the beginning. Consequently, at the time of fusion, all four membrane halves are particle-deficient, three totally and one nearly so. (It would seem likely that rare particles persisting in the zone of fusion would have formerly resided in the outer half of the outer acrosomal membrane.) Our firm impression is that adjacent regions of both membranes lose their particles before fusion, and that particle deficiency in one membrane alone is insufficient to bring about the event of fusion. Certainly, in the liposome-plasma membrane unions, no particles are apparent in any of the four hemi-layers involved, the two native membrane layers and the two synthetic ones.

Let us first consider the loss of globular and elongated particles. It is generally accepted that particles examined in freeze-fracture preparations represent membrane-intercalated, protein-containing structures existing in a fluid lipid phase of the membrane. The translational mobility of these elements depends upon many factors: the intrinsic liquidity of the membrane lipids as determined by their phase-transition temperature, the length and degree of saturation of fatty acid chains, the amount of cholesterol, the mobility of attached surface receptors, and the extent of their association with cytoplasmic proteins (3, 23, 28, 35).

Unquestionably, we observe different classes of particles in freeze-fracture in the spermatozoan plasma membrane; particles differing in proportions or other aspects of the foregoing factors which govern their mobility. Since we cannot yet discriminate among the diverse particle groups in the equatorial segments of the acrosomal and plasma membranes of this system at the moment, we are unable to fully comprehend their functions or mode of departure from the fracture plane. Happily, there are a few hints.

The accentuation of fibrillar profiles palisaded around the clearing ellipses in the E faces of the acrosomal and plasma membranes affords us one clue. Although the observation per se fails to explain the particle loss, it does suggest that either filamentous microstructures are somehow involved with the disappearance of particles from the fracture plane, or that the usually globular particles, if they are susceptible to deletion, sustain an elongating deformation during the fracturing process. Thin sections provide no substantial implication favoring one or the other possibility; but, considering parallel situations in other systems, the former is the more attractive. The insertion of filamentous proteins may be a widespread phenomenon in membranes (3, 23, 28). Whatever the mechanism may be, our observations in this region are most consistent with a straightforward deletion of particles (i.e., the extraction of some protein structures).

After the acrosomal and plasma membranes become one, there is also the possibility of particle redistribution. After fusion, the exposed interior of the acrosomal membrane reveals a high particle density, whereas the density of small particles in the postacrosomal segment of the plasma membrane diminishes. The one or two strands of large
particles on the postacrosomal (plasma membrane) side of the fusion line (as in Fig. 7) may be a large-particle eddy from the acrosomal membrane, whereas the crest of a forward-flowing, small-particle wave leaves a trough behind, cleared as in Figs. 8b, 9a—this clearing, perhaps, in anticipation of fusion with the oocyte membrane. In the guinea pig, as in other mammals, it is this postacrosomal segment which initially participates in gamete fusion, although the terminal portion of the acrosomal membrane may also take part in this function (24).

Preceding the acrosome reaction, particle accretion into distinctive clusters in front of the striated ring could also be partly responsible for the overall diminution in particle density in the plasma membrane postacrosomal segment. But these clusters seem too distant to influence the sites of fusion; more likely, they serve another purpose.

Whether moved by perturbations originating on the inner or outer surface of the membrane, or set in motion by the establishment of continuity between particle-rich and particle-poor membrane regions, the net result is the loss of protein-containing structures from these segments of the spermatozoan membranes. Their absence precedes fusions.

Extensive, insightful discussions of possible functions of the persisting large particles intermingled with the cleared areas, the role of calcium ions, and the molecular mechanisms of the event of fusion itself are all cogent aspects of numerous recent papers (1, 2, 5, 6, 16–18, 26, 27, 32, 33, 37). Our morphological data lead us to favor the view (1, 2, 17, 18) that the development of smooth interfaces between phospholipid bilayers is the pervading feature of incipient fusion. With the addition of calcium ions, membranes thus prepared, unite. While the geometric positioning of specific particles such as "rosettes" may be helpful in determining these sites in other systems (7, 26, 27, 32, 33), we did not observe them here.

The possible incorporation of liposomes, or the exchange of liposome lipids at the physiologically prepared barren spots of native membranes, may be taken as a supportive finding that the clearing of the plasma membrane is indeed a primary implication of receptivity to fusion. At the time of the acrosome reaction, the outer surface of the plasma membrane is seemingly as well-disposed for fusion as the inner surface, a state which results in momentary, focal secretion-absorption coupling. In this instance, these observations have some pragmatic intimations. Fertilization will not normally occur if the spermatozoon does not undergo an acrosome reaction (4, 6, 38). Preventing the reaction by stabilizing the membrane so that it cannot clear, or by adding lipids which might be incorporated by the sperm but would not permit the ultimate fusion with the egg, might lead to novel forms of fertility control. Likewise, the receptiveness of lipid vesicles might be utilized as a test of the sperm's preparedness for fertilization, a practical concern in assessing the functional competence of spermatozoa in man.

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