THE AFLAGELLATE SPERM OF CALLIANASSA CALIFORNIENSIS

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The Aflagellate Sperm of *Callianassa californiensis*

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INTRODUCTION

Ghost shrimp Callianassa californiensis Dana (1854) (Thalassinoidea, Anomura, Crustacea) are intertidal residents of estuaries and bays from Alaska to Baja California. Despite their common name, Callianassa are more closely related to crayfish, lobsters, and crabs than to shrimp. Their muddy burrows are well known to fishermen who use Callianassa as bait for croaker, turbot, (Morris et al., 1983) and sturgeon, and to aquaculturists whose oyster beds the shrimp disrupt with their constant digging (MacGinitie and MacGinitie, 1968; Morris et al., 1983). Studies have been written on the ecology, physiology, and larval dispersal of Callianassa (MacGinitie and MacGinitie, 1968; Felder et al., 1986; Griffis and Chavez, 1988; Johnson and Gonor, 1982) yet little is known regarding reproduction, especially gamete development, function, and ultrastructure. Because of their economic value and phylogenetic relationships to other decapods, reproduction in Callianassa is of interest. In this study, findings regarding the ultrastructure and acrosome reaction of the mature sperm of Callianassa californiensis are reported and compared to selected studies of sperm of other decapod crustaceans. Callianassa sperm share some of the peculiarities of the

aflagellate, non-motile sperm of other decapods which have stimulated the curiosity of researchers (Clark et al., 1984; Dudenhausen and Talbot, 1982; Moses, 1961).

While "typical" animal sperm are elongate cells with a head, midpiece, and flagellar tail, decapod sperm are usually spherical, aflagellate, and possess spikes or processes which radiate from the nucleus or cell body. Typical sperm swim toward the egg propelled by undulations of the whip-like flagellum; most crustacean sperm are immotile (Clark et al., 1984; Talbot and Summers, 1978). The chromatin of more typical sperm is condensed; mature decapod sperm chromatin is uncondensed and fibrillar (Shigekawa and Clark, 1986). These decapod characteristics are shared with all but three classes of crustaceans, the Cirripedia, Mystacocarida, and Branchiura which have more typical sperm (Adiyodi and Adiyodi, 1983; Brown, 1970). Baccetti contends that aflagellate sperm are an evolutionary response to internal fertilization or passive delivery of sperm in spermatophores in species in which the sperm are deposited directly on the egg mass. The axoneme within the flagellum of passively delivered sperm has become aberrent, reduced in motility, or lost in some species (Baccetti, 1986).

In the order Decapoda are two suborders, Pleocyemata and Dendrobranchiata (Barnes, 1987). The former includes the infraorders Brachyura (true crabs); Anomura (sand crabs and hermit crabs); Astacidea (lobsters and crayfish); Palinura (spiny lobsters); and Caridea (most shrimps). The sperm of animals in these groups are usually spherical with numerous radial processes. The members of the suborder Dendrobranchiata, with the single infraorder Penaeidea, produce sperm which bear a single anterior spike.

Sperm Ultrastructure

Brachyuran sperm, exemplified here by Libinia emarginata, the spider crab, and Callinectes sapidus, the blue crab, are characterized by a cup-shaped nucleus which surrounds a large and complex acrosome. Only the electron dense apical cap of the acrosome is free of nuclear material. Central to the acrosome is the acrosomal tubule, which in Callinectes is contained in a central canal. The acrosomal tubule includes fibrous material at its apex and granular material at its base (Brown, 1966). In Libinia the distal acrosomal tubule is filled with "large microtubules" (Hinsch, 1969). Around the tubule in both species the acrosome consists of layers of material of varying electron density. In Callinectes the central canal is enveloped in a

filamentous layer of "acrosomal rays" within a layer of "large microtubules". In both Callinectes and Libinia a "thickened ring" is found at the base of the canal or tubule. In Libinia two centrioles are located within the tubule near the thickened ring. Below the thickened ring in both species a membranous lamellar area called the central region separates the acrosome from the nucleus. Both Callinectes and Libinia have radial processes whose nuclear content has been affirmed by positive Feulgen staining (Brown, 1966; Hinsch, 1969). In Libinia, microtubules (22-27 nm) originate at the centrioles and enter the nucleus through "pores"; they traverse the nucleus and extend the length of the four processes. Three processes project radially from the cell; the fourth, the posterior median process, projects posteriorly, perpendicular to the other three (Hinsch, 1969). In Callinectes sperm, which have eight processes, the nuclear material is loosely packed (Brown, 1966).

Most sperm of Astacidea and Palinura are similar to brachyuran sperm; they are roughly spherical, possess radial processes, and are non-motile. Unlike brachyuran sperm the acrosome is not centrally located; it is anterior to the nucleus rather than within it. The numbers of radial arms

emanating from the nucleus range from zero in Cherax tenuimanis and C. albidus to 15-20 in Pacifastacus leniusculus (Beach and Talbot, 1987; Dudenhausen and Talbot, 1982). In Pacifastacus leniusculus and in the palinurans Panulirus argus and P. guttatus the processes contain microtubules which originate in the nucleus and extend to the ends of the processes (Dudenhausen and Talbot, 1982; Talbot and Summers, 1978). The nucleus is uncondensed and contains microtubules. A characteristic of these sperm is the membrane lamellar complex or body in the region lateral to the acrosome and the nucleus. This structure is composed of stacks of convoluted membranes. Although they are degenerate or absent in most astacideans, centrioles have been found in this area in Cherax albidus and Panulirus (Beach and Talbot, 1987; Talbot and Summers, 1978). Mitochondria are found in the lamellar complex in Cherax, Pacifastacus, and Panulirus, although they are degenerate or absent in other species. For example, in the crayfish Procambarus clarkii the mitochondria are engulfed by sustentacular cells before the sperm mature (Moses, 1961). The lamellar area may be analogous to the central region of brachyuran sperm. The acrosome is usually complex, containing areas of varying electron density and an inner or

subacrosomal area, possibly comparable to the tubule or canal of brachyurans. In Panulirus and Pacifastacus there is a crystalline or crystalloid configuration within the acrosome (Talbot and Summers, 1978; Dudenhausen and Talbot, 1982). The acrosome and nucleus are bounded by their own membranes; the plasma membrane encloses the entire sperm.

The anomuran most closely related to Callianassa whose sperm have been studied is the mole crab Emerita talpoida. Its sperm resemble astacidean sperm more closely than sperm of brachyurans. The sperm are spherical, with a large uncondensed nucleus, and have three to nine arms which are Feulgen negative. The acrosome is anterior to the nucleus (Barker and Austin, 1963).

The sperm of members of the infraorder Dendrobranchiata differ from sperm of the infraorder Pleocyemata reviewed above. The sperm of the penaeid shrimp Sicyonia ingentis are representative of this group. In these unistellate sperm an anterior spike proceeds from a central cap which encompasses the acrosomal vesicle and the subacrosomal region. Posterior to the central cap is the nucleus, surrounded by a thin layer of cytoplasm, and without a nuclear membrane. The radially arranged components of the acrosomal vesicle are the anterior

granule, a membranous region called the membrane pouches, and the coiled anterior spike (Clark et al., 1981). Actin has been identified as a component of the spike in Sicyonia brevirostris and other related species (Brown et al., 1976). In the subacrosomal area a cylindrical formation, the extended saucer, rises from a crystalline substance; it spreads radially into "petals" below the posterior acrosomal membrane. The extended saucer is surrounded by a granular region. Below the extended saucer lies the crystalline lattice, a screen-like formation alternately electron lucent and electron dense (Clark et al., 1984), reminiscent of the crystalline formations found in the acrosomal area of Panulirus and Pacifastacus (Talbot and Summers, 1978; Dudenhausen and Talbot, 1982). The electron dense nuclear plate separates the acrosomal area from the nucleus. The acrosomal membrane which encloses the anterior granule, the membrane pouches, and the spike has fused with the plasma membrane to form a pentalaminar membrane around the acrosome; the nucleus and small amount of cytoplasm are bound peripherally by the plasma membrane and apically by the nuclear plate. Within the nucleus are membrane lamellar bodies and uncondensed, fibrillar chromatin (Shigekawa and Clark, 1986). As in the astacidean Procambarus the

mitochondria have been sloughed into the extracellular matrix (Moses, 1961; Shigekawa and Clark, 1986).

The sperm of Cirripedia, Mystacocarida, and Branchiura do not conform to this model of aflagellate sperm bearing radial processes. The filiform sperm of barnacles resemble typical sperm having a flagellum with the standard 9+2 axoneme pattern as well as mitochondria and a distal centriole. Glycogen reserves are stored along the flagellum and a secretory or protoplasmic vesicle lies next to the nucleus (Adiyodi and Adiyodi, 1983; Brown, 1970). The interstitial Mystacocarida also have "typical" thread-like, flagellate sperm (Adiyodi and Adiyodi, 1983.) Branchiurans, such as Argulus sp., have lengthy, helical sperm whose elongated organelles stretch almost the length of the sperm. A trough-shaped structure which may be an acrosome also parallels the length of the sperm. The flagellum contains an axoneme with the standard 9+2 pattern of microtubules (Adiyodi and Adiyodi, 1983; Brown, 1970).

Other unusual crustacean sperm are those of the members of the class Branchiopoda, including the order Anostraca (fairy shrimp) and the suborder Cladocera (water fleas). These animals have aflagellate sperm which may

reach the eggs using pseudopods. This aberrant sperm apparently has no acrosome (Adiyodi and Adiyodi, 1983).

Acrosome Reaction

In decapod sperm the acrosome reaction has been observed in the brachyurans Callinectes and Libinia, the astacidean Homarus americanus, and in the penaeid Sicyonia. In Callinectes and Libinia sperm, whose reaction is similar, the acrosome reaction begins when the apical cap of the acrosome contacts and binds to the egg investments or chorion. The apical cap opens up or everts, allowing the expansion and elongation of the acrosome through the cap into the chorion. The acrosomal area surrounding the tubule everts and enters the chorion; the acrosomal tubule follows, making contact with the egg plasma membrane or oolemma. The sperm membrane, which is now the exposed inner posterior acrosome membrane, fuses with the oolemma. Until the acrosome and its tubule are embedded in the chorion the nucleus remains above the chorion. After membrane fusion the nucleus enters the egg cytoplasm (Brown, 1966; Hinsch, 1971).

In Sicyonia the acrosome reaction occurs in two phases, first, the exocytosis of the acrosomal contents; then the formation and extension of the acrosomal filament.

The reaction begins when the spike contacts and binds to the vitelline envelope. The spike begins to depolymerize, and the pentalaminar membrane dehisces and folds back, releasing enzymes packed in the acrosomal vesicle and membrane pouches. The spike disintegrates and the sperm binds to the glycocalyx of the egg. While the sperm is bound to the egg, jelly is released from cortical crypts in the egg's surface engulfing the sperm (Clark et al., 1984). During the second phase of the reaction the extended saucer lengthens, forming an acrosomal filament which increases the distance between the crystalline lattice and the petals at the end of the filament. The filament grows to approximately 10 um in length and contains numerous "tubular-like structures" which appear continuous with the crystalline lattice (Griffin et al., 1988).

Experimental approach

Feulgen staining, observation with the light and electron microscopes, and ionophore induced reactions are some of the techniques commonly employed in studies of sperm. In this study these techniques and identification of mitochondrial activity, extraction of membranes, immuno-gold labeling, and x-ray analysis have been employed and are described briefly below.

Nuclear staining

Feulgen staining has been used to identify nuclear material in numerous species. In the Feulgen reaction alcohols and glycerols in nuclear material are oxidized to aldehydes; the aldehydes react with Schiff reagent and this material is stained a purple-red color (Humason, 1972). In Callinectes and Libinia positive Feulgen staining has identified the nucleus and the continuity of the nucleus with the radial processes (Brown, 1966; Hinsch, 1969). In Panulirus the nucleus is Feulgen positive and although the processes are contiguous with the nucleus, they are negative for the Feulgen stain (Talbot and Summers, 1978). Barker and Austin (1963) found the radial processes of Emerita talpoida to be negative to Feulgen stain. In this study Feulgen staining was used to identify the nucleus before and after the acrosome reaction, and to assess the presence of nuclear material in the radial processes.

Pyruvate dehydrogenase activity in mitochondria and membrane lamellar bodies

In flagellate sperm, mitochondria are located near or around the tail to provide energy for movement. In the mature sperm of decapod crustaceans the activity and in some

cases the presence of mitochondria is questionable. In Cherax mitochondria are present but they lack cisternae and may not be functional (Beach and Talbot, 1987). Mitochondrial remnants are found in the lamellar region of Pacifastacus sperm (Dudenhausen and Talbot, 1982). In Callinectes, Procambarus, and Sicyonia the mitochondria have been sloughed off and are not present in mature sperm (Brown, 1966; Moses, 1961; Shigekawa and Clark, 1986). In some species the mitochondrial functions may have been assumed by the central region or membrane lamellar complex (Brown, 1966; Beach and Talbot, 1987). Mitochondria are found in Callianassa in the cytoplasm peripheral to the nucleus where membrane lamellar bodies are also located. If either of these organelles is actively producing energy, it should contain pyruvate dehydrogenase. To detect this enzyme the sperm are treated with pyruvate, copper sulfate, and potassium ferrocyanide. If active, the pyruvate dehydrogenase will react with the copper sulfate and potassium ferrocyanide to produce copper ferrocyanide. The copper ferrocyanide will be visible with transmission electron microscopy as electron dense plaques of approximately 0.02 um within the organelle (Nestorescu et al., 1973).

Microtubule identification

Microtubules have been identified in the radial processes and in the nucleus of several decapod genera including Pacifastacus, Panulirus, and Libinia, but not in Emerita or in Sicyonia. To insure that microtubules are positively identified, if present in Callianassa, an extraction technique and an immuno-gold labeling procedure were used. Extraction with detergent disrupts hydrophobic associations of the bilayer of the plasma membrane and membranes of organelles (Alberts et al., 1989). Not only are the plasma membrane, nuclear envelope, and organelle membranes broken apart, but the endoplasmic reticulum is destroyed. This releases membrane bound proteins and makes the cytoskeleton visible to electron microscopy. This technique was used to remove cellular membranes and to disclose the microtubules.

Immunogold labeling of tubulin (the structural protein of microtubules) was employed to locate and identify microtubules. Primary antibodies to sea urchin tubulin raised in mouse and secondary anti-mouse antibodies raised in goat were used. The secondary antibodies (goat anti-mouse antibodies) were conjugated to gold particles. If tubulin is present in the sperm, when the primary antibodies

are placed on the sample they should bind to the tubulin. The secondary antibodies should bind to the primary antibodies, and the tubulin-antibody complex may be viewed with the transmission electron microscope. If present, the gold particles are revealed as electron densities gathered around the tubulin (Langanger et al., 1984).

Electron dense granules

Energy dispersive X-ray analysis was used to determine the composition of electron dense particles found in reacted sperm (see acrosome reaction, below). In conjunction with an electron microscope this technique analyzes the electron emissions of the sample. When the sample is bombarded with an electron beam from the microscope, electrons in the sample are disrupted and energy in the form of x-rays is released. The energy released is characteristic of the particular element releasing it, allowing the element to be identified (Postek et al., 1980).

Acrosome reaction

The calcium ionophore A23187 has been used successfully to induce acrosome reactions in animals ranging from echinoderms to mammals and the induced reaction is considered to mimic the natural event (Talbot and Chanmanon,

1980; Clark et al., 1981). This divalent ionophore transports extracellular calcium across the sperm plasma membrane triggering the reaction (Talbot et al., 1976, Decker et al., 1976). In decapod sperm Clark et al. (1981) and Talbot and Chanmanon (1980) used ionophore A23187 to induce the acrosome reaction in Sicyonia ingentis and the American lobster Homarus americanus respectively. In the current study, ionophore A23187 was used to induce the acrosome reaction in Callianassa.

METHODS AND MATERIALS

Specimens of Callianassa californiensis were collected from Bodega Bay Harbor and the Hayward shoreline of San Francisco Bay. For transmission and scanning electron microscopy sperm, spermatophores, and sections of the vas deferens and testis were removed from mature males, fixed in 2.5% glutaraldehyde buffered in sea water (pH 7.2) for 30 to 90 min, washed twice in sea water, and post-fixed in 1% osmium tetroxide with sea water buffer for 30 min. Additional specimens were fixed in Karnovsky's fixative (Karnovsky, 1965). The specimens were rinsed in distilled water and dehydrated in a graded ethanol series. For transmission electron microscopy (TEM) the material was embedded in Spurr's resin (Spurr, 1969) or L R White (London Resin Company), and sections were cut with diamond or glass knives on a Reichart OM-U2 Ultramicrotome. The sections were stained with 8% aqueous uranyl acetate for 20 min, lead citrate (Reynold's) for 2 min and examined on a Hitachi HS-8 or Zeiss 902 transmission electron microscope. For scanning electron microscopy (SEM) specimens were fixed, washed, and dehydrated as described above. They were then critical point dried in a Polaron critical point drier using CO_2, sputter-coated with 35 nm of gold/palladium with a

Hummer VII sputter-coater, and observed with a Hitachi S-570 or Amray 1810 SEM.

Phase contrast and Nomarski differential interference contrast observations were performed on an Olympus BH2 compound microscope.

Nuclear staining

One half micron sections of unreacted mature sperm and ionophore reacted mature sperm were treated with Feulgen stain using an adaption of the procedure described by Humason (1972). Sections were rinsed and hydrolyzed in 5 N HCL, stained in Schiff's reagent for 2 hr at 60 degrees C, and then washed, rinsed, and counterstained in fast green for 10 min at 60 degrees C. They were viewed with an Olympus BH2 microscope, and photographed using Kodachrome 64 film.

Pyruvate dehydrogenase activity in mitochondria and membrane lamellar bodies

To determine if the mitochondria were active or if the membrane lamellar bodies were performing mitochondrial functions, the sperm were treated with pyruvate, copper sulfate, and potassium ferrocyanide following the protocol recommended by Nestorescu et al. (1973). The vas deferens

was dissected into 0.5 mm units and divided into experimental and control groups. The experimental material was incubated in a solution of 0.1 M sodium citrate, 30 mM copper sulfate, 5 mM potassium ferrocyanide, 40 mM magnesium chloride, 0.2 M sucrose, and 12 mM sodium pyruvate buffered in 0.05 M sodium cacodylate in seawater. The control group was treated with the same solutions without the sodium pyruvate and copper sulfate. After 20 min of incubation both treatments were rinsed in sodium cacodylate buffer and fixed in 2.5% glutaraldehyde (with sodium cacodylate buffer) for 1 hr and postfixed in 1% osmium tetroxide (sodium cacodylate buffer) for 1 hr. Following dehydration in ethanol and infiltration, the samples were embedded in Spurr's resin, sectioned, and viewed with an Hitachi HS-8 transmission electron microscope.

Identification of microtubules by extraction and immunogold labeling techniques

In order to locate microtubules and to establish their presence in the nuclear processes and elsewhere in the sperm, an extraction fixation was performed according to the procedure described by Schliwa and van Blerkom (1981). Half millimeter sections of the vas deferens were gently homogenized and briefly centrifuged with a Beckman Microfuge

E. The supernatant containing the sperm was drawn off and pelleted at 15,000 rpm for 4 min. The pellets were washed in PHEM extraction buffer (60 mM 1,4 piperazine diethylsulfonic acid [Pipes], 25 mM N-2-hydroxyethylpiperazine N 1-2-ethanesulfonic acid [HEPES], 10 mM EGTA, and 2 mM $MgCl_2$, pH 6.9), followed by treatment with PHEM buffered Triton X-100. Three different replicates were exposed to the treatment for one, three, and five minutes respectively. The replicates were fixed in 1% glutaraldehyde in PHEM buffer for 1 hr, and post-fixed in 0.1% osmium tetroxide for 1 hr. The material was dehydrated in ethanol, embedded in Spurr's resin, and viewed on a Hitachi HS-8 electron microscope.

To identify tubulin, freshly extracted spermatophores were homogenized and pelleted in a Beckman Microfuge E for approximately 4 min. They were fixed for 30 min with 2% paraformaldehyde with 0.5% glutaraldehyde made up in 0.05 M sodium cacodylate with sea water buffer. After two washes in cacodylate buffered seawater, the sperm were post-fixed for 30 min in 1% osmium tetroxide in cacodylate buffered seawater, washed in distilled water, dehydrated in a graded ethanol series, infiltrated and embedded in L R White. After sectioning, control and experimental sections

were placed on nickel grids. For immunogold labeling the protocol described by Langanger et al. (1984) was followed. The samples were blocked (to prevent non-specific binding) with a saline solution of 10% rabbit serum and 20% fetal calf serum in phosphate buffered saline (PBS) for 5 min, and rinsed with FCS/PBS (fetal calf serum/phosphate buffered saline). The experimental grids were treated with the primary antibody, polyclonal mouse anti-beta-tubulin with 10% FCS/PBS, and 0.1% Tween 20 for 1 hr. The control grids received 10% FCS/PBS and 0.1% Tween 20. The grids were washed with 10% FCS/PBS, and the secondary antibody, goat anti-mouse IgG conjugated to 15 nm gold particles, diluted 1:30 with 10% FCS/PBS plus 0.4% gelatin, and 0.1% bovine serum albumin was applied for 1 hr at room temperature. The grids were washed with PBS, post-stained with uranyl acetate and lead citrate, and observed with the Hitachi HS-8 microscope.

Electron dense granules

To identify the composition of the electron dense granules observed in ionophore reacted sperm, resin embedded thin sections were subjected to x-ray micro-analysis using a Kevex Analyst 8000 coupled with a Jeol 200 CX STEM at the

National Center for Electron Microscopy, Lawrence Berkeley Laboratories.

Acrosome reaction

To induce the acrosome reaction, the ionophore A23187 (20 mM in 100% ethanol) was added to normal fresh sperm isolated in seawater using a procedure adapted from Talbot and Chanmanon (1980). A control group was treated with 100% ethanol. The sperm were observed and photographed using an Olympus BH2 light microscope. Sperm treated with ionophore A23187 were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, and processed for TEM and SEM.

RESULTS

The mature sperm of Callianassa californiensis are approximately 4 um in diameter and roughly spherical in shape. The most prominent features are the acrosome and the nucleus with radial processes extending outwardly from it. The acrosome, dominating the upper third of the cell, consists of an electron dense acrosomal cap and a less dense, granular, subacrosomal area. The cap region is smooth surfaced and consists of homogeneous, darkly staining (osmiphilic) material; there is a central depression at the apex of the acrosomal cap (Figs. 1 and 2). The border of the acrosomal cap and nucleus is irregular (Fig. 2). The subacrosomal area lies within the acrosomal cap and above the nucleus. It is composed of granular or flocculent material, interspersed with rods or filaments. The acrosome, including the subacrosomal area, is bounded by the acrosomal membrane. Posterior to the subacrosomal area the acrosomal membrane appears folded, extending into the subacrosomal area (Fig. 3). The nucleus lies below the acrosome and subacrosomal area. In the nucleus the chromatin appears uncondensed and fibrous. Darkly staining dense particles, measuring 25 to 70 nm, are scattered throughout the nucleoplasm. In some areas a narrow layer of

cytoplasm is peripheral to the nucleus; in other areas the nuclear membrane is contiguous with the plasma membrane. Within the cytoplasm, which is most abundant at the acrosome-nuclear junction, are mitochondria, membrane lamellar bodies, and small electron dense inclusions (Figs. 1 and 4). The mitochondria, with few and irregularly spaced cristae, are clustered together; the membrane lamellar bodies are less numerous. In some sperm, the membrane lamellar bodies appear as sheets of stacked membranes, folded or convoluted. In others the lamellae appear thicker and have a honeycomb pattern (Figs. 9 and 10). Although most organelles are situated at the junction of the nucleus and acrosome, some mitochondria and membrane lamellar bodies are found posterior to the nucleus. No centrioles have been identified. The nucleus is bounded by a nuclear membrane which appears in many sperm to be discontinuous below the subacrosome (Fig. 3). Projecting outward from the nucleus are eight or nine slender processes with an approximate diameter of 120 to 200 nm and length of 15 to 22 um. The radial processes contain tubular structures which originate in the nucleus. A nipple-like prominence interrupts the cell surface where the processes emerge (Figs. 5 and 6). Because the processes are fragile and break during treatment

their number and length cannot be determined with complete accuracy. The plasma membrane, which encloses the entire cell, is roughly textured in the nuclear area (possibly a fixation artifact) but smooth around the acrosome (Fig. 5).

Nuclear staining

The nucleus stained positive with Feulgen stain. The stain covered the cell below the acrosome; only a small area around the perimeter of the nucleus contained cytoplasm and organelles which stained with fast green counterstain. The acrosome, already dark, stained darker (Fig. 7) and the subacrosomal area stained lightly with the fast green (data not shown). The radial processes, which in Libinia emarginata (Hinsch, 1969) and Callinectes sapidus (Brown, 1966) have been found to contain nuclear material, did not react positively to the Feulgen stain.

In sperm which had undergone the acrosome reaction, the area of positive staining increased (Fig. 8). Because the acrosomal cap opens at the apex and the nuclear material flows through the opening, ballooning against the posterior acrosomal membrane and forming an inflated sphere of nuclear material, reacted sperm are approximately one and a half times the size of unreacted sperm. The acrosomal cap material forms a constricting ring around the junction

between the nucleus and the newly inflated area (see below for a complete description). The expanded nuclear area stained with Feulgen stain, indicating that the nuclear material had surged into the enlarged area. Green staining organelles and cytoplasm were distributed around the perimeter of the nuclear material. The material of the acrosomal cap, which stained darkly in unreacted sperm, was negative for Feulgen stain, absorbing instead the green counterstain (Fig. 8).

Pyruvate dehydrogenase activity in mitochondria and membrane lamellar bodies

In the experimental treatment, densities of copper ferrocyanide are evident in the membrane lamellar bodies but not the mitochondria (Fig. 9). This may be compared to the control treatment in which no densities appear in either mitochondria or membrane lamellar bodies (Fig. 10); treated mitochondria from the experimental group have the same appearance as in the control treatment.

Identification of Microtubules by Extraction and Immunogold Labeling Techniques

Extraction treatment with the detergent Triton X-100 removed membranes from the acrosome exposing a network of

tubular structures reminiscent of the swirls observed in the anterior cap of Pacifastacus leniusculus (Dudenhausen and Talbot, 1982). These structures are most evident at the central depression at the apex of the acrosome, at the inside edges next to the subacrosomal space, and at the periphery where the acrosome borders on the nucleus (Fig. 11). This material has only been seen using this treatment and may be a fixation artifact. The subacrosomal area contains fibers or filaments which measure approximately 5 nm in diameter but whose length cannot be determined (Fig. 12).

In the nucleus of extracted sperm the chromatin is dispersed and granular; interspersed in the chromatin are tubules or filaments with a diameter of approximately 16 to 19 nm and variable length (Fig. 13). The diameter of these tubules is identical to the diameter of tubules in the radial processes. In cross-section tubules from the radial processes appear as electron dense rings with electron lucent centers. Although the arrangement of tubules within the radial processes may not be consistent, in one sperm four inner tubules were seen surrounded by a ring of ten tubules (Fig. 14). The number of tubules observed within a process ranged from four to twenty. Tubules are also seen

in the radial processes of sperm treated with ionophore A23187 and in untreated sperm (Figs. 1 and 15).

In the experimental group of sperm treated with immunogold label to identify beta-tubulin, gold label was found scattered in the nucleus of the sperm, but no filaments or tubules resembling microtubules were observed (data not shown). Gold label was not observed in the radial processes, nor did the filaments located in the subacrosomal space label with gold. The gold label did not bind to the control sperm (data not shown).

Electron dense granules

X-ray analysis of the electron dense inclusions found in the nucleus and mitochondria of ionophore reacted sperm disclosed that inclusions in the nucleus and expanded nuclear area contain high percentages of magnesium phosphate and calcium phosphate (Figs. 15 and 16). By atomic percent the nuclear inclusions were 50% phosphorus, 24% magnesium, and 15% calcium (Table 1). The mitochondria contained less magnesium and little calcium; by atomic percent the mitochondria were 15% magnesium and 5% calcium. The mitochondria contained no phosphorous. The densities in mitochondria were composed mostly of osmium, which was used

in fixation (22%), and silica (15%) which is commonly found in marine organisms (Fig. 17, Table 2).

Acrosome reaction

The sequence of events in the acrosome reaction has been reconstructed from observations of sperm induced by the ionophore A23187, sperm spontaneously undergoing the acrosome reaction, and sperm encounters with eggs (Figs. 18 and 19). When a sperm encounters an egg or responds to the ionophore the central depression of the acrosomal cap opens, allowing the subacrosomal material to extrude (Figs. 20 and 21). As the reaction continues the acrosomal cap rolls back and the opening at the apex enlarges. The acrosomal cap material forms a ring above the nucleus. The subacrosomal contents escape and the nuclear material flows through the acrosomal ring, ballooning outward (Figs. 22 and 23). The (inner) posterior acrosome membrane which became exposed when the acrosomal cap rolled back is now the leading edge of the cell and it contains the nuclear material expanding against it. The sperm increases in size approximately 1.5 times. As the reaction is carried to its conclusion the entire nucleus and cytoplasm flow through the ring, everting or "exploding" as has been described in other decapods by

early investigators (Figs. 24 and 25), (Brown, 1966; Binford, 1913; Terni, 1937).

The nucleus of the reacted sperm stained positive for Feulgen stain as did the enlarged area indicating that the chromatin had expanded into it (Fig. 8).

DISCUSSION

The extraction treatment with the detergent Triton X-100 revealed a network of membranes or tubules in the acrosome which resemble those described in the acrosome of Pacifastacus: "The apical cap is composed of stacks of parallel lamellae which form alternating electron dense and electron lucent bands; these lamellae are often arranged in concentric whirls..." (Dudenhausen and Talbot, 1982). This reticulum also resembles the membrane lamellar complex lateral to the acrosome and nucleus of the crab Carcinus maenas (Pearson and Walker, 1975). More experimentation needs to be completed in Callianassa to determine if this is a coagulation response of lipids in the acrosome to the extraction procedure or if a reticular network underlies the acrosomal material. The subacrosomal area, with its flocculent and granular material and the presence of rods or filaments (Fig. 3), is reminiscent of that of Cherax albidus and C. tenuimanus which have a network of fibers as well as electron dense granules in this region (Beach and Talbot, 1987). These rod shaped bodies may be hydrolytic enzymes and lysins which are released during the acrosome reaction to lyse the egg chorion (Talbot and Chanmanon, 1980; Brown, 1966).

Nuclear staining

Although the radial processes of Callianassa are bounded by the nuclear membrane as well as the plasma membrane, these processes, unlike those of Libinia and Callinectes (Hinsch, 1969; Brown, 1966), do not stain with Feulgen stain. In studying Callinectes Brown (1966) noted that the Feulgen staining of the processes did not extend to their extremities, but stopped a short distance from the body of the sperm. Brown speculated that the small amount of nuclear material in the distal processes may not be enough to cause the Feulgen reaction. This may be the case in Panulirus (Talbot and Summers, 1978), Homarus (Talbot and Chanmanon, 1980), and the mole crab Emerita talpoida (Barker and Austin, 1963), all of whose processes, like those of Callianassa, tested negative for Feulgen stain (Fig. 8).

In Callianassa tubules are sometimes visible within the radial processes; these tubules measure approximately 16 to 19 nm, whereas microtubules measure 25 nm (Alberts et al., 1989). Microtubules are usually organized by centrioles or basal bodies, but neither of these has been found in Callianassa. In Libinia, where the tubules in the radial processes have been identified as microtubules by their diameter of 25 to 27 nm, the processes clearly emanate

from centrioles. They originate below the acrosomal tubule and enter the nucleus through nuclear "pores", pass through and exit the nucleus, and project outward from it bound by both nuclear membrane and plasma membrane (Hinsch, 1969). In the sperm of the crayfish Pacifastacus, in which centrioles have not been identified, Dudenhausen and Talbot (1982) argue that the radial processes (whose tubule diameter is 25 nm) are organized by the nuclear envelope. The processes originate in folds of the nuclear envelope posterior to the subacrosomal space and radiate tangently from it. Meek and Moses (1961) found tubules organizing from the nuclear membrane of aging crayfish spermatocytes, and Dudenhausen and Talbot (1982) have observed microtubules polymerize from the nuclear envelope of disturbed Pacifastacus spermatocytes. In Callianassa tubules are faintly visible in the nucleus before the processes emerge from the cell (Fig. 1). However, tubules have not been located around the nuclear membrane below the subacrosomal area where they are thought to originate in Pacifastacus. In SEM micrographs (Figs. 5 and 6) the processes are seen to emerge from folds in the plasma membrane.

Barker and Austin (1963) counted three to nine nuclear processes on Emerita. On Callianassa sperm which

seem least damaged by fixation eight or nine processes can be counted.

Pyruvate dehydrogenase activity in mitochondria and membrane lamellar bodies

The results of this experiment were striking. While the mitochondria did not pick up the copper ferrocyanide, the membrane lamellar bodies did, staining heavily with electron dense particles (Fig. 9). This suggests that the mitochondria are indeed inactive and that the membrane lamellar bodies participate in cellular respiration as suggested by Brown (1966) and Beach and Talbot (1987). However, further confirmation of this activity is needed before the membrane lamellar bodies can be assumed to be active in cell respiration.

Identification of microtubules by extraction and immunogold techniques

It was hoped that extraction and immunogold treatments would reveal microtubules originating in the nucleus and radiating outward through the processes as in Libinia, Callinectes, Pacifastacus, and other decapods (Hinsch, 1969; Brown, 1966; Dudehausen and Talbot, 1982; Talbot and Summers, 1978). Instead, a tubular structure

with a diameter smaller than microtubules, approximately 16 to 19 nm rather than 25 nm, has been disclosed (Figs. 13 and 14). These tubules support the radial processes, as do the microtubules in other decapods, and may have a similar arrangement as microtubules in other decapods, but their source and other functions are unknown. Immunogold labeling failed to identify these structures as tubulin.

Electron dense granules

Because calcium and magnesium enter the sperm during the ionophore induced acrosome reaction (Alberts et al., 1989; Talbot et al., 1976) it is not surprising that large amounts were found by x-ray analysis in reacted sperm. Internal calcium is usually sequestered in the endoplasmic reticulum and mitochondria (Alberts et al., 1989). In reacted Callianassa the calcium and magnesium densities appear on the periphery of the acrosomal ring and in the cytoplasm. Clark et al. (1981) report that external calcium must be present in the medium for the acrosome reaction to occur in the shrimp, Sicyonia. Talbot and Chanmamon (1980) suggest that calcium plays a role in the acrosome reaction of the lobster, Homarus, as an increase in the number of spontaneous reactions occur if the calcium concentration of the medium is elevated.

Acrosome Reaction

In the light microscope Callianassa sperm appear as translucent spheres which display no movement of their own. When Callianassa sperm and eggs bind, a cone shaped projection extends from the spherical sperm cell and contacts the chorion of the egg (Fig. 19). This "cone" may be the ring formed by the acrosomal cap during exocytosis seen in the scanning and transmission electron microscopes. The radial processes extend outward and a few degrees away from parallel to the egg. They appear to take no part in attachment of the sperm to the egg. In Callinectes Brown (1966) observed that the processes first attach to the chorion of the egg. In Callianassa the processes may balance and orient the sperm so that the acrosomal area contacts the egg surface. The widest part of the acrosomal cone or ring is wider than the diameter of the sperm body. The sperm cell flattens coming closer to the egg cell membrane, and finally disappears within the egg leaving only a slight elevation on the egg's surface.

Ionophore A23187 has been used to produce an acrosome reaction in decapods such as Homarus and Sicyonia (Talbot and Chanmanon, 1980; Clark et al., 1981), and animals as diverse as sand dollars and guinea pigs (Talbot

et al., 1976). Ionophore induced reactions mimic naturally occurring reactions, and the two appear to be identical. Numerous Callianassa sperm which have reacted spontaneously in response to immersion in sea water and fixative materials have been observed and their reaction is comparable to the ionophore reaction. Sperm reacted and bound to eggs have not been examined with the electron microscope, but appear similar in the light microscope to ionophore reacted sperm.

Decapod eggs are enclosed in two layers of secretions which the sperm must penetrate before they can fertilize the egg (Adiyodi and Adiyodi, 1983). The lengthy and complex acrosome reaction has a twofold purpose: to penetrate the egg layers and to insert the sperm nuclear contents into the egg.

Before the two-phased acrosome reaction begins the mature Callianassa sperm has undergone changes which will facilitate exocytosis of the subacrosomal material and eversion of the nucleus. The anterior nuclear membrane is discontinuous below the subacrosomal area (Fig. 3) facilitating the surge of nucleoplasm into and through the subacrosomal space during the nuclear eversion phase. This condition is shared with the lobster Homarus americanus, the crab Callinectes sapidus, and the shrimp Sicyonia ingentis

whose nuclear membranes are also discontinuous between the nucleus and the acrosome (Talbot and Chanmanon, 1980; Brown, 1966; Shigekawa and Clark, 1986). Chromatin usually condensed in immature decapod sperm is decondensed in mature Callianassa sperm (Figs. 1 and 20) as it is in Sicyonia (Shigekawa and Clark, 1986) and Homarus (Talbot and Chanmanon, 1980), allowing the pliable chromatin to flow through the ring formed by the acrosome cap during the exocytotic phase. The posterior acrosomal membrane has proliferated, folding into the subacrosomal space, enabling it to receive and contain the swelling nucleoplasm (Figs. 3 and 22-24).

The exocytotic phase begins at the apex of the acrosomal cap where the central depression is located and the cap material thinnest. Upon contact with ionophore or egg investments the sperm plasma membrane and acrosomal membrane probably vesiculate creating an opening at the central depression as in Homarus (Talbot and Chanmanon, 1980) and the horseshoe crab Limulus (Tilney, 1985). The acrosomal cap opens, rolls back, and forms a ring around the subacrosomal area, exposing the interior acrosomal material and subacrosomal area to the external environment or egg chorion. Instead of appearing dense and homogeneous as it

had before, the acrosomal cap material appears flocculent and fibrous as the ring forms. A change in this substance is evidenced by its response to Feulgen stain; whereas the acrosome of unreacted sperm stained darkly, the externalized acrosome of reacted sperm stains with the green counterstain (Figs. 8 and 9). Like the membrane pouches of Sicyonia the cap material may contain species-specific recognition proteins which bind the sperm to the egg's glycocalyx (Clark et al., 1981). Exocytosis releases the contents of the subacrosomal area which disperse and contact the outer investments of the egg. The subacrosomal area is likely to be composed of or contain lytic enzymes which lyse the egg's glycocalyx, making way for sperm entry and phase two of the acrosome reaction (Talbot and Chanmanon, 1980).

As the acrosomal cap rolls back forming the acrosomal ring, the nuclear material surges through it, enlarging the cell by approximately 1.5 times. As in Homarus this may be facilitated by hydration of nuclear material (Talbot and Chanmanon, 1980). The posterior (inner) acrosome membrane which has become the leading edge of the sperm can now contact the plasmalemma of the egg. This membrane may contain proteins which facilitate fusion of the sperm and egg membranes.

CONCLUSION

Callianassa sperm share the unusual characteristics typical of decapod sperm. Like sperm of related species Callianassa sperm are spherical, non-motile, and lack flagella. Their mitochondria have few cristae and appear to be inactive in mature sperm. Extending from the nucleus are radial processes whose function is unclear. These processes are supported by tubular structures. Within the nucleus the chromatin is uncondensed. Like the nuclei of crayfish and lobsters Callianassa sperm nuclei are surmounted by an electron dense acrosome; in crab sperm the acrosome is surrounded by the nucleus. Lateral to the acrosome in Callianassa, crayfish, and lobsters are membrane lamellar bodies which have been implicated in cellular respiration (Beach and Talbot, 1987). In Callinectes Brown (1966) suggested that this function is performed by the lamella of the central region. In Sicyonia membrane lamellar bodies occur on the periphery of the nucleus (Shigekawa and Clark, 1986), but no such function has been ascribed to them.

The acrosome reaction in Callianassa sperm resembles that of other decapods as far as is known. The acrosome encloses a subacrosomal area, comparable to the acrosomal tubule in crabs (Hinsch, 1969, Brown, 1966), which is

released or thrust forward during the exocytotic phase of the acrosome reaction. During the nuclear eversion phase the nuclear material surges through an opening in the apex of the acrosome. This is similar to the reaction of Callinectes (Brown, 1966) and Libinia (Hinsch, 1969) but distinguished from the reaction of the shrimp Sicyonia (Griffin et al., 1988) in that no acrosomal filament is formed.

Callianassa sperm are distinct from other decapod sperm in a variety of ways. No microtubules have been located either in the nucleus or in the radial processes; instead the processes are supported by tubules whose diameter is approximately 16 to 19 nm. Membrane lamellar bodies in Callianassa are fewer or not as extensive as in other decapods, and are located posterior to the nucleus as well as lateral to the acrosome. This research has indicated that the lamellar bodies may be active in respiration, although more research should be completed in this area. The acrosomal membrane surrounds the acrosomal cap and also encloses the subacrosomal area in Callianassa; the subacrosomal area in Sicyonia (Shigekawa and Clark, 1986), in the crayfish Cherax (Beach and Talbot, 1987), and in Pacifastacus (Dudenhausen and Talbot, 1982) is separated

from the acrosome by the acrosomal membrane. While they bear similarities to all decapod sperm, Callianassa sperm more closely resemble the sperm of crayfish and lobsters than the sperm of crabs and penaeid shrimps.

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APPENDIX

Figure 1. Cross-section of mature Callianassa californiensis sperm. Acrosome (A), subacrosomal area (SA), nucleus (N), mitochondria (M), and radial process (P) are visible.
Magnification = 26,000X
Bar = 1 um

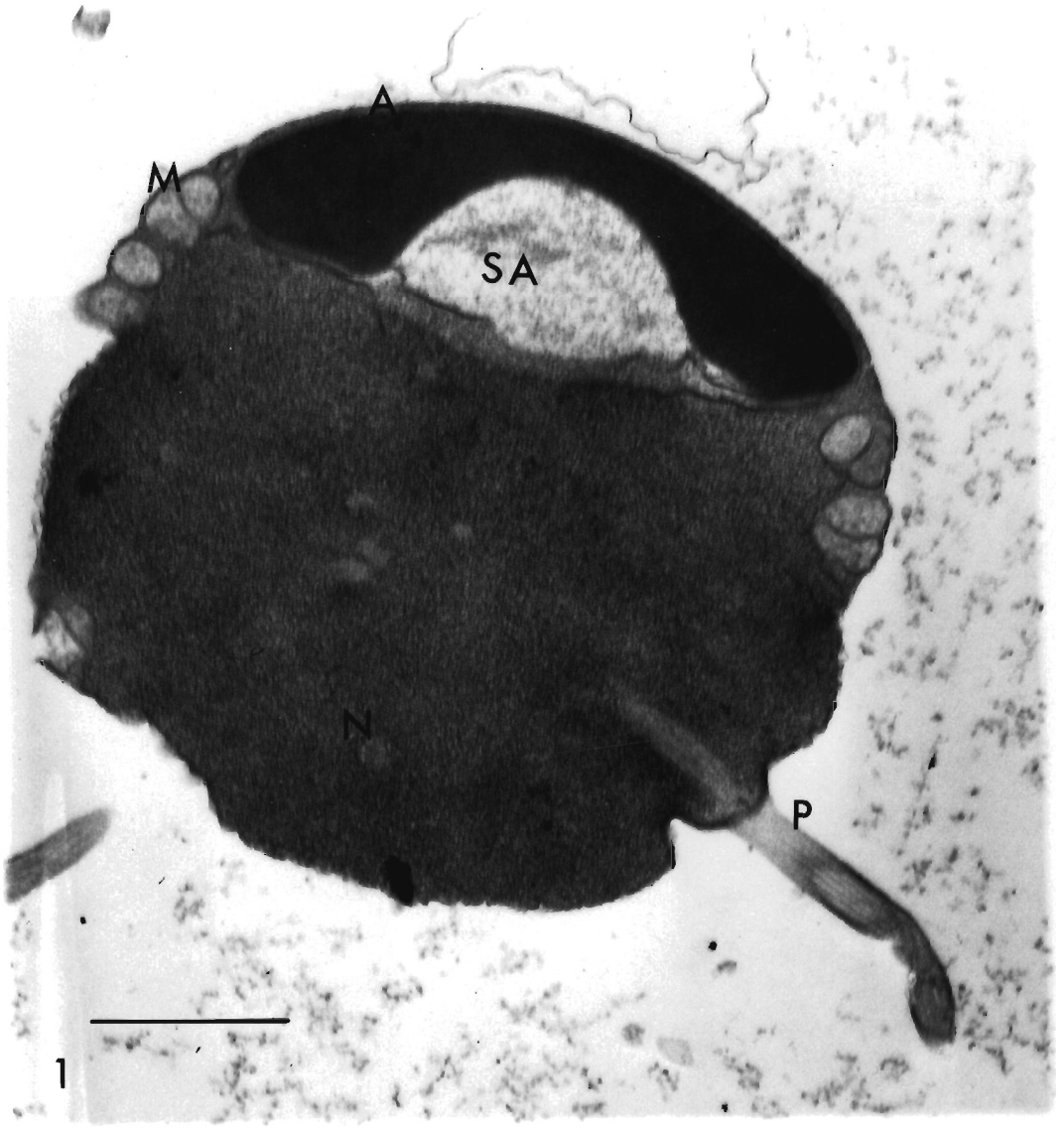

A
M
SA
N
P
1

Figure 2. SEM micrograph of Callianassa californiensis sperm showing the acrosome (A), central depression (D), nucleus (N), and radial processes (P).
Magnification: 21,000X
Bar = 1 um

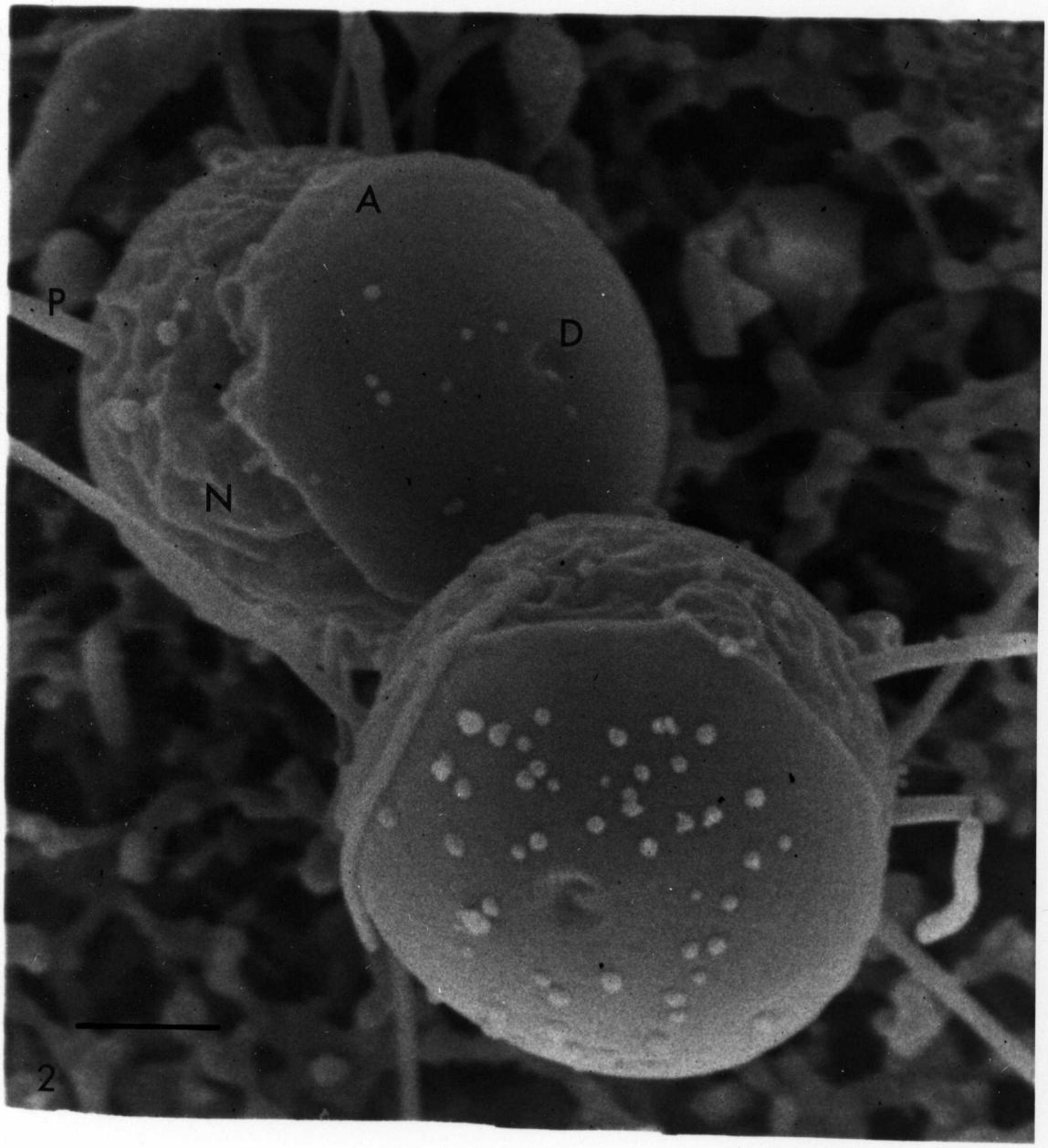

A
P
D
N
2

Figure 3. Subacrosomal area (SA). Note folded acrosomal membrane (AM), discontinuous nuclear membrane (arrow), and rods or filaments in subacrosomal area.
Magnification: 42,000X
Bar = 0.5 um

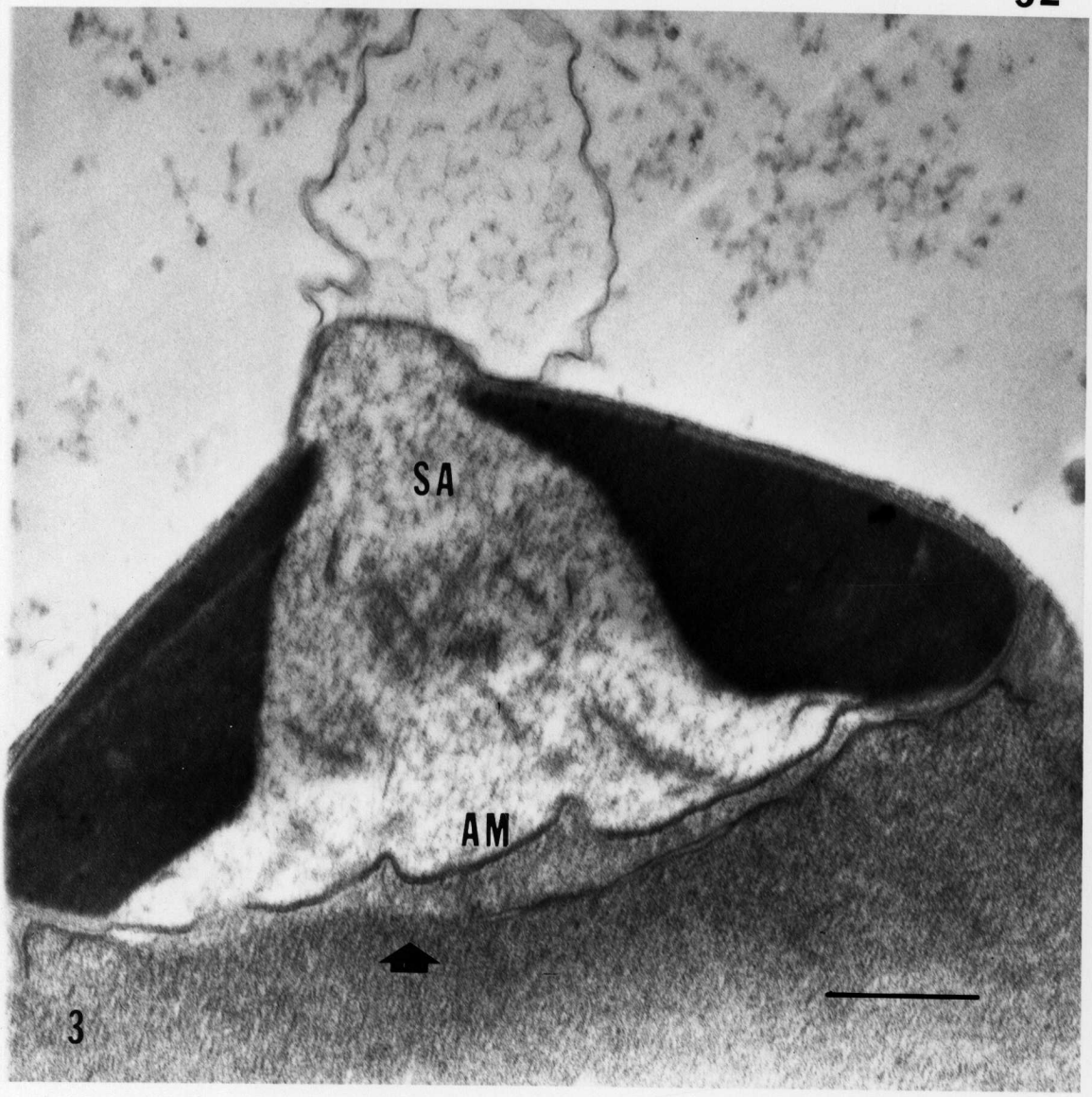
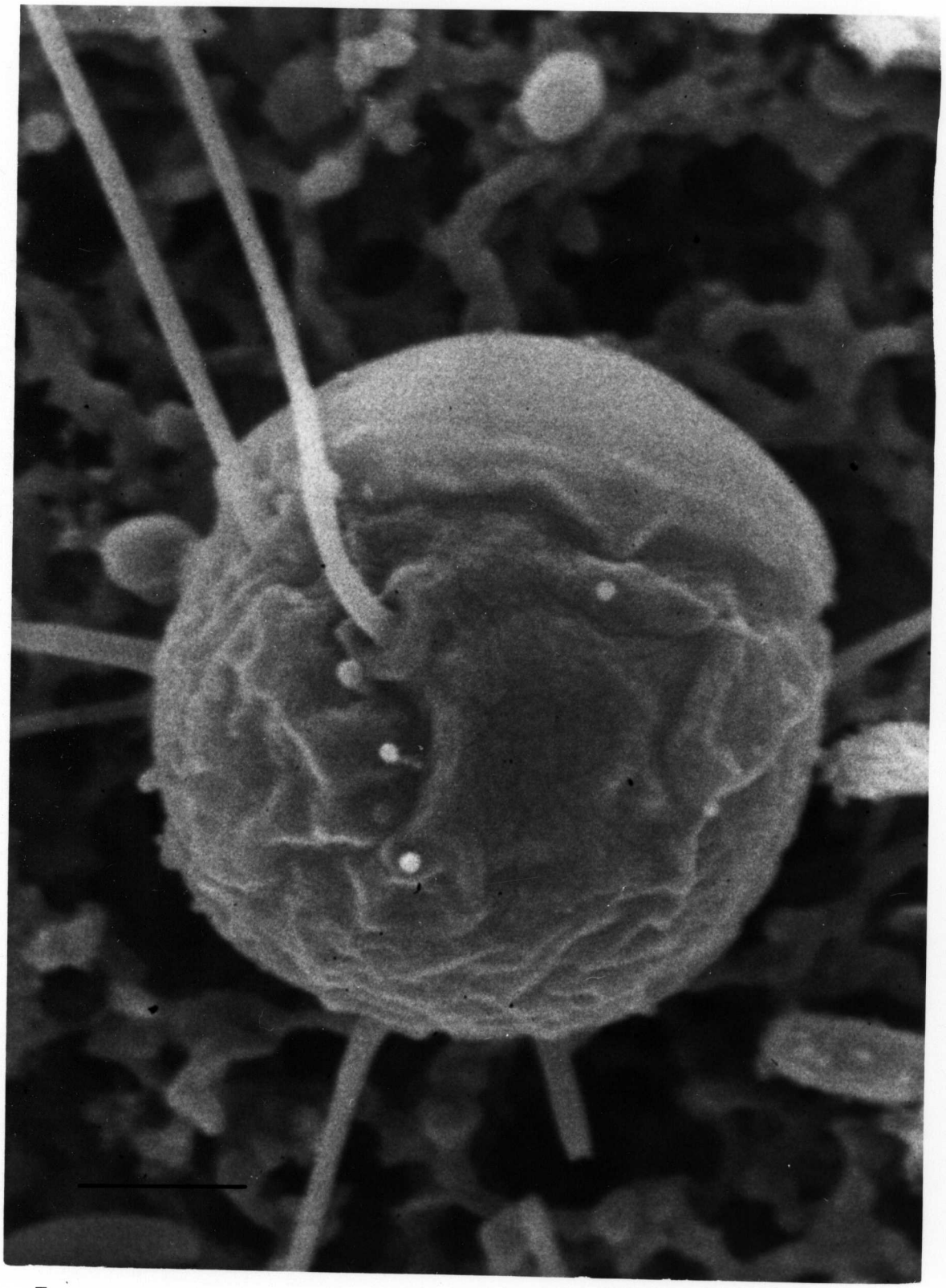

SA
AM
3

Figure 4. Callianassa sperm with membrane lamellar body (MB).
Magnification: 28,000X
Bar = 1 um

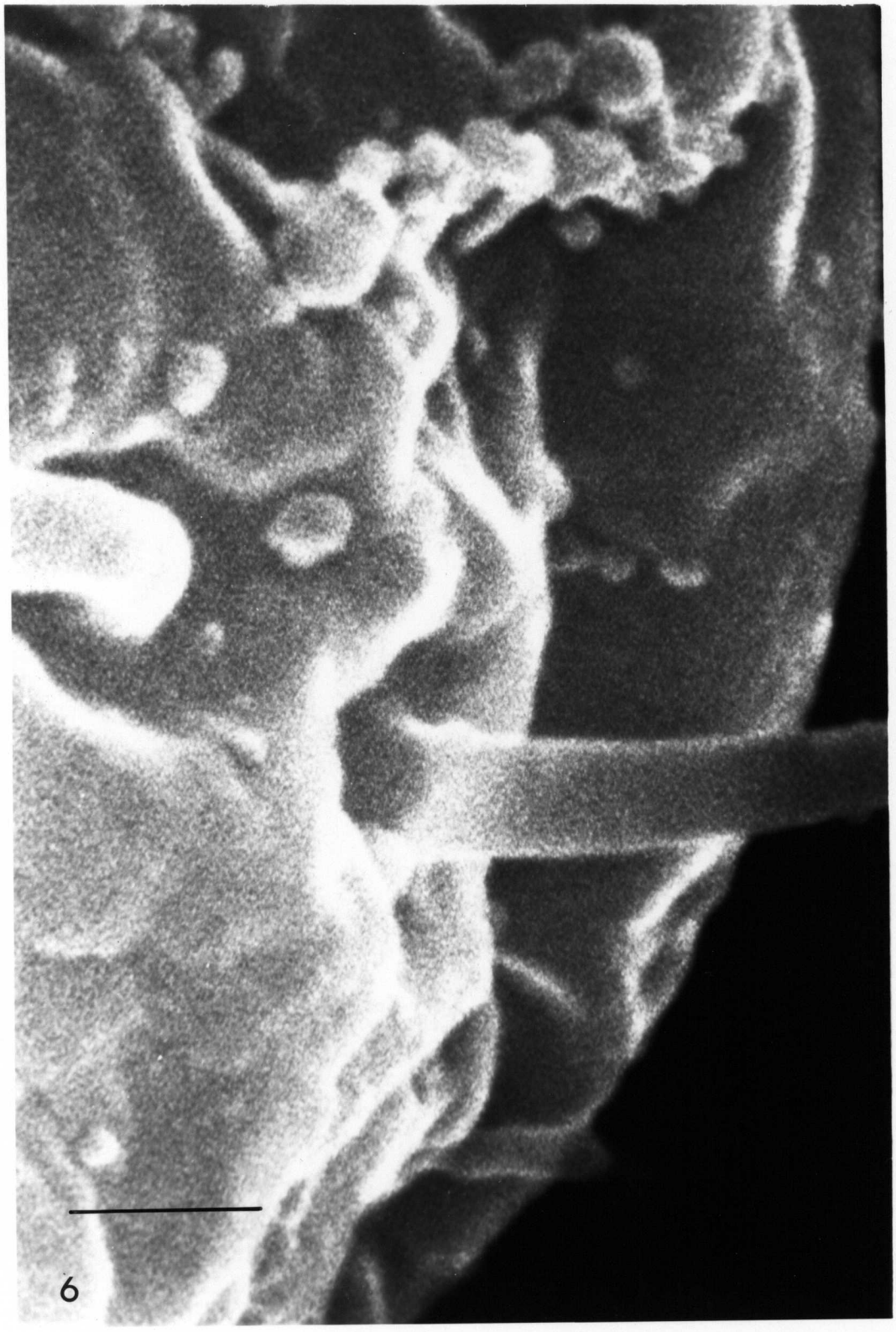
4

Figure 5. Radial processes emerge from sperm cell.
Magnification: 28,000X
Bar = 1 um



5

Figure 6. Radial processes emerge from hollow in raised area on cell surface.
Magnification: 120,000X
Bar = .25 um



6

Figure 7. Feulgen stained sperm. Nuclear material (N) stains pink; acrosome (A) stains darkly. Other areas stain with green counterstain.
Magnification : 2,100X
Bar = 5 um

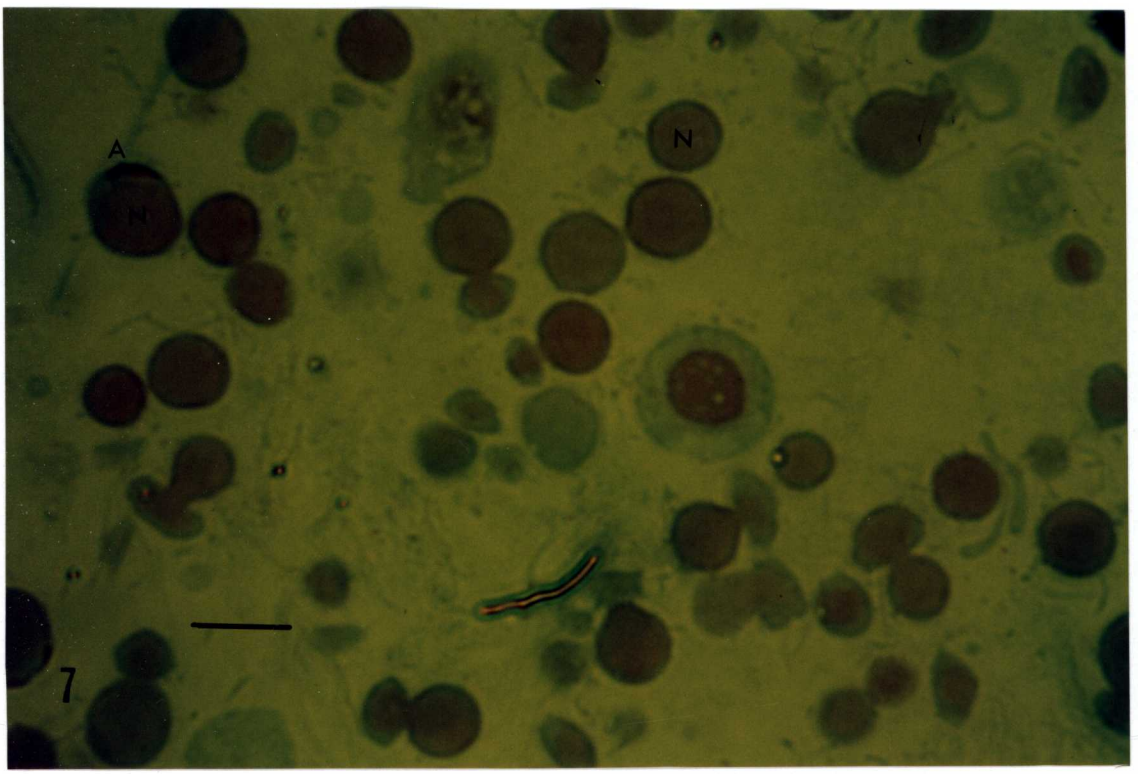

A
N
N
7

Figure 8. Feulgen stained reacted sperm. Nuclear material (N) extends through acrosomal ring in reacted sperm (arrow). Acrosome (A).
Magnification: 2,100X
Bar = 5 um

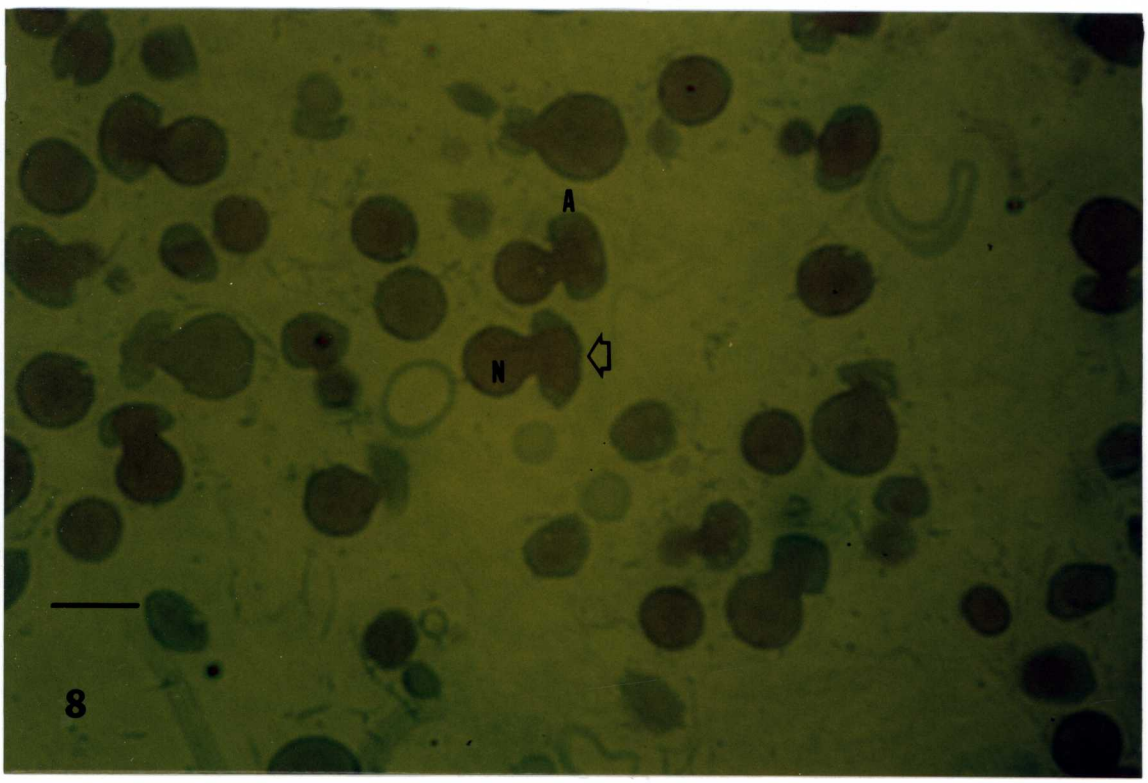

A
N
8

Figure 9. Sperm treated with potassium ferrocyanide and copper sulfate to detect pyruvate dehydrogenase activity. Note fine granules of copper ferro-cyanide within membrane lamellar bodies (MLB). Membrane lamellar bodies have honeycomb appearance. Mitochondria (M).
Magnification: 38,000X
Bar = 0.5 um

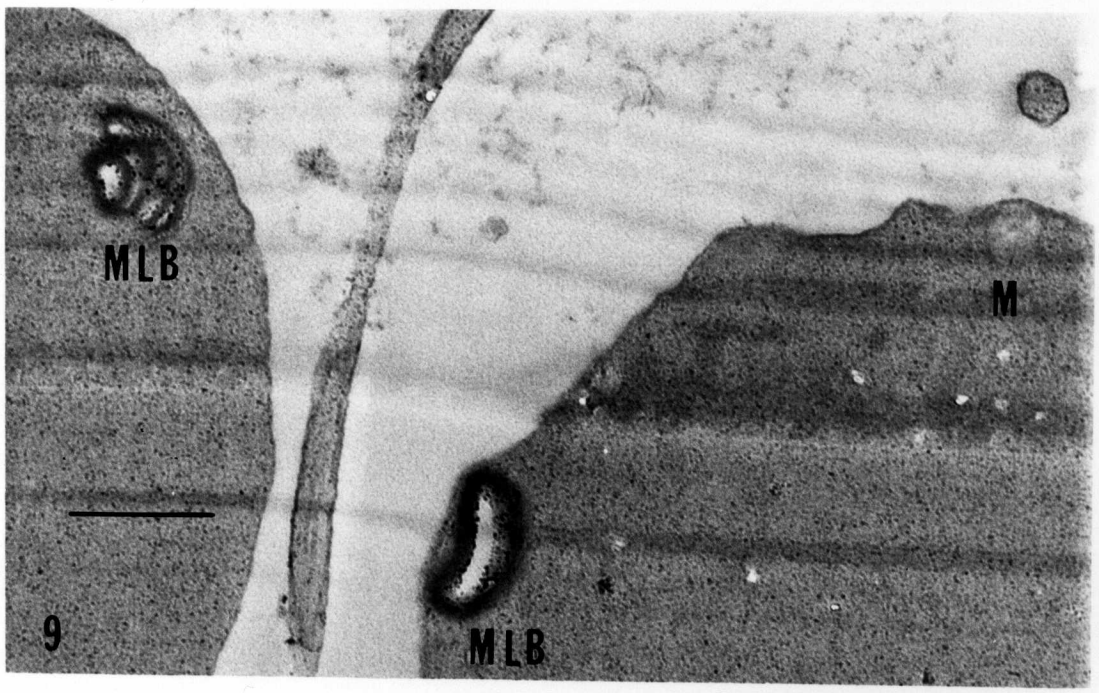

MLB
M
9
MLB

Figure 10. Sperm treated with potassium ferrocyanide as control for pyruvate dehydrogenase activity. Compare to Figure 9. Membrane lamellar body (MLB), mitochondria (M).
Magnification: 53,000X
Bar = 0.5 um

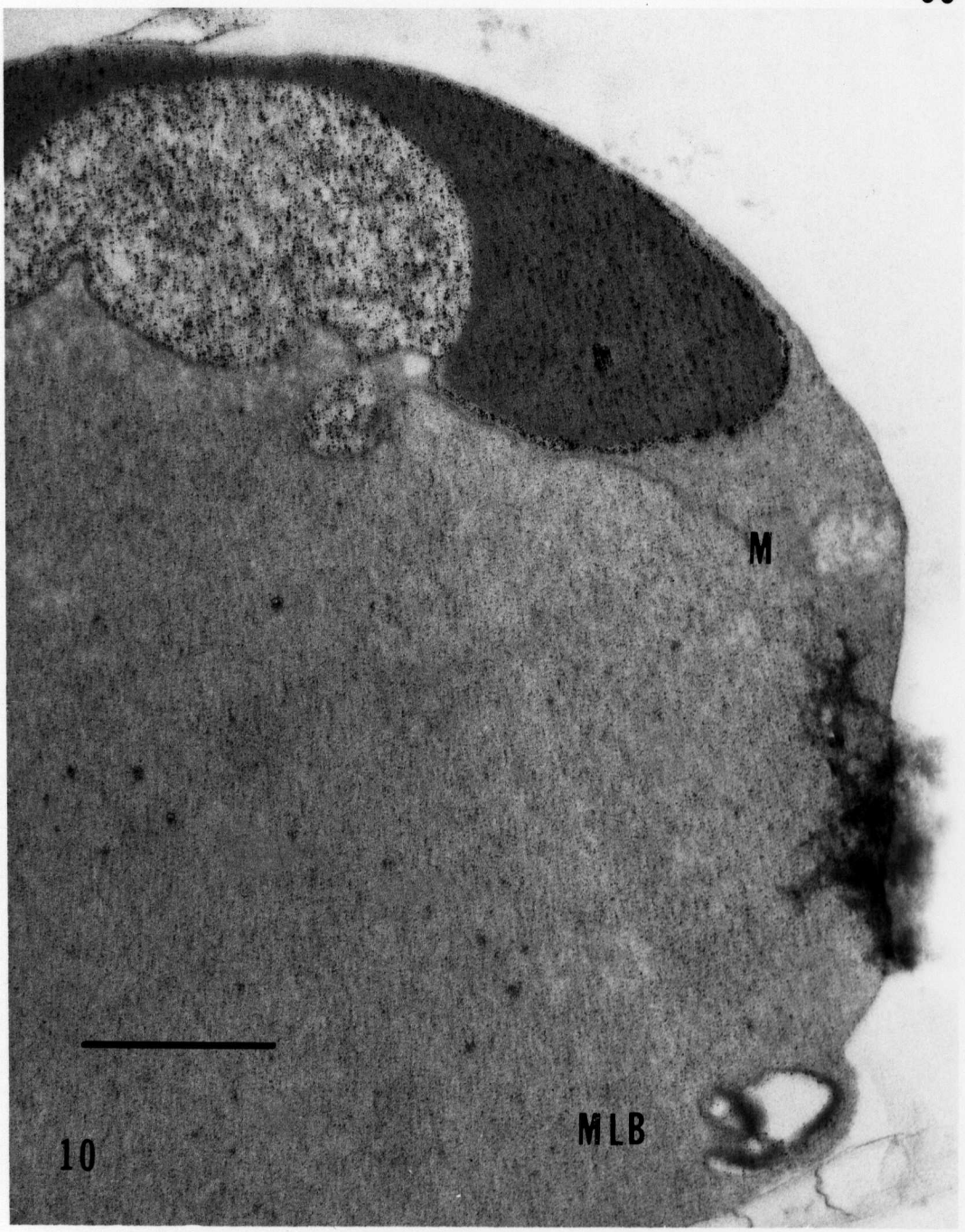
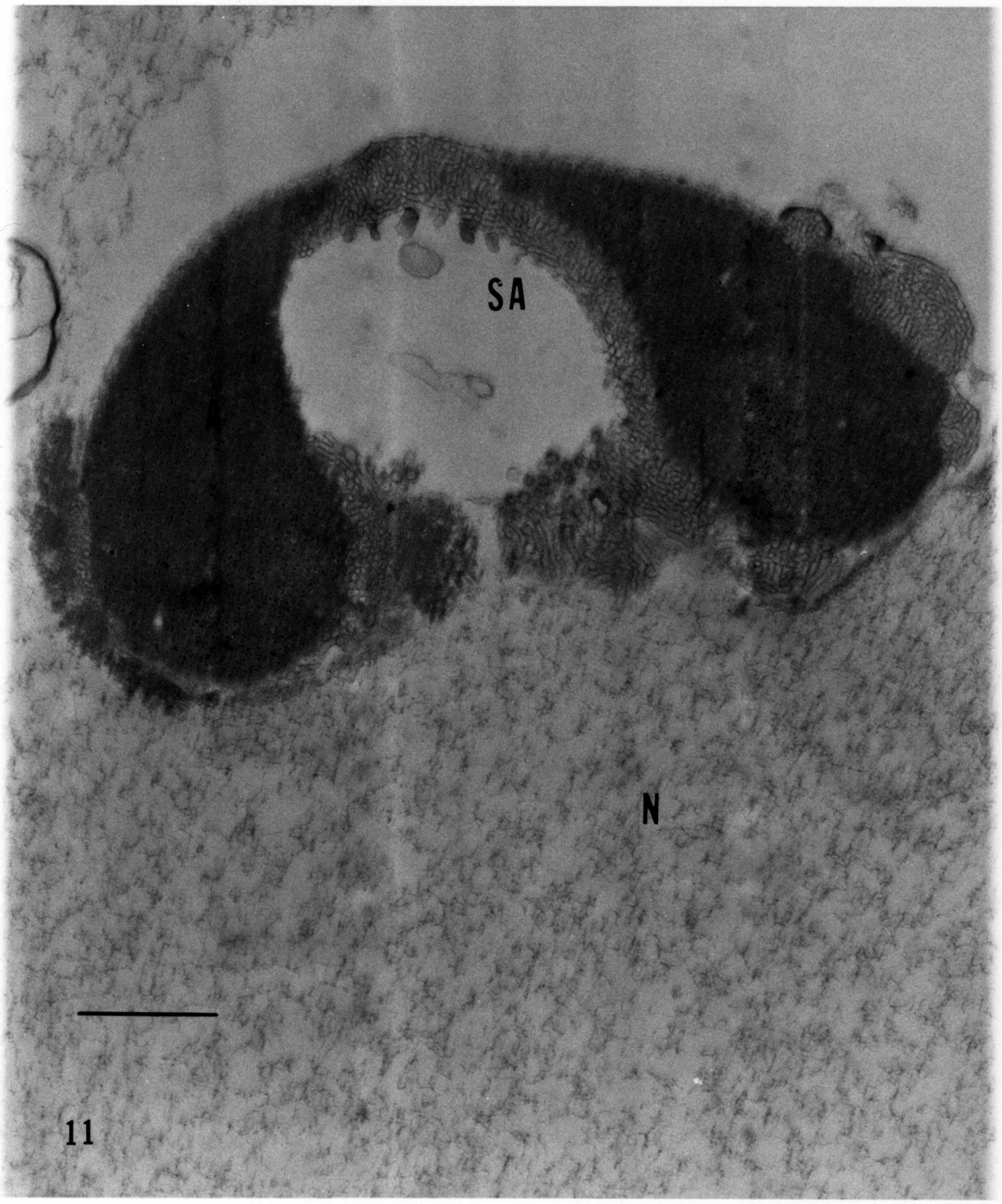

M
MLB
10

Figure 11. Extracted sperm. Note absence of nuclear and acrosomal membranes. Acrosome (A), nucleus N), subacrosomal area (SA).
Magnification: 42,000X
Bar = 0.5 um



11

Figure 12. Extracted sperm. Note fine filaments in subacrosomal area (SA). Acrosome (A), nucleus (N), subacrosomal area (SA).
Magnification: 80,000X
Bar = 0.25 um

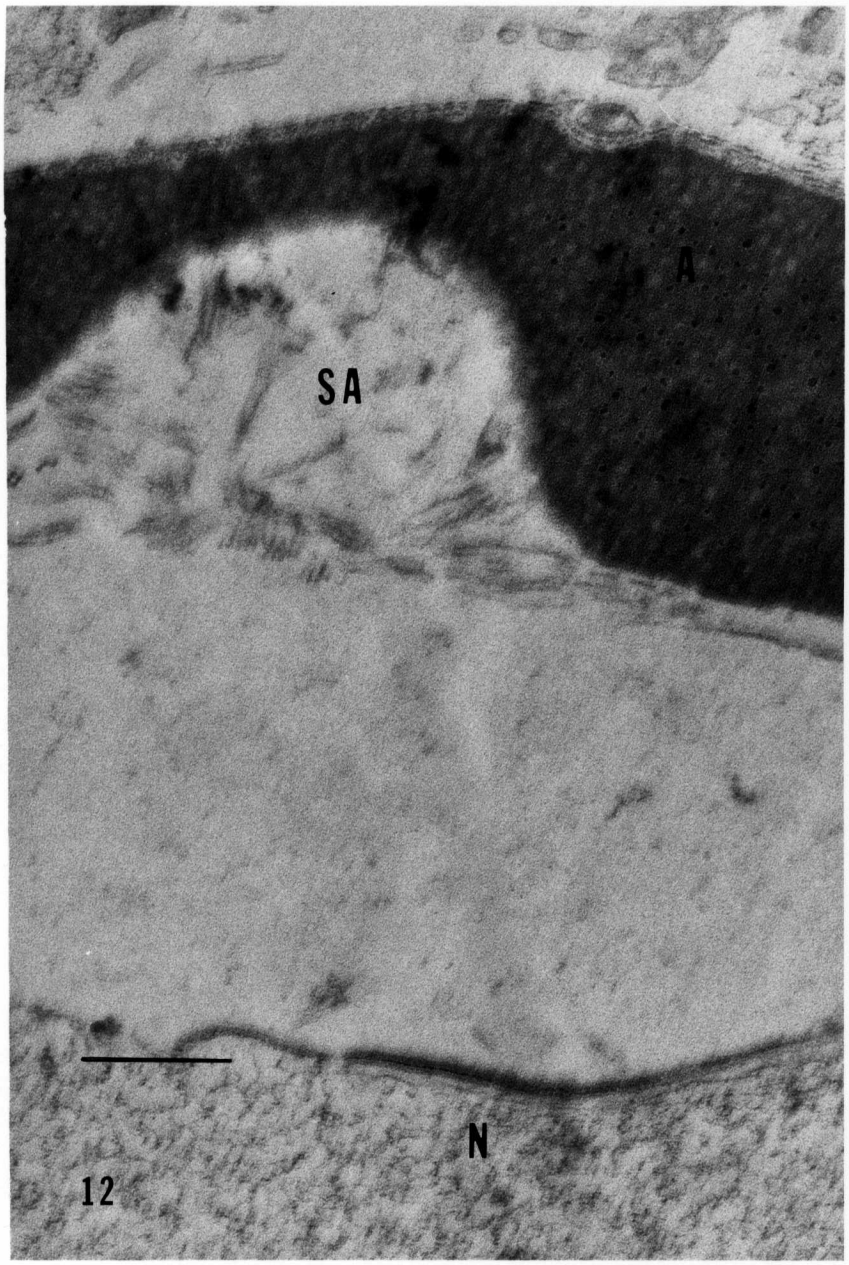
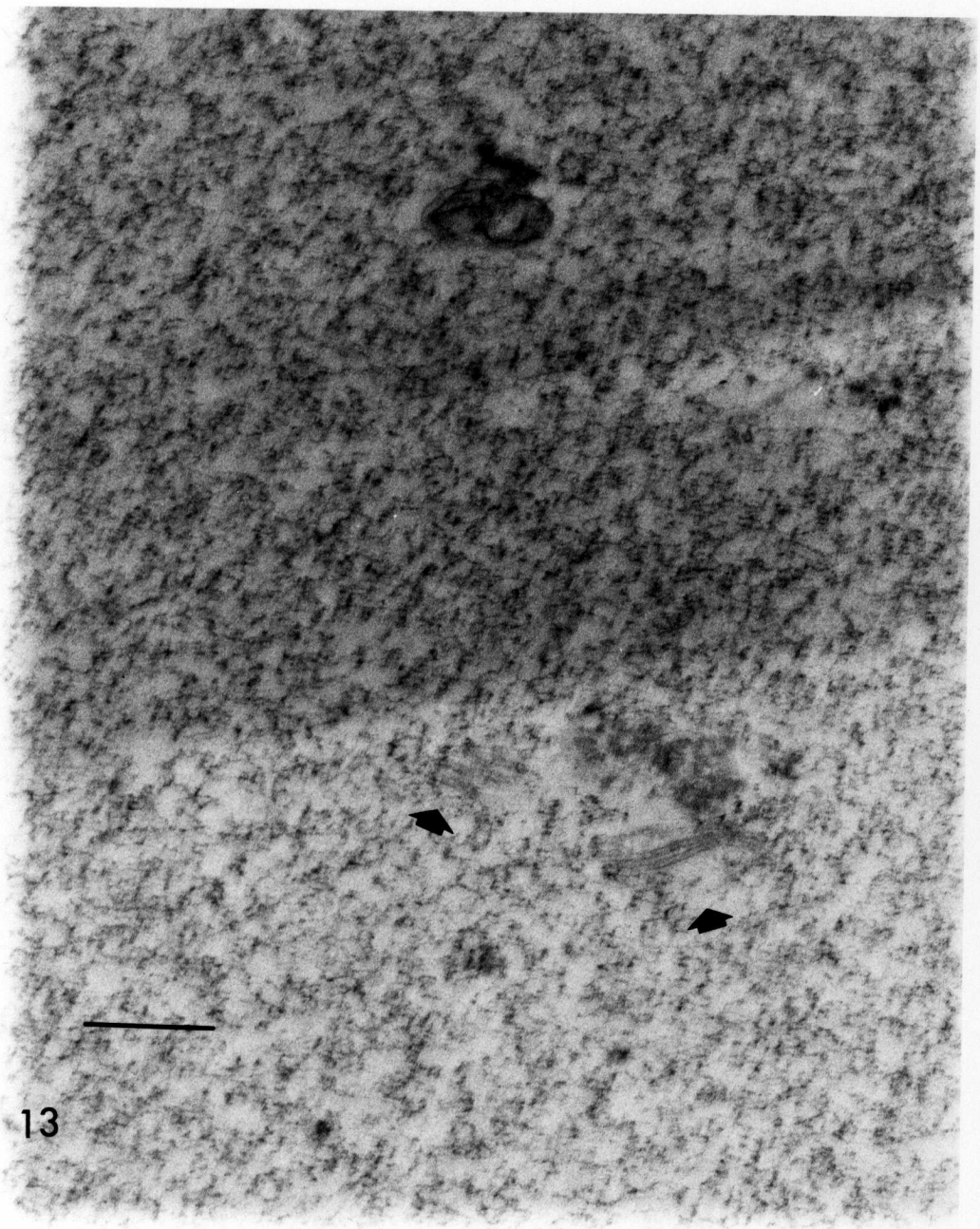

A
SA
N
12

Figure 13. Extracted sperm. Filaments measuring approximately 16 to 19 nm are seen in the chromatin (arrows).
Magnification: 74,000X
Bar = 0.25 um



13

Figure 14. Extracted sperm. Tubules of approximately 16 to 19 nm are seen in longitudinal section and cross-section within chromatin (arrows).
Magnification: 47,000X
Bar = 0.25 um

Inset: Cross-section of radial process.
Magnification: 86,000X

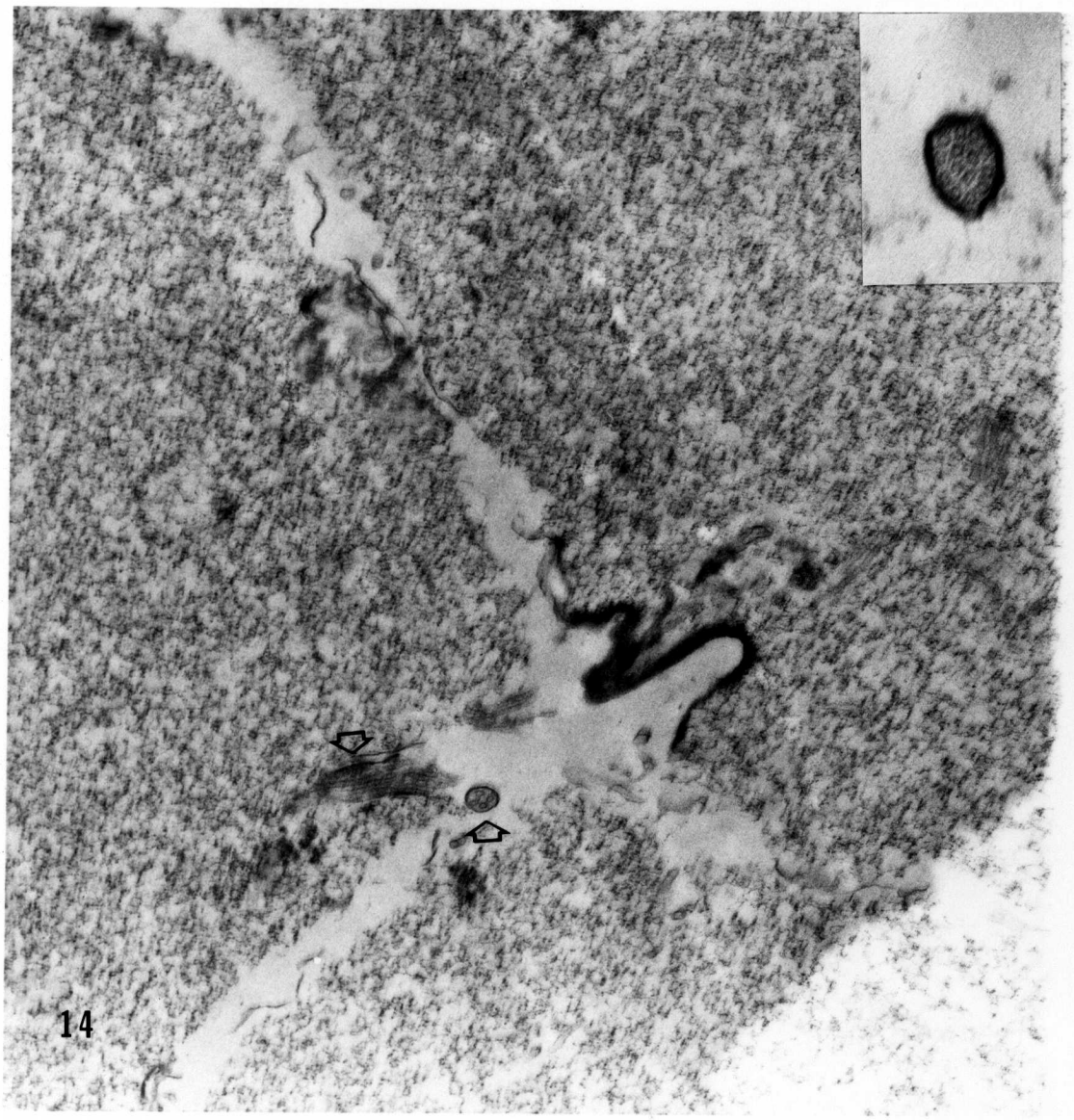

14

Figure 15. Ionophore reacted sperm. In reacted sperm the acrosome (A) appears flocculent instead of dense. Small electron dense granules contain high percentages of magnesium phosphate and calcium phosphate (See text). Nucleus (N), expanded area of nucleus (E).
Magnification: 34,000X
Bar = 0.5 um

Inset: Tubules in radial process (arrow).
Magnification: 27,000X

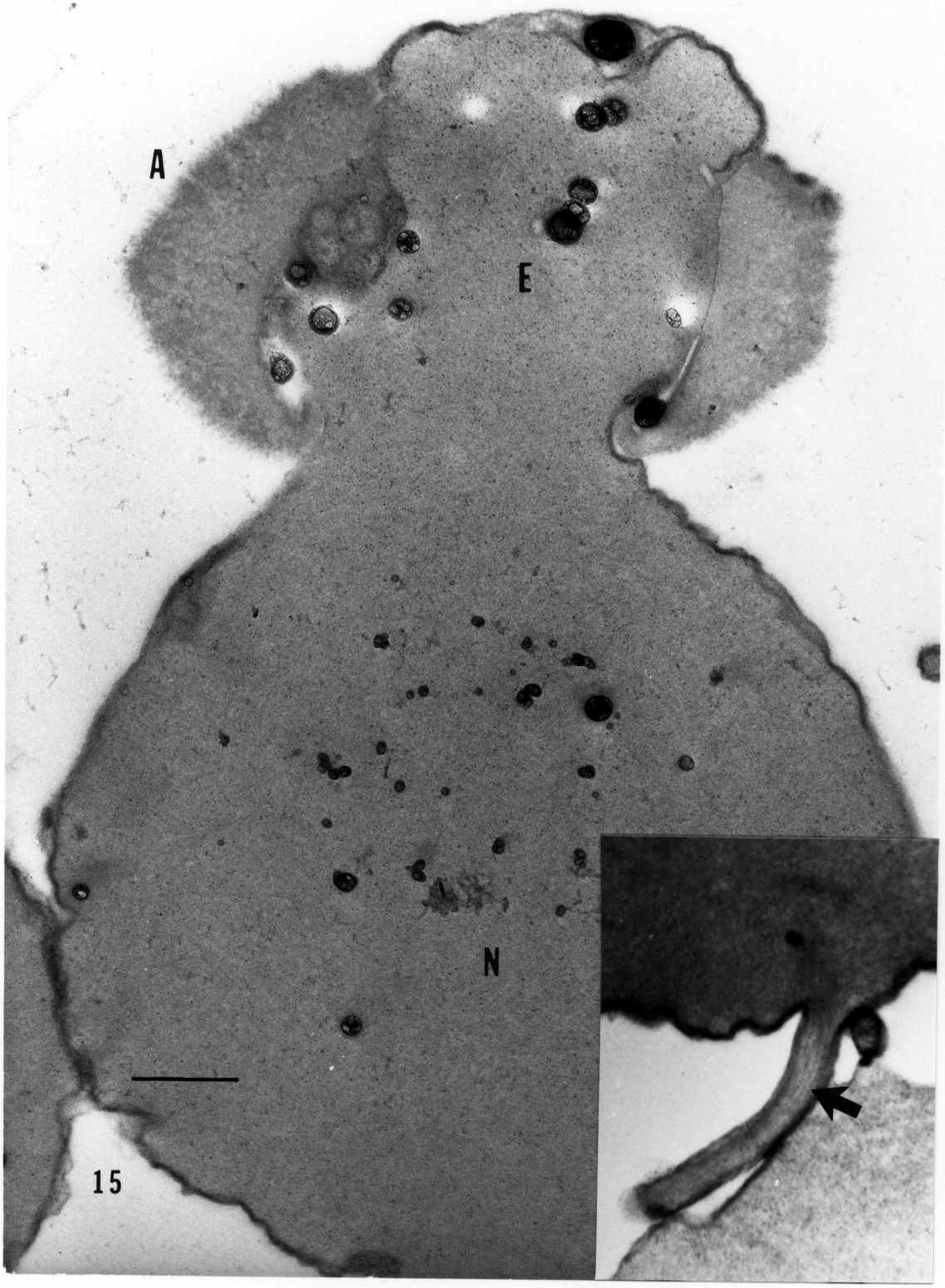
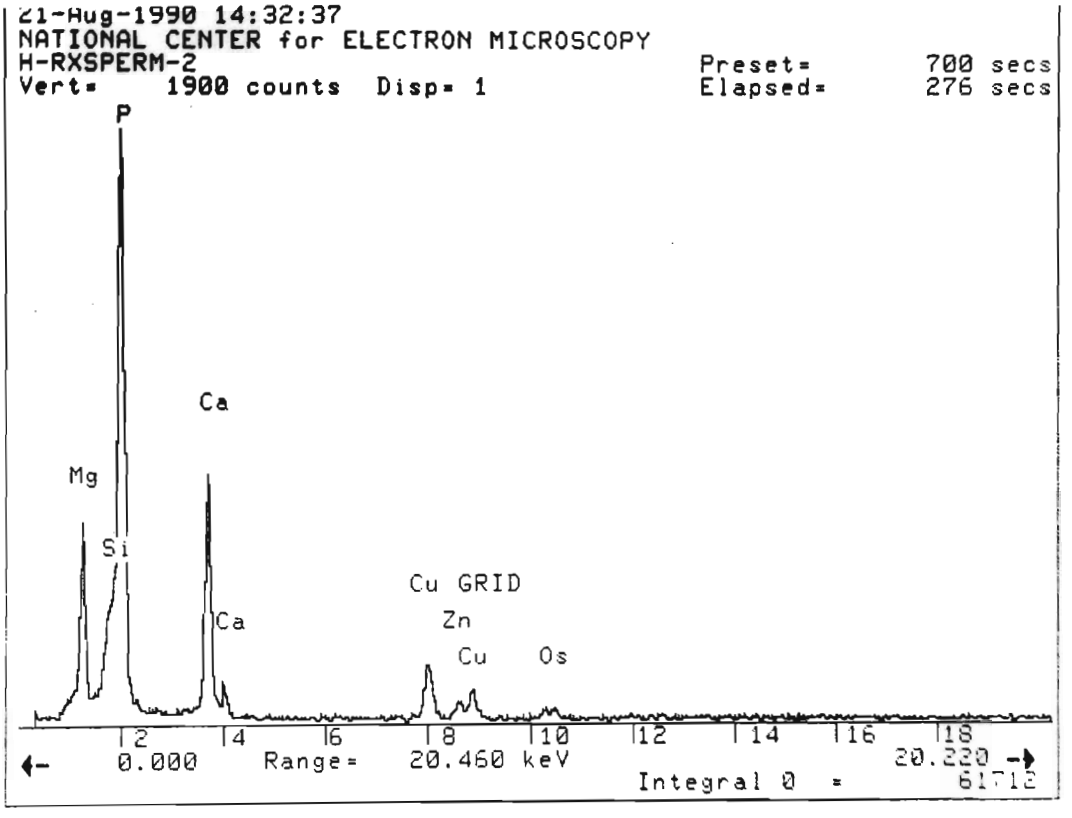

A
E
N
15

Figure 16. Energy dispersal x-ray analysis of electron dense granules in nuclear area of reacted sperm indicates high quantities of magnesium phosphate and calcium phosphate.


21-Aug-1990 14:32:37
NATIONAL CENTER for ELECTRON MICROSCOPY
H-RXSPERM-2
Vert= 1900 counts Disp= 1
Preset= 700 secs
Elapsed= 276 secs
P
Ca
Mg
Si
Ca
Cu GRID
Zn
Cu
Os
2
4
6
8
10
12
14
16
18
0.000
Range= 20.460 keV
20.220
Integral 0 =

16

Table 1. Energy dispersal x-ray analysis indicates atomic and weight percent of elements found in electron dense granules in sperm.

Aug. 21, 1990 H-RXSPERM-2

THIN FILM ANALYSIS
(THEORETICAL K-FACTORS)

Accelerating voltage	200.0 KeV
Beam - sample incidence angle	72.0 degrees
Xray emergence angle	90.0 degrees
Xray - window incidence angle	0.0 degrees
Window thickness	7.5 microns

ELEMENT & LINE	K-FACTOR
Mg Ka	1.4319
Si Ka	1.0000
P Ka	1.0074
Ca Ka	0.9158
Zn Ka	1.5733

ELEMENT & LINE	WEIGHT PERCENT	ATOMIC PERCENT	PRECISION * 2 SIGMA	INTENSITIES
Mg Ka	18.77	23.94	0.34	14.67
Si Ka	9.06	10.01	0.20	10.15
P Ka	49.72	49.78	0.47	55.26
Ca Ka	18.80	14.55	0.26	22.98
Zn Ka	3.65	1.73	0.17	2.60
TOTAL	100.00			

* ABSOLUTE PRECISION OF WEIGHT PERCENT

Figure 17. Graph of x-ray analysis of elements found in mitochondria indicates low amounts of calcium, magnesium, and phosphate were found in mitochondria.

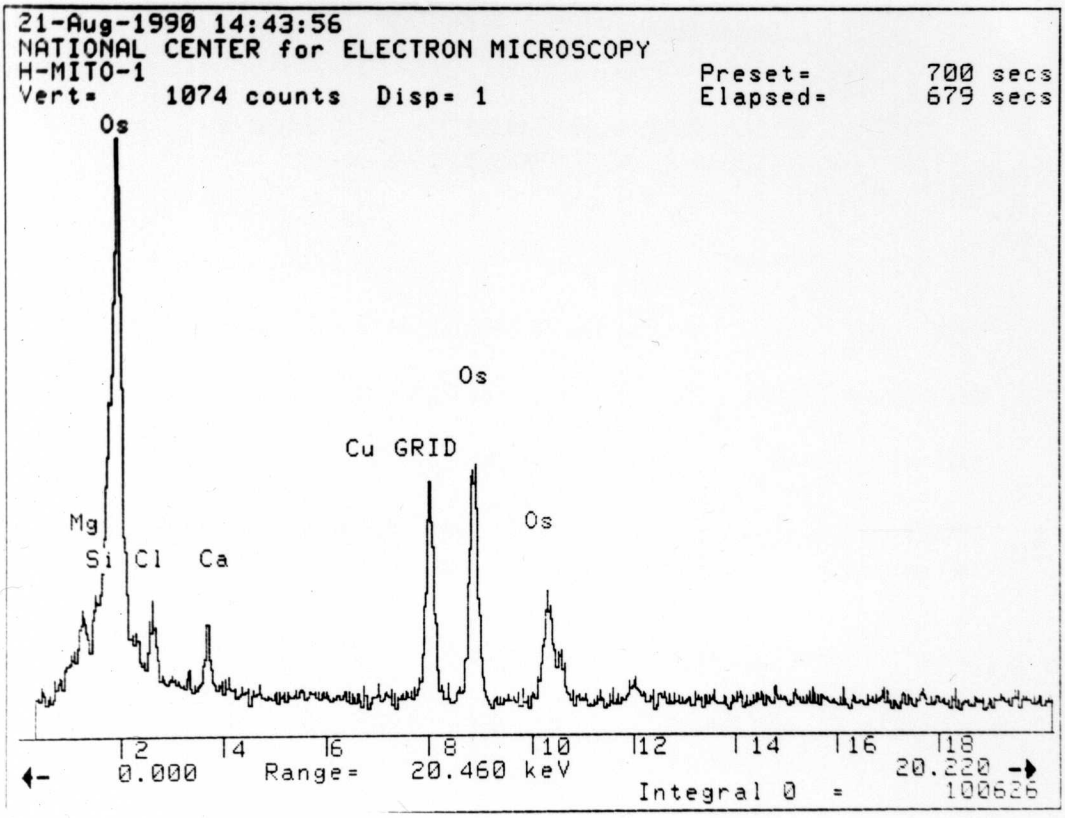

21-Aug-1990 14:43:56
NATIONAL CENTER for ELECTRON MICROSCOPY
H-MITO-1
Vert= 1074 counts Disp= 1
Preset= 700 secs
Elapsed= 679 secs
Os
Os
Cu GRID
Os
Mg
Si
Cl
Ca
2
4
6
8
10
12
14
16
18
0.000
Range= 20.460 keV
20.220
Integral 0 = 100626

Table 2. X-ray analysis of atomic and weight percents of elements found in mitochondria of sperm.

Aug. 21, 1990 H-MITO-1

THIN FILM ANALYSIS
(THEORETICAL K-FACTORS)

Accelerating voltage	200.0 KeV
Beam - sample incidence angle	72.0 degrees
Xray emergence angle	90.0 degrees
Xray - window incidence angle	0.0 degrees
Window thickness	7.5 microns

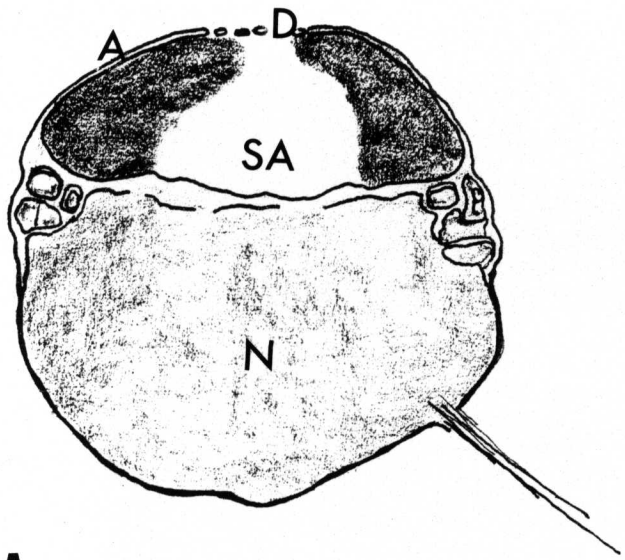
ELEMENT & LINE	K-FACTOR
Mg Ka	1.4319
Al Ka	1.1818
Si Ka	1.0000
S Ka	0.9991
Cl Ka	0.9795
Ca Ka	0.9158
Os La	3.7632

ELEMENT & LINE	WEIGHT PERCENT	ATOMIC PERCENT	PRECISION * 2 SIGMA	INTENSITIES
Mg Ka	5.60	14.80	0.27	1.55
Al Ka	5.01	11.95	0.23	1.68
Si Ka	14.55	33.31	0.34	5.77
S Ka	3.17	6.36	0.18	1.26
Cl Ka	3.83	6.94	0.17	1.55
Ca Ka	2.94	4.72	0.15	1.27
Os La	64.90	21.93	0.94	6.84
TOTAL	100.00			

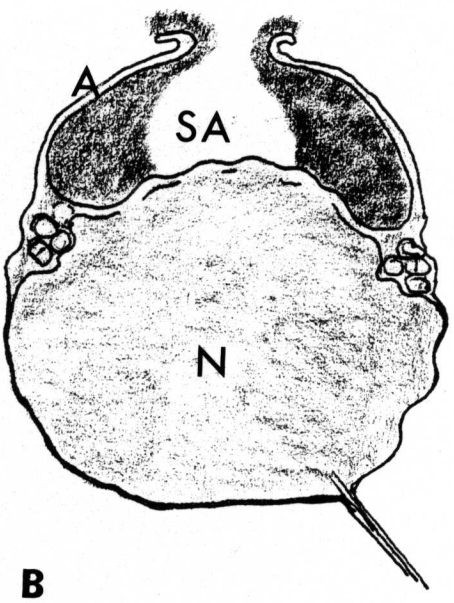
* ABSOLUTE PRECISION OF WEIGHT PERCENT

Figure 18. Schematic drawings of acrosome reaction. Acrosome (A), expanded nuclear area (E) central depression (D), nucleus (N), subacrosomal area (SA).

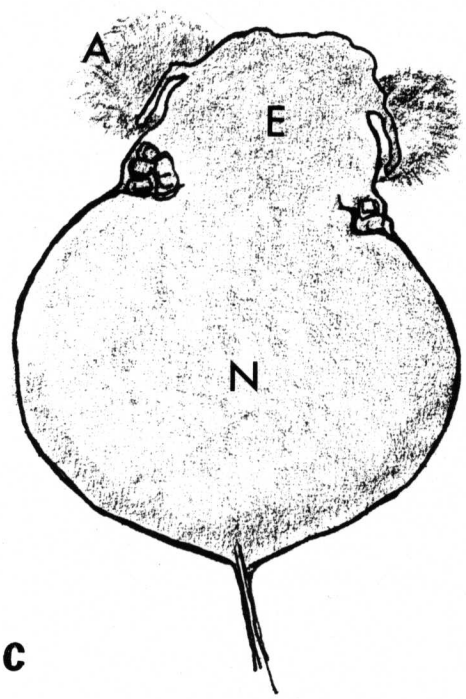
A. Initial phase, opening of the central depression.
B. Release of contents of subacrosomal area.
C. Acrosome forms ring above nuclear area (arrow) and nuclear area expands.
D. Nuclear material pushes through acrosomal ring. Beginning of sperm eversion.



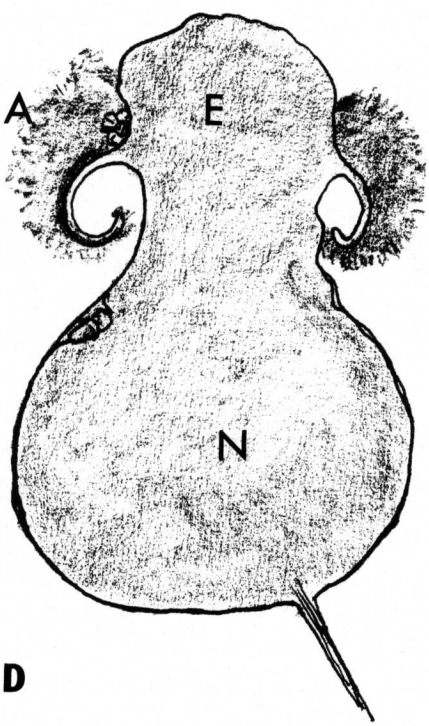
A



B



C

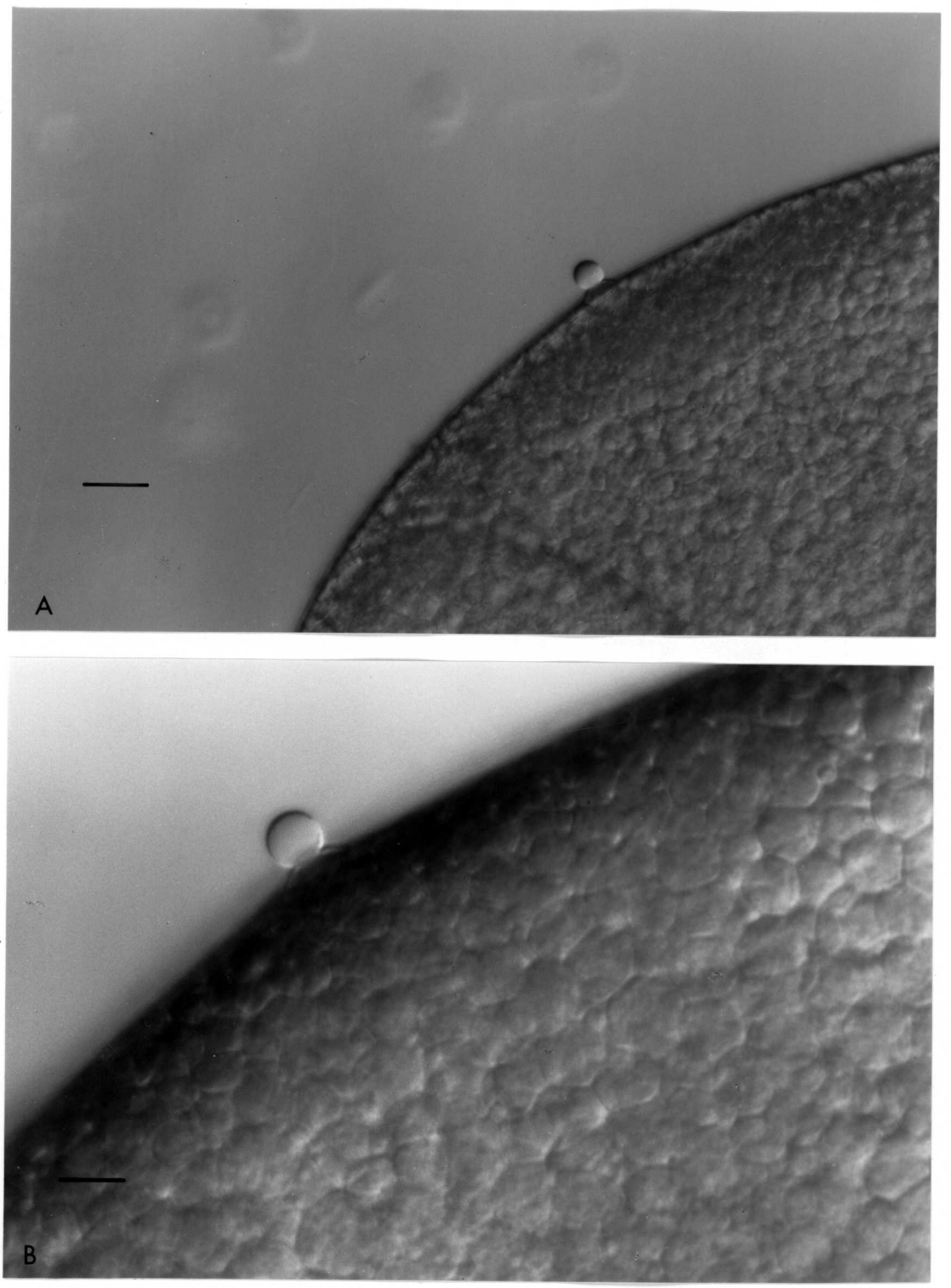


D

Figure 19. Acrosome reaction of sperm in presence of egg.

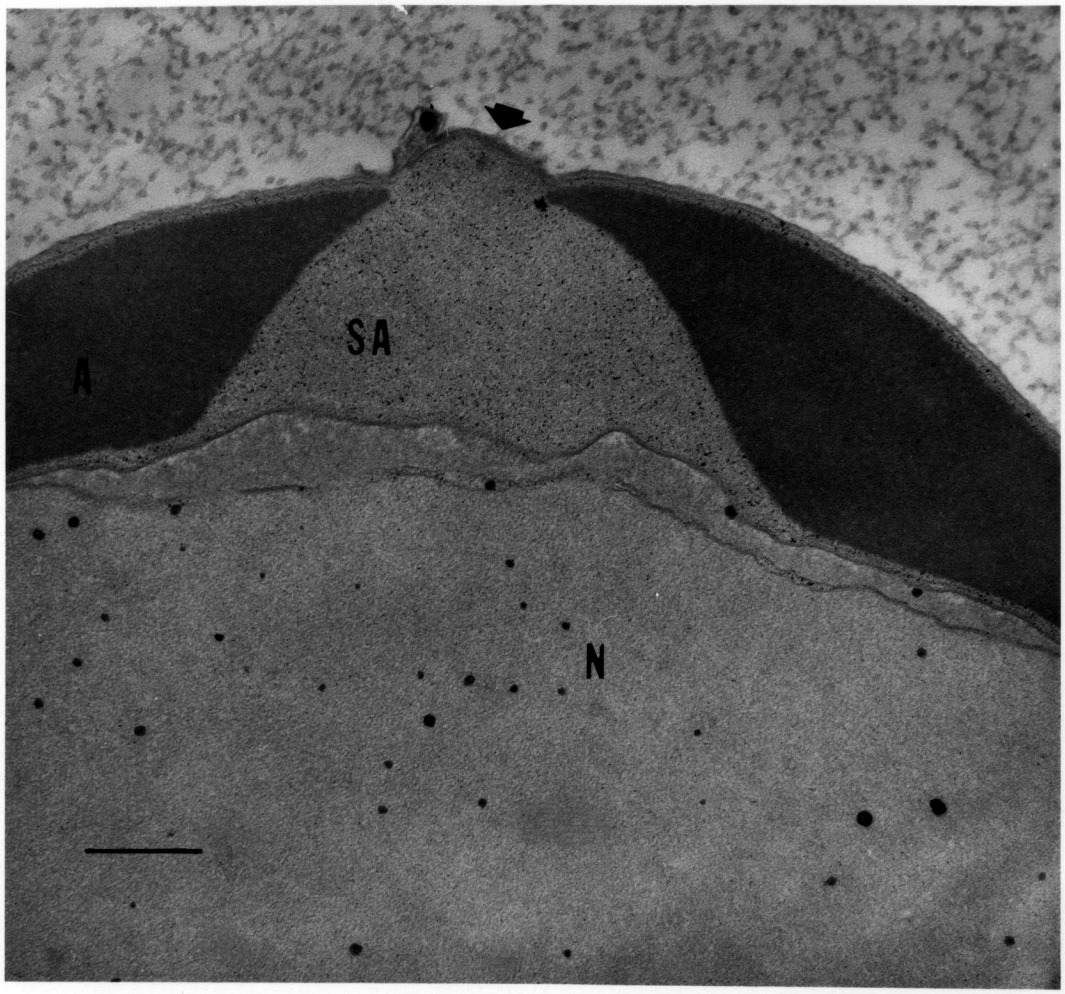
A. Sperm has undergone acrosome reaction in response to contact with egg. Radial processes are faintly visible at side of nucleus.
Magnification: 1000X
Bar = 10 um

B. "Cone" projecting from nucleus to egg may be ring formed by acrosome above the nucleus. Sperm appear to sink into egg.
Magnification: 2000X
Bar = 5 um



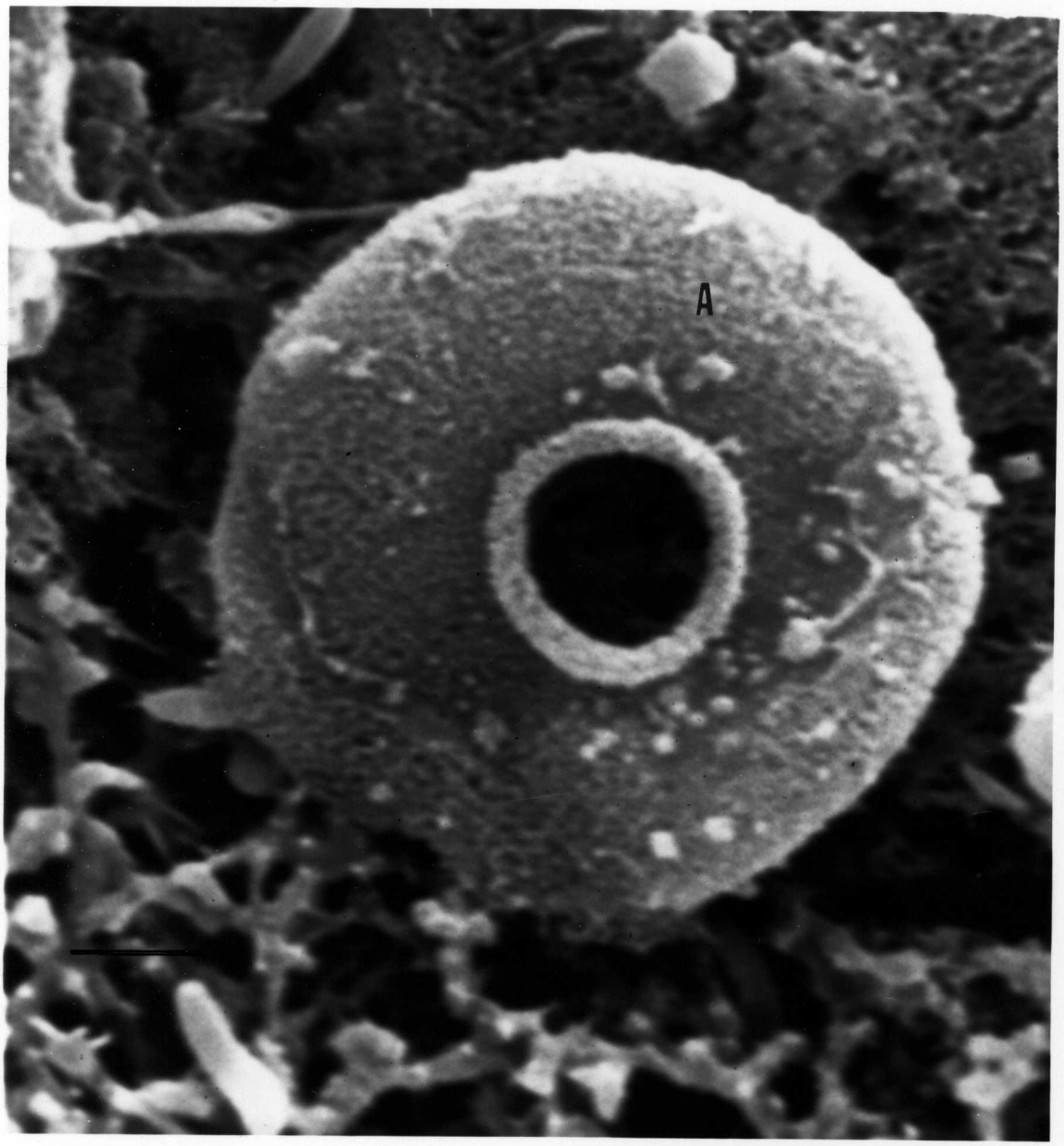
19

Figure 20. Ionophore reacted sperm showing opening of central depression and escaping subacrosomal material (arrow). Acrosome (A), nucleus (N), subacrosomal area (SA).
Magnification: 57,000X
Bar = 0.25 um



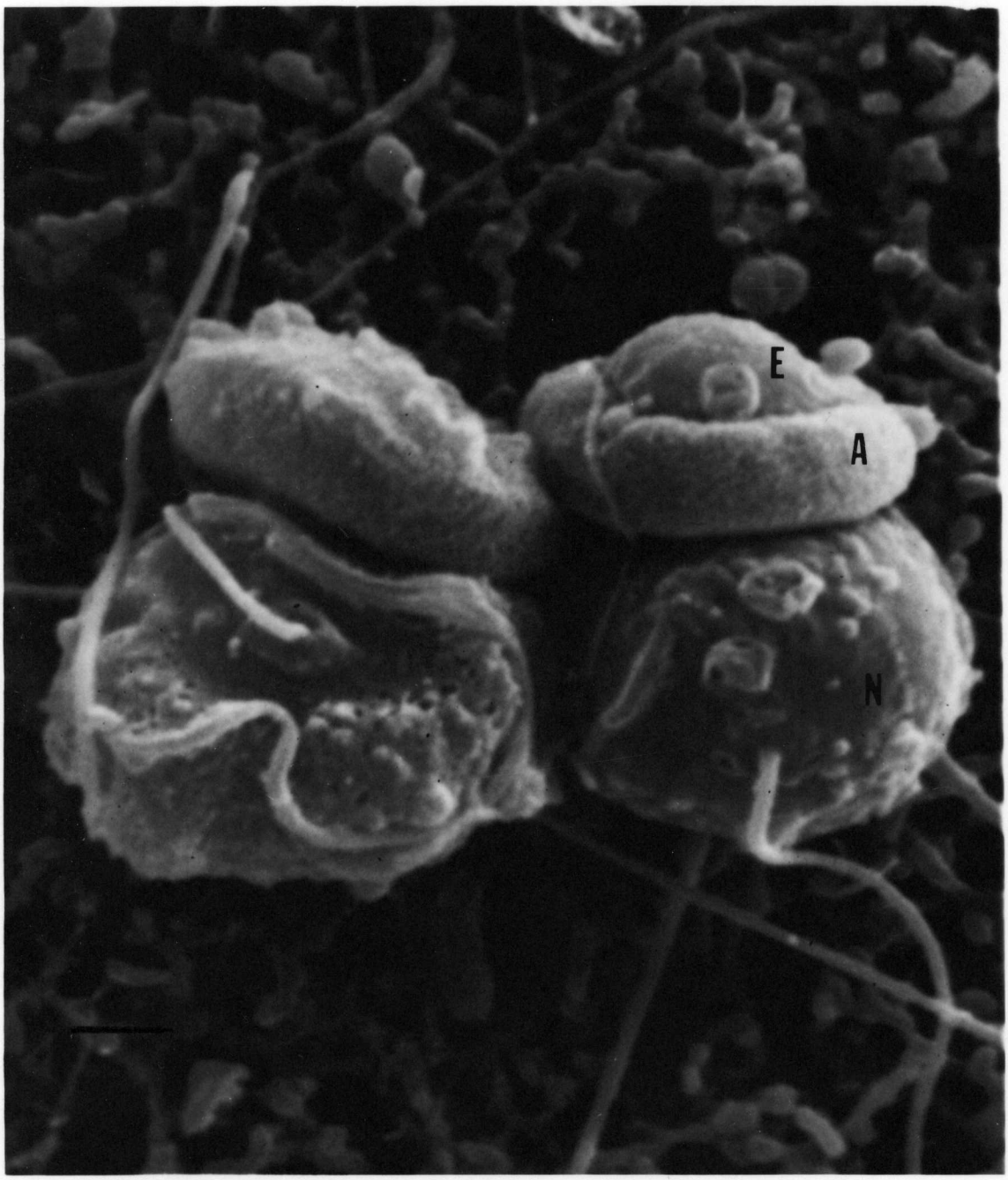
20

Figure 21. Central depression opens in ionophore reacted sperm. Acrosome (A).
Magnification: 19,000X
Bar = 1 um



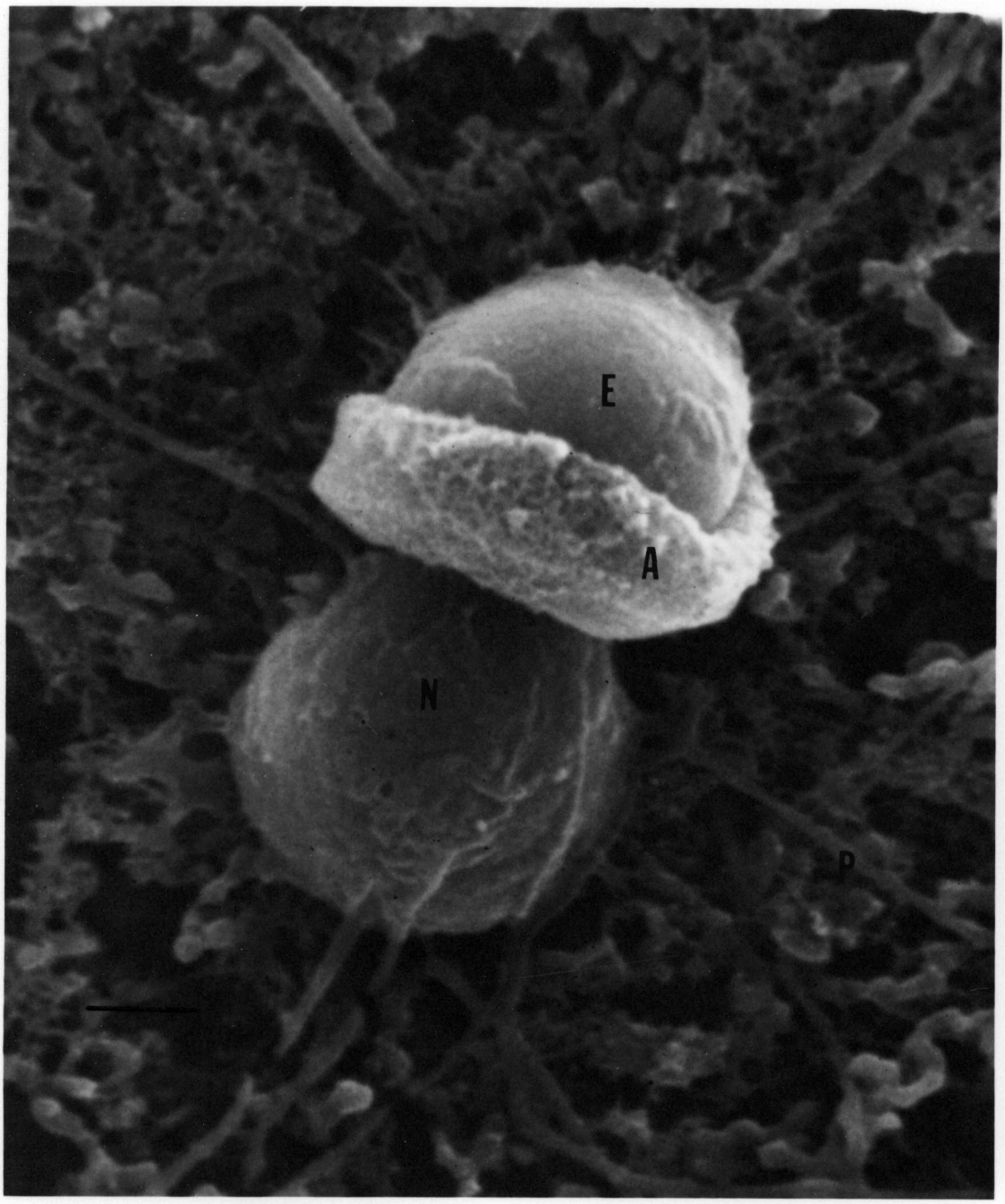
21

Figure 22. Acrosome opens forming ring, and nuclear area expands in ionophore reacted sperm. Acrosome (A), expanded nuclear area (E), nucleus (N), radial processes (P).
Magnification: 16,000X
Bar = 1 um



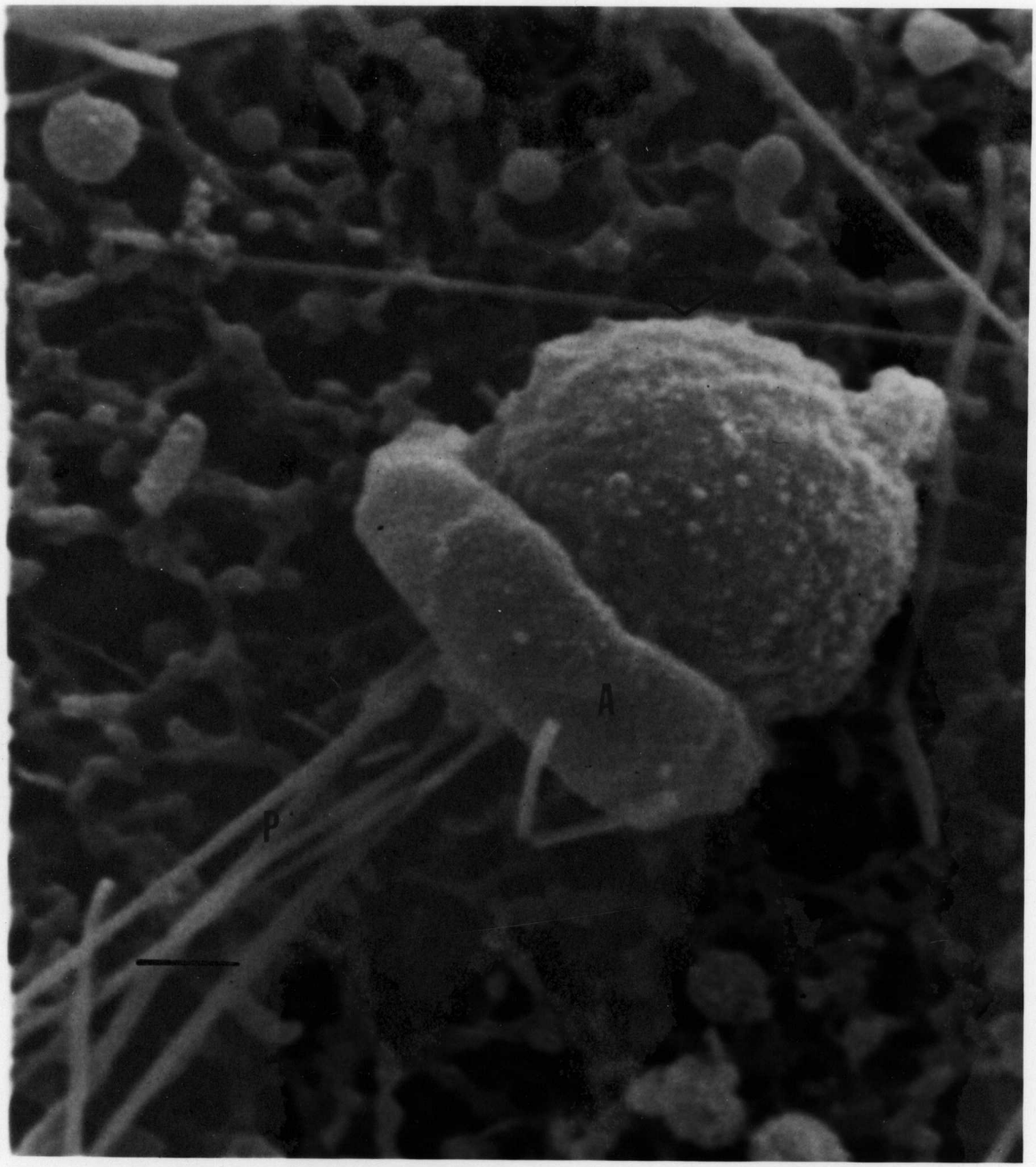
22

Figure 23. Nuclear area expands through acrosomal ring in ionophore reacted sperm. Acrosome (A), expanded nuclear area (E), nucleus (N), radial processes (P). Magnification: 15,800X Bar = 1 um



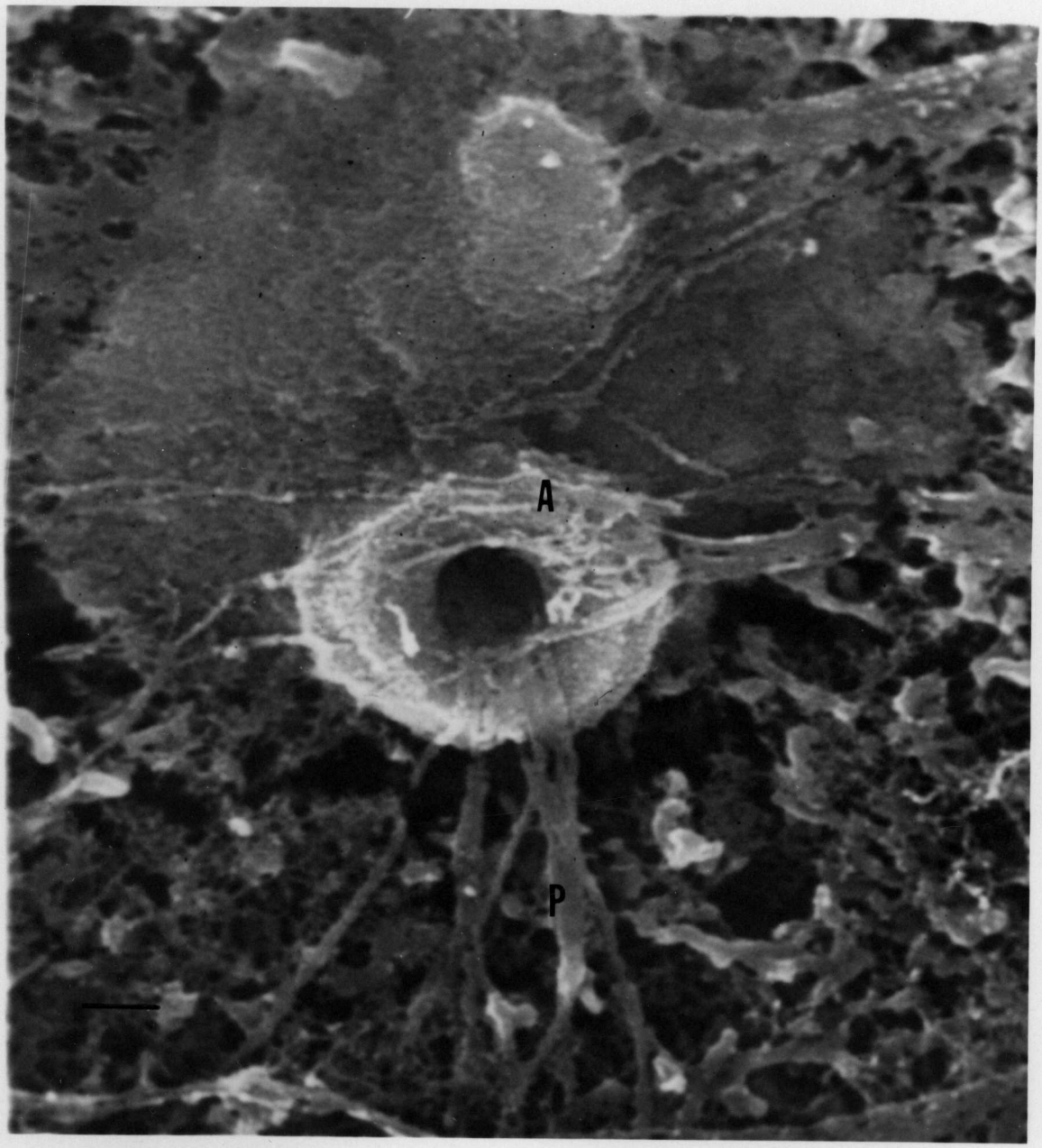
23

Figure 24. In everted reacted sperm posterior acrosomal membrane has become outer membrane of cell (arrow). Radial processes (P).
Magnification: 15,000X
Bar = 1 um



24

Figure 25. In last phase of reaction sperm is completely everted, and nuclear material has gone through acrosomal ring. Acrosome (A), radial processes (P).
Magnification: 12,000X
Bar = 1 um



25