BIOLOGY OF REPRODUCTION 23, 637–645 (1980)

Evaluation of the Purity of Boar Sperm Plasma Membranes Prepared by Nitrogen Cavitation

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ABSTRACT

Quantitative electron microscopy is used to provide new information regarding the purity and the amounts of plasma membrane originating from various regions of the surface of the boar spermatozoon when such membranes are prepared by nitrogen cavitation and sucrose density centrifugation. These data show that the plasma membranes originate primarily from the head of the spermatozoon; they also show that contamination of these plasma membranes by acrosomal and mitochondrial membranes is negligible. Immunolocalization, using plasma membrane vesicles and divalent IgG directed against plasma membrane antigens, shows that plasma membrane vesicles bind extensively to intact sperm plasma membranes but rarely bind to the outer acrosomal membrane. These new data, taken together with that which has been previously reported, indicate that high pressure gas cavitation provides a simple means for obtaining undamaged, highly purified sperm plasma membranes.

INTRODUCTION

The need for a method that provides plasma membranes of high purity from spermatozoa is of special importance in view of the many observations which indicate that the sperm surface membrane is altered during maturation in the male and during capacitation and fertilization in the female (Bedford and Cooper, 1978). However, experimental evidence demonstrating that a particular membrane preparation is pure is often limited to one or two criteria such as the purification ratio of enzymes presumed to be located solely in the membrane, and/or the homogeneity of the preparation when examined by electron microscopy. Our report on the utility of gas cavitation procedures to prepare plasma membranes from boar and human spermatozoa (Gillis et al., 1978) relied heavily on these criteria. We have since carried out new experiments to assess the purity of these membranes. This report presents the results of a study using quantitative electron microscopy to determine the amount of plasma membrane and other membranes from different regions of the boar spermatozoon that are released after gas cavitation. The observations show that these membranes are not contaminated with acrosomal or mitochondrial membranes.

Accepted July 16, 1980.
Received March 3, 1980.

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MATERIALS AND METHODS

Semen was collected from normal, adult Yorkshire, Duroc and crossbred boars and washed in a buffer as previously described (Gillis et al., 1978). The preparation of plasma membranes has been modified slightly from that described in the earlier report. Boar sperm (10^9–10^{10} cells) were washed in phosphate buffered saline (pH 7.4), resuspended in a volume of ∿100 ml, and placed in a Parr Bomb. The sperm were subjected to nitrogen pressures of either 500 lbs/in^2 (PSI), 650 PSI or 950 PSI for 10 min. The sperm were extruded into a small volume of buffer containing unneutralized EDTA, and the pH was adjusted to 5.0 to inhibit proteolysis; the final EDTA concentration was 1 mM. To ensure further the inhibition of proteolysis, buffers contained the antiproteases aprotinin (500 units/ml) and/or phenylmethylsulfonyl fluoride (1 mg/ml). To isolate membrane vesicles, the cavitate was washed at 4°C once at 1000 × g (10 min) and then the supernatant was washed by centrifugation at 6000 × g for 10 min at 4°C. The resultant supernatant (free of intact sperm) was centrifuged at 100,000 × g (4°C, 30 min) to pellet the membrane vesicles. Membranes were separated by sucrose density centrifugation in discontinuous gradients as described in Gillis et al. (1978) using a three-step gradient containing 1.0 M, 1.30 M,

and 1.57 M sucrose. Membranes used to obtain antiserum were obtained from the top of the 1.0 M sucrose.

Antisera (IgG) immunoglobulins against plasma membranes were prepared by inoculation of New Zealand White rabbits with 100–200 μg of membrane vesicles at 2–3 week intervals for 12 months. The initial injections used complete Freund's adjuvant; all subsequent injections used the incomplete adjuvant. Rabbits were bled from ear arteries. The IgG fraction was obtained from heat-inactivated serum by chromatography on DEAE Afigel Blue (Bio-Rad Corp., Richmond, CA). Control animals were treated the same way, but plasma membranes were omitted from adjuvants.

Sperm or membrane vesicle pellets were prepared for electron microscopy as follows: pellets were transferred to 2.5% glutaraldehyde in cacodylate buffer (pH 7.4). After 30 min, the glutaraldehyde-fixed material was transferred to vials containing 1% osmium tetroxide, 1.5% potassium ferrocyanide (Russell and Burguet, 1977). The tissue was dehydrated with increasing concentrations of ethanol, infiltrated with propylene oxide and embedded in Araldite. Sections showing silver or silver-gray interference colors were prepared, stained, and viewed on a Phillips 201 electron microscope.

Quantitative estimates of the plasma membrane removed from different areas of the sperm surface were carried out as follows: scale models of the boar spermatozoon were constructed from clay (∿61 cm in length). Paper of uniform weight was used to cover the entire surface. In constructing the model, the proportions and approximate three-dimensional shape of each of the segments (principal segment, equatorial segment, and postacrosomal region of the head; midpiece and principal piece of the tail) were measured from low magnification electron micrographs of freeze-fractured boar spermatozoa that showed these regions clearly. The paper from each segment was cut out and weighed and compared to the total weight to estimate the percentage surface area represented by that segment. At least four different fields from electron micrographs from the cavitates obtained at 650 PSI and 950 PSI were examined using a millimeter ruler to determine the fraction of membrane that had been disrupted (i.e., was missing) in a given segment on a particular spermatozoon.

The fields selected were chosen by arbitrarily dividing the thickness of the pellet into four layers. Micrographs were taken of each layer. The fields from which they were taken were not previewed except roughly to exclude areas with heavy contamination folds and low sperm density. In the quantitation procedure, it was assumed that "hits" of spermatozoa were random and that all parts of the spermatozoon were represented on the micrographs.

The measurements were averaged for a particular region. These averages and the percentage of the total surface area represented by each segment provided the means for estimating the amount of membrane originating from each segment of the boar sperm surface in a given population of vesicles.

RESULTS

To determine the origin of membranes released from spermatozoa by nitrogen cavitation at the pressure routinely used in our preparative procedure (650 PSI), electron micrographs of cavitated spermatozoa were prepared and are shown in Figs. 1–4. Figure 1 is typical of the type of survey micrograph used to quantitate the loss of plasma membranes after cavitation. Sperm cavitated at lower pressure appear similar to those shown in Figs. 1–4. The striking feature of the micrographs obtained at 500 PSI and 650 PSI is that only the plasma membrane is missing from the cavitated sperm. The outer acrosomal membrane, distinguishable by its "fuzzy" outer coat (Russell et al., 1980), does not vesiculate and remains in place. At the higher magnification (Figs. 2–4) the typical pattern of rupture at 650 PSI is seen. The plasma membrane overlying the principal segment of the head is missing, and there is some removal of plasma membranes overlying the equatorial (Fig. 2) and postacrosomal (Fig. 4) region of the head, but both the outer and inner acrosomal membranes are retained. Longitudinal and cross sections of the flagellum show that the plasma membrane is missing in some segments of the tail, but there is never evidence of rupture of the mitochondrial membranes (Fig. 3). A qualitatively similar pattern was observed when a lower gas pressure (500 PSI) was used.

Sperm that were cavitated at higher pressure (950 PSI) are shown in Fig. 5. At this nitrogen pressure the outer acrosomal membrane was removed along with major portions of the plasma membrane of the head. When the intact outer acrosomal membrane (Fig. 6) and one which has been freed from its sperm attach-

FIG. 1. Survey micrograph of the type used for the quantitative studies, showing boar sperm cavitated at 650 PSI. The head regions of the sperm cells are largely deficient in plasma membrane; however small portions of this membrane (arrows) can usually be found along a portion of the postacrosomal sheath (pas). The outer acrosomal membrane (curved arrows) is present (although perhaps perforated since the acrosomal contents are largely absent) and appears "puffed out" over the principal segments, but retains its typical spacing in the equatorial segment. Cross sections of some flagella show an intact plasma membrane; in others the plasma membrane is missing (asterisks) or partially removed. × 17,000.

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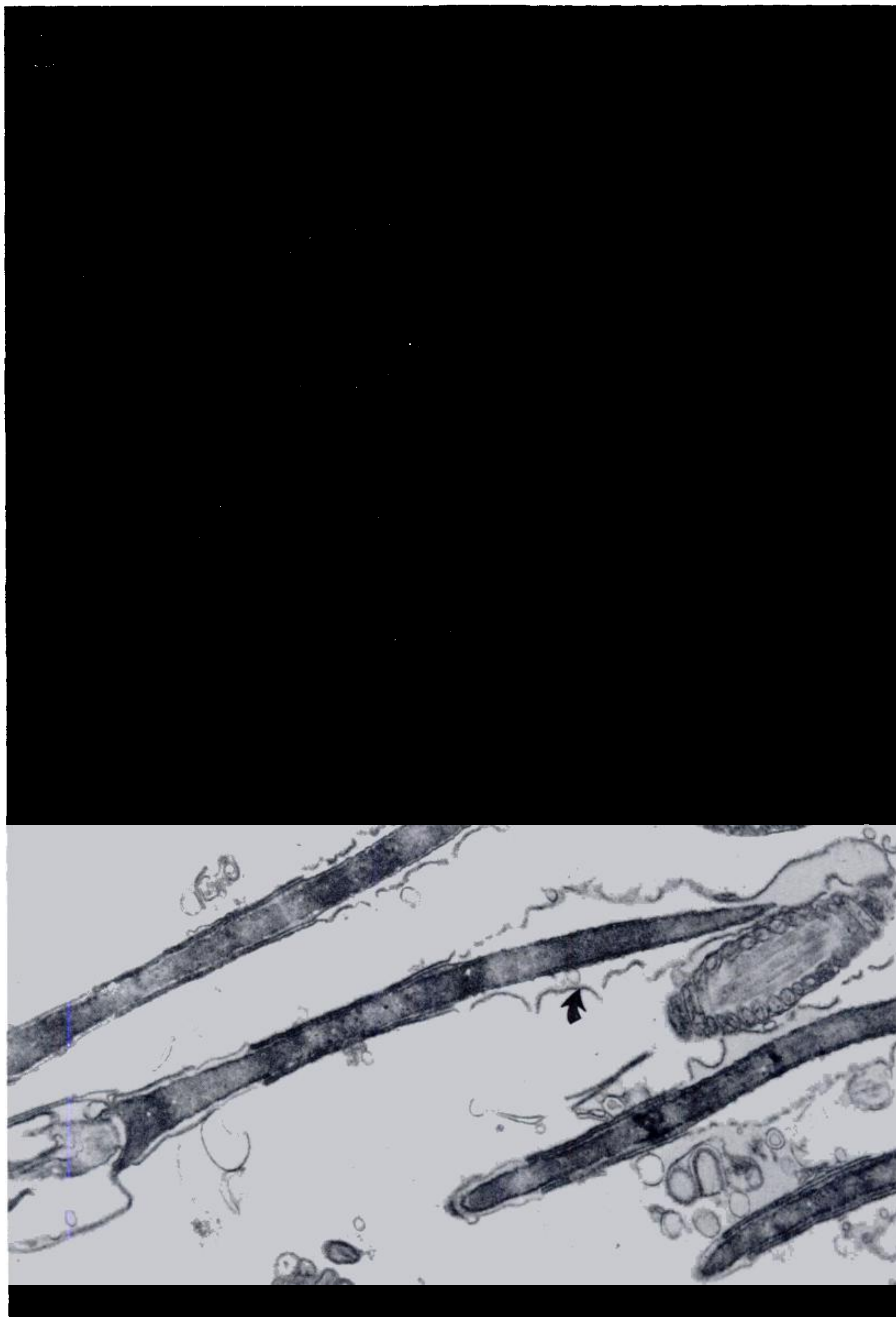

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ments (Fig. 5) are compared, both appear similar. This membrane is easily identified in sucrose gradients where it sediments at high sucrose density (∿1.57 M) and may be identified by its characteristic fuzzy appearance (Fig. 7). Note that most of the membrane does not vesiculate under the conditions of these experiments, as do plasma membranes which sediment at a lower density. An aggregation of such membranes at the 1.57 M sucrose interface is shown in Fig. 8.

To obtain a better estimate of the contribution of the various areas of the spermatozoon surface to the membrane vesicle population, quantitative analyses of electron micrographs of cavitates obtained at 650 PSI and 950 PSI were made (as described in Materials and Methods), and results are shown in Table 1. Virtually all the plasma membrane overlying the principal segment of the heads was liberated at both cavitation pressures. Membrane yields from the postacrosomal segment were slightly increased at the higher cavitation pressure. Plasma membrane yields from the principal piece and midpiece of the flagellum were also increased at the higher pressure. Despite the greater surface area of the flagellar plasma membrane, even at the highest pressure used in these experiments, the majority of membranes in the vesicle population originated from the head. From the Table, one can calculate that 51% of the plasma membrane was removed at a cavitation pressure of 650 PSI; of this, 78% originated from the head. At a pressure of 950 PSI, 61% of the plasma membrane was removed and 68% originated from the head. Note also that at the lower cavitation pressure the outer acrosomal membrane was present in more than 98% of the spermatozoa. In the four different low magnification fields examined, only one spermatozoon showed a portion of the outer acrosomal membrane to be missing. Most of the acrosomal membrane over the principal segment of the head, however, was missing at the higher cavitation pressures. Usually the outer acrosomal membrane remained at the equatorial segment. This is possibly due to the presence of bridging structures, which hold the acrosomal membranes together in this region (Russell et al., 1980).

The observations quantitated here for a single cavitate were qualitatively similar in other cavitates examined at the same pressure. Indeed, qualitative examination of cavitates at lower gas pressure suggests, as do these quantitative estimates, that the relative proportion of flagellar plasma membrane in vesicle populations may be inversely related to the gas pressure. The obvious significance of this will be discussed below.

In another type of experiment we determined whether antibodies raised against plasma membranes were specific for plasma membranes or whether they would react with other membrane types. To do this, washed sperm were mixed with antimembrane IgG and allowed to agglutinate; membrane vesicles were then mixed with the sperm suspension. Extensive agglutination was induced by the divalent antibodies. This bridging interaction involved the plasma membrane of intact sperm with other sperm, sperm with vesicles, and vesicles with other

FIG. 2. Typical boar sperm cavitated at 650 PSI. In some regions where plasma membrane on sperm heads and flagellum is present, it is indicated by small arrows. The outer acrosomal membrane (curved arrows) is present and over the sperm head. × 20,000.

FIG. 3. Longitudinal section of a 650 PSI cavitate flagellum showing regions of intact and missing (indicated by solid triangles) plasma membrane. Compare this 650 PSI cavitate with the 950 PSI cavitate of Fig. 5. Mitochondria of the principal piece of the flagellum are not disrupted by this procedure. × 25,000.

FIG. 4. The caudal region of this sperm head is typical of most 650 PSI cavitated sperm in that the plasma membrane (small arrows) is present overlying only a portion of the postacrosomal sheath (pas). The remainder of the sperm head lacks this membrane. × 54,000.

FIG. 5. Typical boar sperm cavitated at 950 PSI showing all or portions of the following membranes: plasma (small arrows), outer acrosomal (curved arrow), and inner acrosomal (arrowhead). Also indicated is the postacrosomal sheath (pas). The plasma membrane extends only a short distance up the postacrosomal sheath, but in this micrograph, atypically, is also seen overlying the equatorial segment. The inner acrosomal membrane is intact throughout, but the outer acrosomal membrane is missing over a portion of the equatorial segment and completely over the principal segment. Compare this 950 PSI cavitated sperm with the 650 PSI cavitated sperm shown in Fig. 2. × 30,000.

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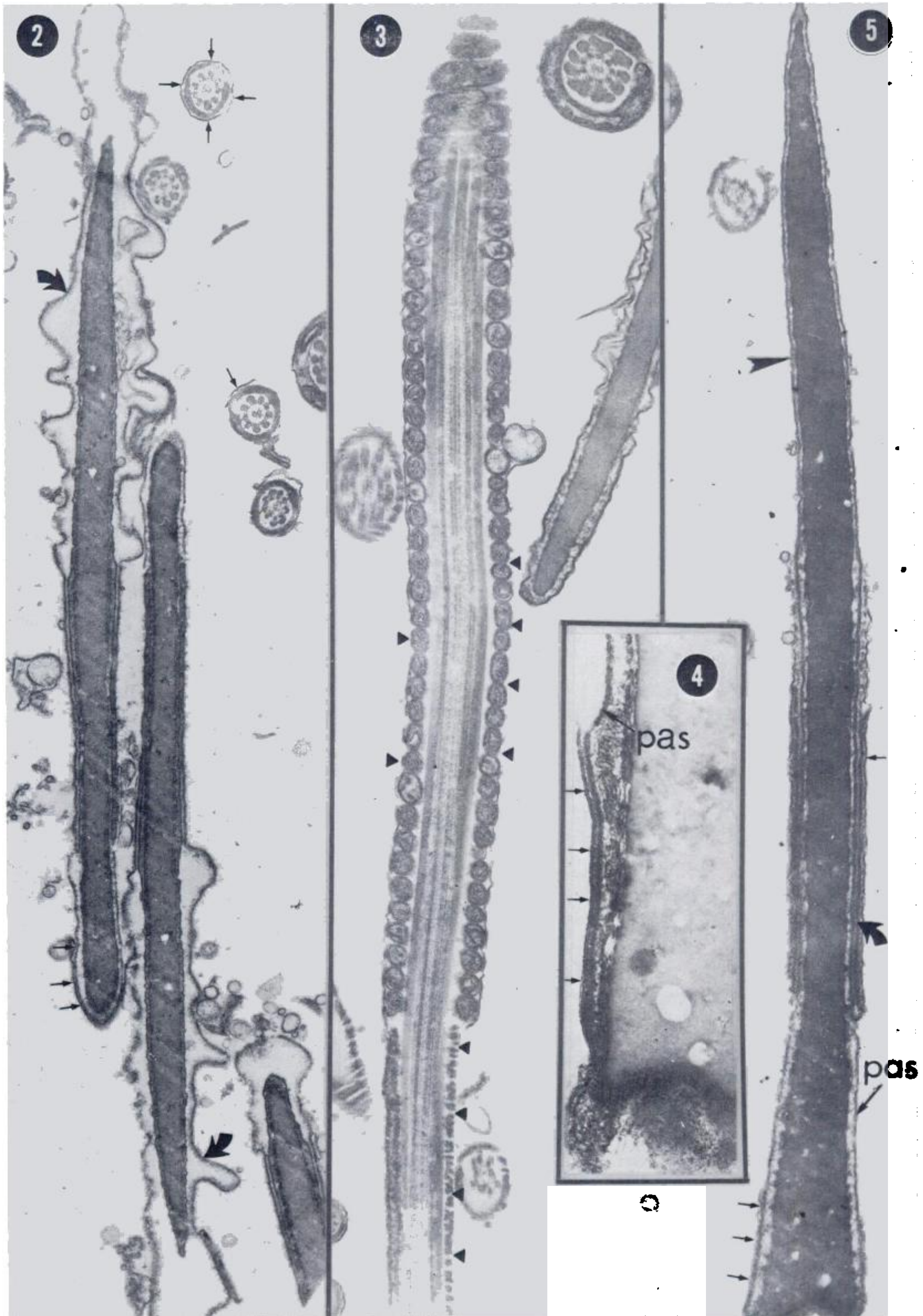

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vesicles (Figs. 9, 10). In those few sperm where the outer acrosomal membrane was exposed, vesicle binding was rarely observed. This was also found to be the case when the inner acrosomal membrane was exposed. This indicates that the acrosomal membrane was not a significant contaminant of plasma membrane preparations used to prepare the antiserum. We note that IgG from control animals does not agglutinate sperm nor does it form immune precipitates with detergent-solubilized plasma membrane as does IgG from immunized animals.

DISCUSSION

Various experimental approaches have now been used to assess the purity of boar sperm plasma membranes isolated by gas cavitation. Using electron microscopy, (Gillis et al. (1978) and Peterson et al. (1980) showed that the membrane vesicles were homogeneous and free of nonmembranous contaminants. Enzymological studies showed the membranes to be enriched approximately eightfold in surface enzyme marker 5′-nucleotidase and more than 20-fold in adenylyl cyclase. In this study new evidence, obtained by electron microscopy, has shown that acrosomal membranes and mitochondrial membranes are not contaminants of the plasma membrane preparations. Antisera raised against plasma membranes recognized only plasma membranes and not acrosomal membranes. The sum of the results of these morphological, enzymological and immunological studies, we believe, satisfy stringent criteria for purity and indicate that the cavitation procedure we have described is suitable for preparing boar sperm plasma membranes virtually free of other membrane types.

The population of homogeneous membrane vesicles, obtained by nitrogen cavitation, sediments between 1.0 M–1.30 M sucrose in discontinuous gradients and forms multiple bands in linear sucrose gradients between these densities. This apparently reflects the lack of uniformity in the density of the plasma membrane in different regions of the sperm surface. Such discontinuities in plasma membrane density are suggested by differences in the protein distribution, as seen in freeze-fracture preparations for electron microscopy, between the plasma membrane overlying the acrosomal and postacrosomal regions of the boar spermatozoon (Russell et al., 1980). Other studies in this laboratory and elsewhere (Friend, 1977) also show unique features in the structure of the flagellar membrane. However, to date, surface membrane markers that could be used to identify these subpopulations of vesicles are not available.

Our original selection of a cavitation pressure (650 PSI) had been somewhat arbitrary, but it was clear that at this pressure plasma membranes do not vesiculate uniformly along the surface of the spermatozoon and that plasma membrane vesicles originate predominantly from the head. Since higher cavitation pressures increase the yield of flagellar plasma membranes, it is conceivable that progressively lower gas pressures may yield progressively more plasma membrane from the head. While it is possible to obtain head plasma membranes (fused with outer acrosomal membranes) by chemical methods (Gillis et al., 1978), a method that minimally disturbs membrane structure would be more desirable. Disruption of the

FIG. 6. Sperm cavitated at 950 PSI and pelleted at high speed. For orientation purposes the nucleus (N), equatorial segment (es), and principal segment (ps) of the acrosome are indicated. The outer acrosomal membrane (curved arrows) appears fuzzy regardless of whether it is attached to the sperm or completely removed (membrane at the lower right of the figure). The inner acrosomal membrane is indicated. × 135,000.

FIG. 7. 1.57 M sucrose interface membranes. Indicated is the unvesiculated outer acrosomal membrane (curved arrow) and nearby vesicles of unknown origin. × 57,000.

FIG. 8. Another example of the 1.57 M sucrose interface membranes showing unvesiculated outer acrosomal membrane (curved arrow) and nearby vesicles of unknown origin. The typical organization of outer acrosomal membrane indicates that this membrane may be isolated by relatively pure form. × 15,000.

FIG. 9. Sperm treated with anti-sperm plasma membrane IgG and subsequently exposed to plasma membrane vesicles. Several sperm in which the intact sperm plasma membranes are decorated with plasma membrane vesicles are shown. Vesicles bind to all regions of the plasma membrane of the heaa and flagellum, although they are less numerous on the latter (arrows). One sperm (asterisk) is lacking a plasma membrane, and the outer acrosomal membrane (oam) is exposed. No vesicles are seen on this membrane.

FIG. 10. Sperm treated with anti-sperm plasma membrane IgG and subsequently exposed to plasma membrane vesicles. Two sperm flagella are shown in cross section. Sperm are bound not only to vesicles (v) but to each other (agglutination). × 102,000.

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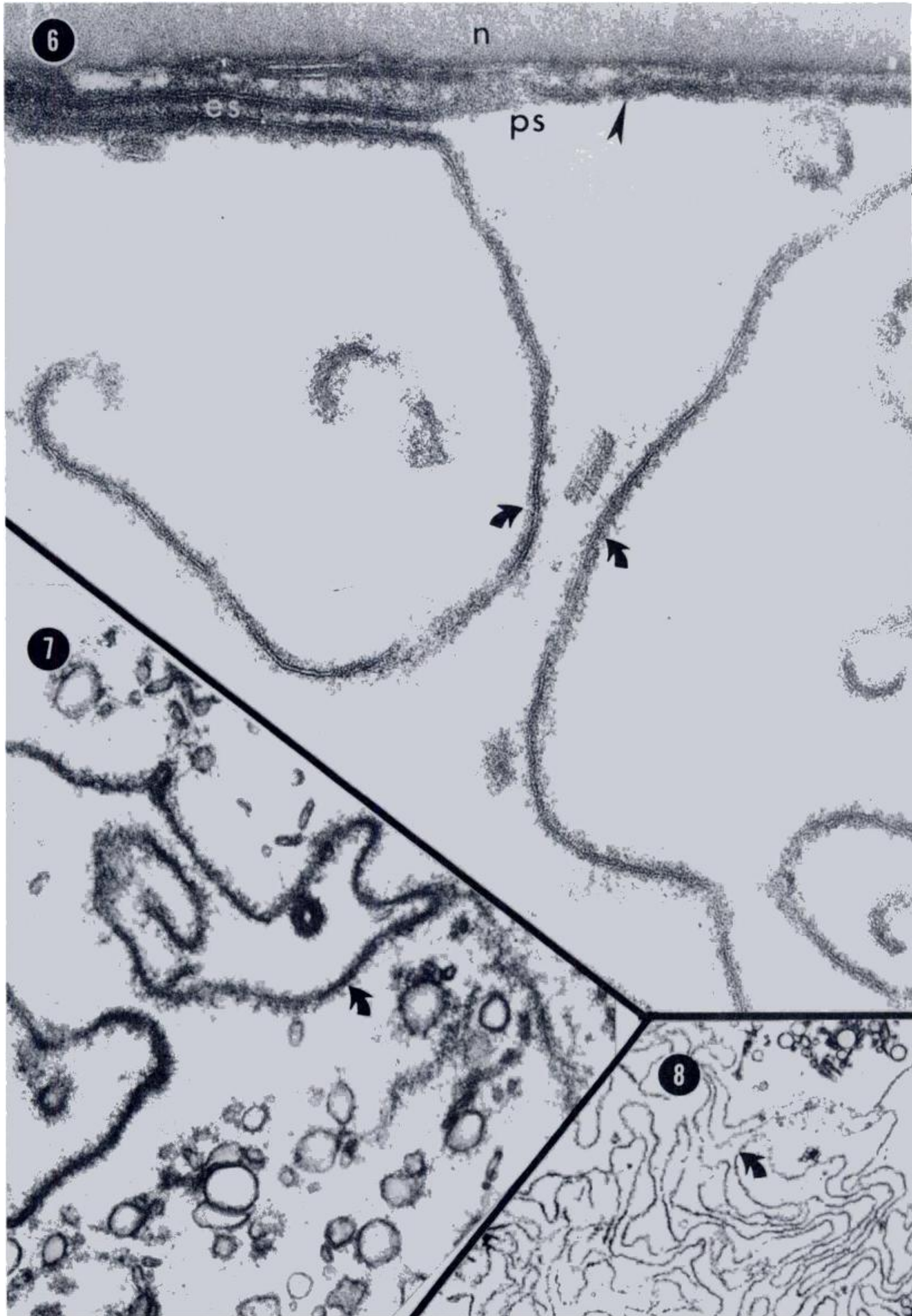

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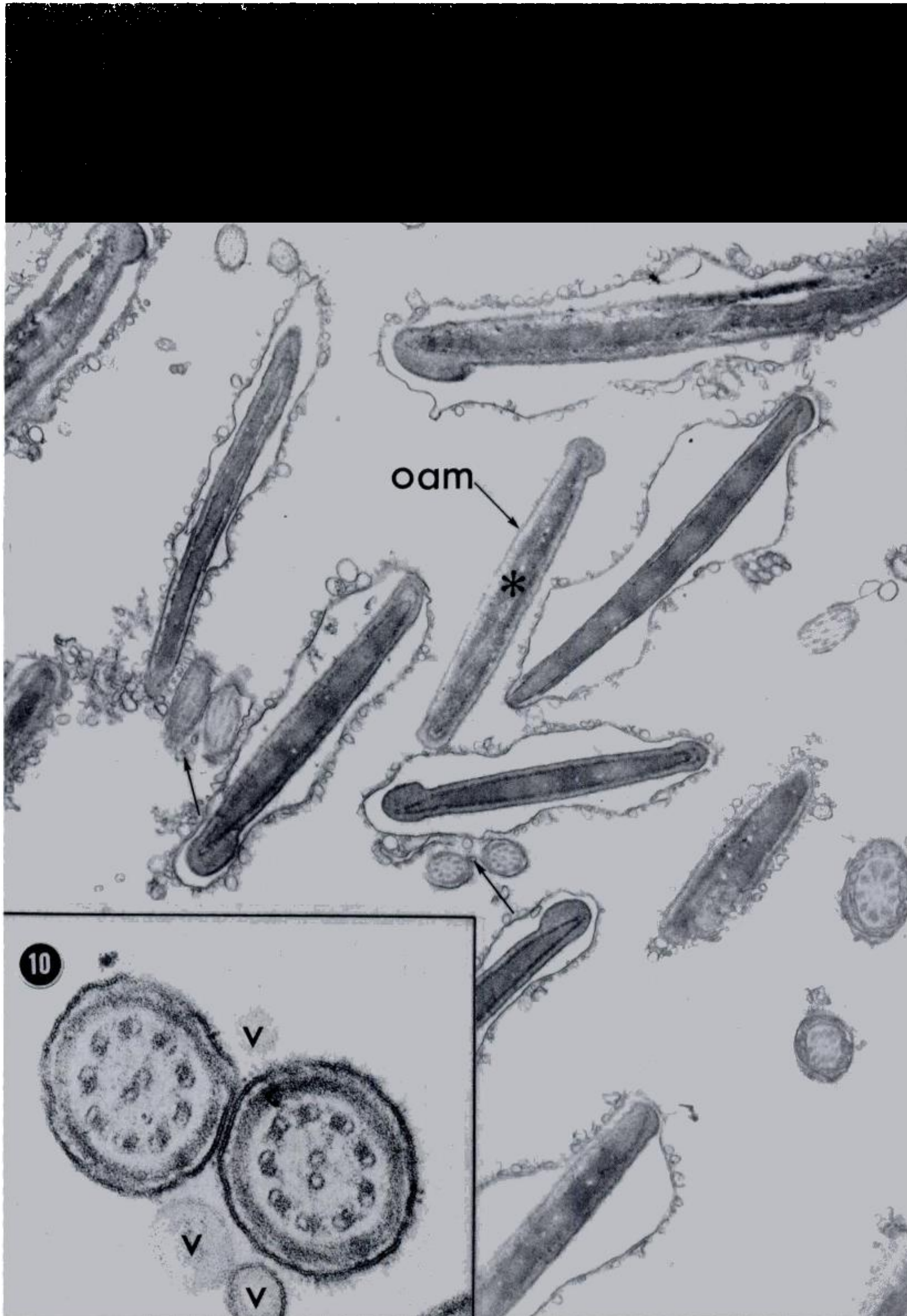

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TABLE 1. Estimated loss of plasma and acrosomal membranes from different regions of the boar spermatozoon after nitrogen cavitation at 650 PSI and 950 PSI.

Segment examined	% Surface area	% Membrane missing	
		650 PSI	950 PSI
Plasma membrane			
Head			
Principal segment	22.6	99.7 ± 0.2 (32)[a]	100.0 ± 0.0 (21)
Equatorial segment	11.0	93.2 ± 2.7 (18)	98.8 ± 0.8 (22)
Postacrosomal region	10.2	70.5 ± 6.8 (16)	76.6 ± 7.4 (11)
Tail			
Midpiece	25.6	17.9 ± 4.3 (28)	34.2 ± 4.4 (46)
Principal piece	30.6	21.6 ± 3.6 (34)	34.4 ± 6.1 (29)
Outer acrosomal membrane			
Principal segment	...	1.3 ± 1.3 (32)	89.7 ± 5.1 (21)
Equatorial segment	...	0.0 ± 0.0 (18)	10.6 ± 6.2 (22)

[a]Numbers in parentheses are the number of individual sperm measured. Values are averages ± SEM.

sperm surface by gas cavitation may provide the means.

The methodologies employed herein have been shown to be valid for boar sperm and to a lesser extent human sperm (Gillis et al., 1978). Caution must be exercised in using these procedures for other species until rigouous tests for purity can be carried out.

ACKNOWLEDGMENTS

The use of the electron microscope facilities at the Springfield campus of Southern Illinois University was greatly appreciated. The excellent technical assistance of Ms. Terri Russell, Mr. Ray Venezia, and Mr. S. Usaha is acknowledged. This study was supported by NIH Grant HD 10947.

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