The Acrosomal Membrane of Boar Sperm: A Golgi-derived Membrane Poor in Glycoconjugates

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ABSTRACT The acrosome is a large secretory vesicle of the sperm head that carries enzymes responsible for the digestion of the oocyte's investments. The event leads to sperm penetration and allows fertilization to occur. Release of the acrosomal enzymes is mediated by the interaction between sperm acrosomal and plasma membranes (acrosome reaction). Biochemical characterization of the acrosomal membrane has been restrained by a lack of methods to isolate uncontaminated fractions of the membrane. Here, we use new methods to expose the membrane to in situ cytochemical labeling by lectin–gold complexes. We study the topology and relative density of glycoconjugates both across and along the plane of the acrosomal membrane of boar sperm. Detachment of the plasma membrane from glutaraldehyde-fixed cells exposed the cytoplasmic surface of the acrosome to the lectin markers; freeze-fractured halves of the acrosomal membrane were marked by "fracture-label" (Aguas, A. P., and P. Pinto da Silva, 1983, *J. Cell Biol.* 97:1356–1364). We show that the cytoplasmic surface of the intact acrosome is devoid of binding sites for both concanavalin A (Con A) and wheat germ agglutinin (WGA). By contrast, it contains a high density of neuraminidase-resistant anionic sites detected by cationic ferritin. On freeze-fractured sperm, the receptors for Con A partitioned with the exoplasmic membrane half of the acrosomal membrane. The Con A-binding glycoconjugates were accumulated on the equatorial segment of the membrane. A low density of WGA receptors, as well as of intramembrane particles, was found on the freeze-fracture halves of the acrosomal membrane. The plasma membrane displayed, in the same preparations, a high density of receptors for both Con A and WGA. We conclude that the acrosome is limited by a membrane poor in glycoconjugates, which are exclusively exposed on the exoplasmic side of the bilayer. Regionalization of Con A receptors on the acrosome shows that sperm intracellular membranes, like the sperm surface, express domain distribution of glycocomponents.

The acrosome, located in the head of sperm, is a Golgi-derived vesicle that caps the distal two-thirds of the nucleus (1). In mammalian spermatozoa, the acrosome is highly variable in shape from species to species, but in all species studied it contains enzymes such as hyaluronidase, acrosin (a trypsin-like proteinase), acid phosphatase, and arylsulphatase (1–8). Structural and cytochemical studies showed that the acrosome originates from the Golgi complex: it is formed by accretion of clathrin-coated vesicles that pinch off from the *trans*-most portion of the Golgi apparatus of differentiating spermatids (9–12).

The membrane of the acrosome plays a central role in the acrosome reaction, a phenomenon necessary for sperm cells to achieve gamete fusion (13–15). The acrosome reaction involves fusion and fission of acrosomal and plasma membranes and results in exocytosis of enzymes responsible for digestion of the oocyte's investments (cumulus oophorus and zone pellucida) (16). Current knowledge on the chemical composition of the acrosomal membrane is limited (17, 18). Conventional biochemical approaches have been hindered by cross-contamination that occurs during isolation of acrosomal membrane fractions (18, 19). Autoradiography and labeling of ultrathin sections lack the resolution necessary to ascribe the components detected by either technique to membrane profiles exclusively (20–22). Cytochemical studies showed that the acrosomal membrane of mammalian spermatozoa con-

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tains a calcium-sequestering lipid (23), different densities of filipin-sensitive cholesterol molecules along the bilayer (24), and appears to be paved by acrosin (25, 26). In hamster sperm, concanavalin A (Con A)[1]-binding sites were reported on its cytoplasmic side (27), an exceptional finding considering the exclusive expression of carbohydrates on the exoplasmic side of biological membranes (28–30).

In this study, we search for the presence, density, and topology of Con A- and wheat germ agglutinin (WGA)-binding sites on the acrosomal membrane of boar sperm cells, by using in situ cytochemical labeling of the membrane by lectin–protein–colloidal gold complexes. Con A binds to mannose and/or glucose (31) residues, whereas WGA binds to sialic acid and, to a lesser extent, to *N*-acetyl-glucosamine (32, 33). Access of the marker to the membrane was obtained by three different experimental procedures: (*a*) routine disruption of the cell surface by hypotonic shock; (*b*) mechanical detachment of the plasma membrane from fixed spermatozoa; (*c*) freeze-fracture of intact cells followed by marking of membrane halves by "fracture-label" (34–37).

MATERIALS AND METHODS

Cells

Numerous samples of raw semen from mature boars, *Sus scrofa* (a generous gift of Dr. Lawrence A. Johnson, United States Department of Agriculture, Beltsville, MD), were centrifuged (500 *g*, 5 min), and the sperm-rich pellets were washed by successive pelleting and resuspension (three times) in Hanks' balanced salt solution or in phosphate-buffered saline (PBS). The spermatozoa were fixed in 1.5% glutaraldehyde in PBS, pH 7.4, at ice bath temperature for 1 h and washed twice in PBS.

Conventional Freeze-Fracture

Fixed boar spermatozoa were glycerol-impregnated and squeezed between two copper disks of a double replica device (38). The samples were frozen in partially solidified Freon 22 and stored in liquid nitrogen. Freeze-fracture was carried out at −140°C in a Balzers 301 apparatus (Hudson, NH). Replicas were obtained by evaporation from a Pt/C source and cleaned in NaClO and distilled water. Nomenclature of fracture faces is according to Branton et al. (39).

Fracture-Label

Fixed and washed boar spermatozoa were impregnated in 30% glycerol in PBS and mixed in a solution of 30% (wt/vol) bovine serum albumin (BSA) in 30% glycerol (in PBS). Small droplets of the mixture were dispersed on precleaned glass coverslips that were superimposed on other coverslips paved with glutaraldehyde (3% in 30% glycerol/PBS). The preparations were frozen in the liquid phase of partially solidified Freon 22 and transferred to liquid nitrogen, where freeze-fracture of BSA–cell gels was performed by mechanical separation of complementary coverslips immersed in liquid nitrogen (40, 41). The specimens were thawed, deglycerinated, and labeled with Con A-peroxidase-gold or WGA-ovomucoid-gold, as described below (see *Cytochemistry*). Washed specimens were fixed in 1% OsO_4 in veronal buffer for 30 min, dehydrated in ethanol, and critical point-dried in ethanol/CO_2 (35). The fractured gels were shadowed with Pt/C in a Balzers 301 apparatus. Replicas of the fracture faces were isolated by digestion of the gel in NaClO and mounted on formvar-coated grids for electron microscopic observation.

Removal of the Plasma Membrane

HYPOTONIC SHOCK: In this experiment, boar spermatozoa were submitted to hypotonic shock (5 min of incubation in distilled water) and washed twice in PBS before fixation in glutaraldehyde. In addition, we also used cells treated as above. The cells were labeled with lectin–colloidal gold complexes, as described below (see *Cytochemistry*).

MECHANICAL DETACHMENT OF THE MEMBRANE: Fixed and washed boar spermatozoa, in suspension in PBS, were mechanically pushed (three times) through a 25 × ⅝-in needle in order to achieve rupture, and partial removal, of the loosely attached plasma membrane of the sperm head. The cells were washed by successive pelleting and resuspension (three times) in PBS and processed for lectin–gold labeling and thin sections (see *Cytochemistry*).

Labeling by Cationic Ferritin (CF)

Fixed and washed spermatozoa, treated as described in the previous section, were incubated in neuraminidase (from *Clostridium perfringens*, type VIII, Sigma Chemical Co., St. Louis, MO) 1 U/ml in PBS, pH 7.2, for 5 min at 4°C followed by 30 min at 37°C. Neuraminidase-treated and control sperm were incubated in CF (1 mg/ml, Miles Biochemicals, Miles Laboratories, Elkhart, IN) in PBS, pH 7.2, at 4°C for 15 min. The samples were washed in PBS and processed, with some of them embedded in a cross-linked matrix of BSA, for thin-section electron microscopy.

Cytochemistry

Samples of isolated cells or BSA–sperm gels were incubated in solutions of Con A (0.25 mg/ml) or WGA (0.25 mg/ml) in Sorensen's phosphate buffer, 4% polyvinylpyrrolidone, pH 7.4, for 1 h at room temperature (Con A) or 37°C (WGA). Controls were preincubated in lectin-competitive sugars—0.4 M methyl-α-D-mannopyranoside (Con A experiments) or 0.4 M *N*-acetyl-D-glucosamine (WGA experiments)—for 15 min, followed by incubation in the lectin, in presence of the sugar, as described above. After washing was done in Sorensen's buffer, the samples were labeled in a colloidal gold–horseradish peroxidase complex (42–44) (Con A experiments) or in ovomucoid-coated colloidal gold (43–45) (WGA experiments) for 3 h at 4°C. All samples were washed in Sorensen's buffer and processed for electron microscopy.

Processing for Thin-Section Electron Microscopy

Cells and gel fragments were postfixed in 2% OsO_4, and in veronal buffer; reduced by the addition of $K_3Fe(CN)_6$ to a final concentration of 1% (46–48); stained *en bloc* with uranyl acetate (5 mg/ml) in the same buffer, pH 5.8; dehydrated in acetone; and embedded in Epon 812. Thin sections were observed by transmission electron microscopy after counterstaining with lead citrate only.

RESULTS

Structure of the Acrosomal Membrane

Replicas of both protoplasmic and exoplasmic freeze-fracture faces of the acrosomal membrane of boar sperm, obtained by conventional methods, showed few intramembrane particles. Structurally, three areas were distinguished in freeze-fracture replicas of the acrosome: (*a*) the equatorial segment, consisting of a hexagonal array of subunits; (*b*) the peripheral swollen rim, formed by parallel linear ridges made up of small rugosities; and (*c*) the main portion of the membrane, limited by the two previous areas, showing a smooth, noncrystalline texture (Fig. 1).

Cytochemistry of the Acrosomal Membrane

We used three different experimental procedures to study the topology of lectin receptors on the acrosomal membrane of boar sperm cells. In all experiments, Con A- and WGA-binding sites were visualized in situ after incubation of glutaraldehyde-fixed spermatozoa in the lectins, followed by labeling with colloidal gold coated with horseradish peroxidase (42–44) and ovomucoid (43–45), respectively. Specificity of lectin labeling was verified by the drastic reduction (70% or more) in the number of colloidal gold spheres observed on acrosomal membranes of sperm samples submitted to lectin incubation in presence of their competitive sugars (α-D-methyl-mannopyranoside for Con A; *N*-acetyl-D-glucosamine for WGA).

[1] *Abbreviations used in this paper:* CF, cationized ferritin; Con A, concanavalin A; WGA, wheat germ agglutinin.

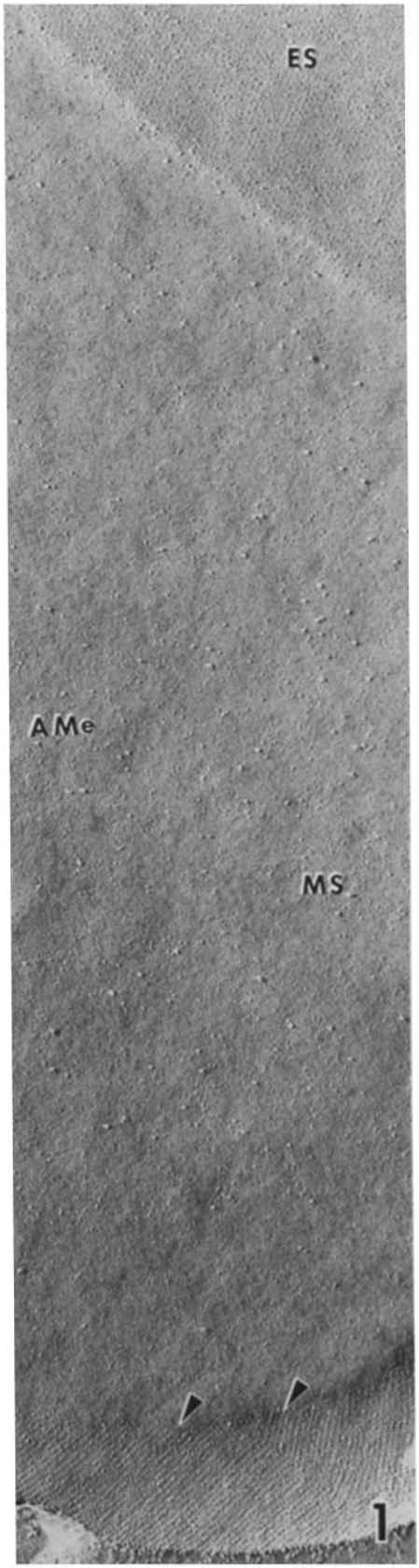


FIGURE 1 Replica of the exoplasmic face of boar acrosomal membrane (*AMe*) obtained by conventional freeze-fracture. The membrane is poor in particles, shows an hexagonal substructure in the equatorial segment (*ES*), and parallel linear ridges made up of rugosities on the peripheral rim of the acrosomal envelope (arrowheads). *MS*, median segment of the acrosome. × 45,000.

LABELING OF MEMBRANE HALVES

Halves of the acrosomal membrane of boar sperm were obtained by freeze-fracture of BSA–spermatozoa gels and fracture-labeled with lectin-colloidal gold conjugates (40, 41, 49). In the Pt/C replicas of critical point-dried specimens, the equatorial segment was formed by a finely granular pattern that was morphologically distinct from the remaining areas of the membrane where a rugose texture was seen (Figs. 2 and 3). The plane of freeze-fracture of spermatozoa in fracture-labeled preparations preferentially followed the plasma membrane. Extensive areas of the protoplasmic half of the acrosomal membrane were particularly difficult to obtain.

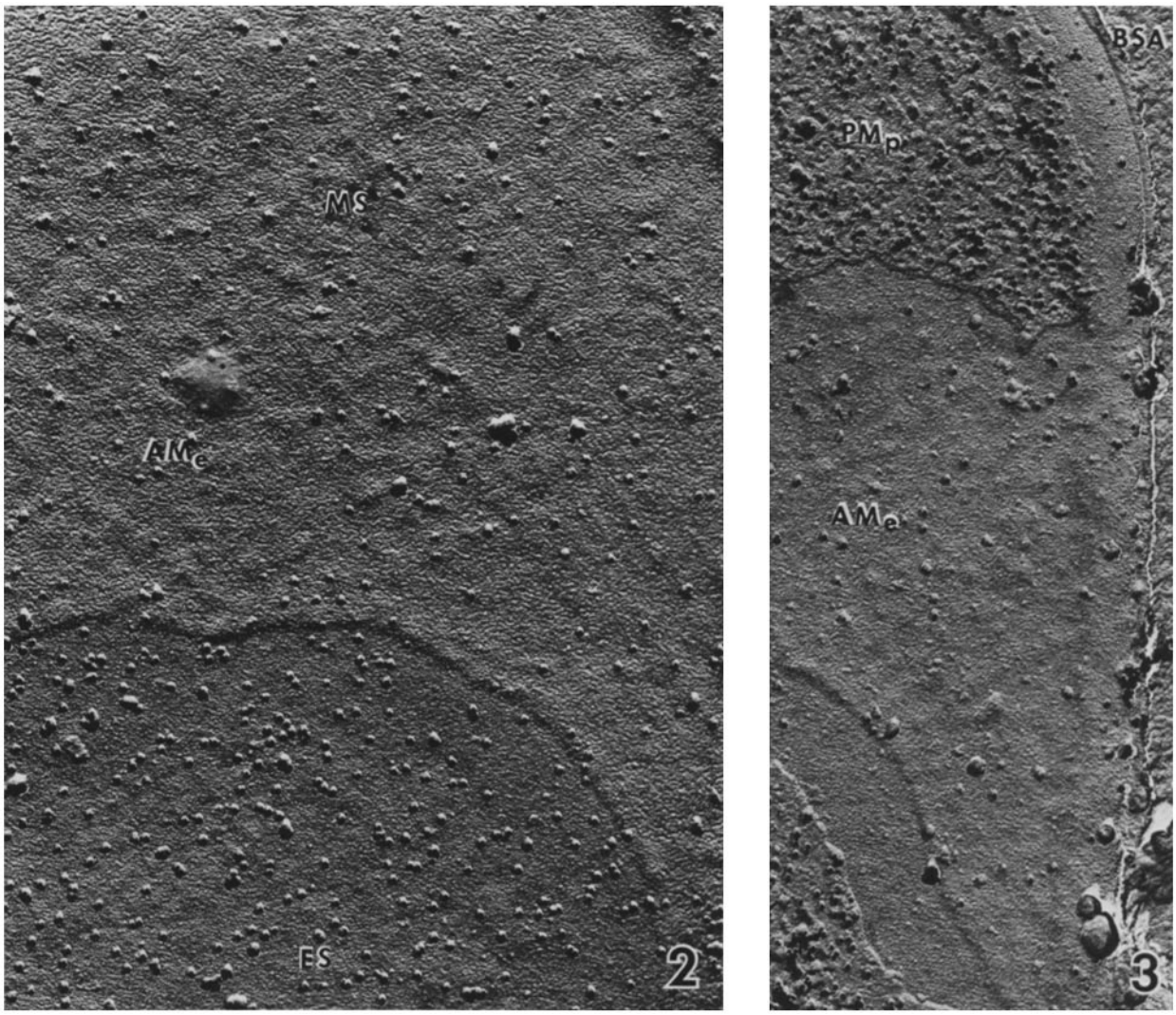
CON A: The exoplasmic membrane half of the acrosomal membrane was moderately labeled by Con A–colloidal gold conjugates (Fig. 2). The density of Con A-binding sites was 2.7 to 3 times higher on the equatorial segment than in the other areas of the acrosome (Fig. 2). The protoplasmic half of the membrane was not significantly labeled by Con A.

WHEAT GERM AGGLUTININ: Both protoplasmic and exoplasmic halves of the acrosomal membrane were weakly labeled by the lectin (Fig. 3). No regional accumulation was disclosed along the freeze-fractured bilayer. Fracture-labeling of plasma membranes by both Con A and WGA was always much denser than that seen on fractured acrosomal membranes (Fig. 3; Figs. 7 and 9 in reference 40).

SURFACE LABELING

AFTER HYPOTONIC SHOCK: Access of particulate cytochemical markers to the acrosome requires rupture of sperm plasmalemma before cell incubation in the labeling solutions. We used hypotonic shock (5 min in water) as the first procedure to induce cell membrane rupture; we also searched for labeling on the rare sperm cells showing spontaneous loss of the plasma membrane in samples not submitted to hypotonic shock. In both specimens, we found that the outer (i.e., cytoplasmic) surface of the acrosome was labeled by both Con A and WGA–gold conjugates (Fig. 4). In all instances, the marker was not closely apposed to the unit-membrane profiles; instead, it was associated with a "fuzzy coat" on the surface of the outer acrosomal membrane. This coat was comprised of filaments of irregular length (8–24 nm) that were perpendicular to the membrane profile. The fuzzy coat was detected by treatment of sperm cells with ferricyanide-reduced osmium tetroxide, a stain for glycoconjugates (46–48). In these preparations, the lumen of acrosomes showed a marked decrease in electron density and was partially filled by a similar fuzzy material. A filamentous material, with the density and texture of the fuzzy coat, was frequently seen in the vicinity of the labeled sperm cells (Fig. 4, arrow). These observations suggested release of the glycoprotein-rich content of the acrosome. Therefore, we reasoned that the lectin labeling found on the cytoplasmic surface of the acrosome might result from contamination of the membrane by adsorption of components originally restricted to the acrosomal lumen.

AFTER MECHANICAL DETACHMENT OF THE PLASMA MEMBRANE: To remove the plasma membrane without damaging the acrosome and releasing its content, we induced mechanical rupture of the sperm head plasmalemma by pushing a suspension of glutaraldehyde-fixed cells through a needle. Because of the aldehyde cross-linking (before cell surface disruption), the acrosomal vesicles did not suffer any structural damage: the shape was maintained, the limiting



FIGURES 2 and 3 Replica of freeze-fractured boar acrosomal membrane labeled by Con A–horseradish peroxidase–colloidal gold. The exoplasmic half (*AMe*) is moderately marked by colloidal gold. The density of the marker is 2.7 to three times higher on the equatorial segment (*ES*) of the membrane half than in its median portion (*MS*). × 40,000. Replica of freeze-fractured boar spermatozoa labeled by WGA–ovomucoid–colloidal gold. The exoplasmic half of the acrosomal membrane (*AMe*) is weakly labeled by colloidal gold whereas the protoplasmic half of the plasma membrane (*PMp*) is heavily labeled. × 31,000.

membrane was intact, and the acrosome lumen displayed its normal, high electron density. In these preparations, we found no labeling of the cytoplasmic side of the acrosomal membrane by both Con A (Fig. 5) and WGA (Fig. 6), whereas the plasma membrane showed labeling by the lectin–gold complexes as reported (40).

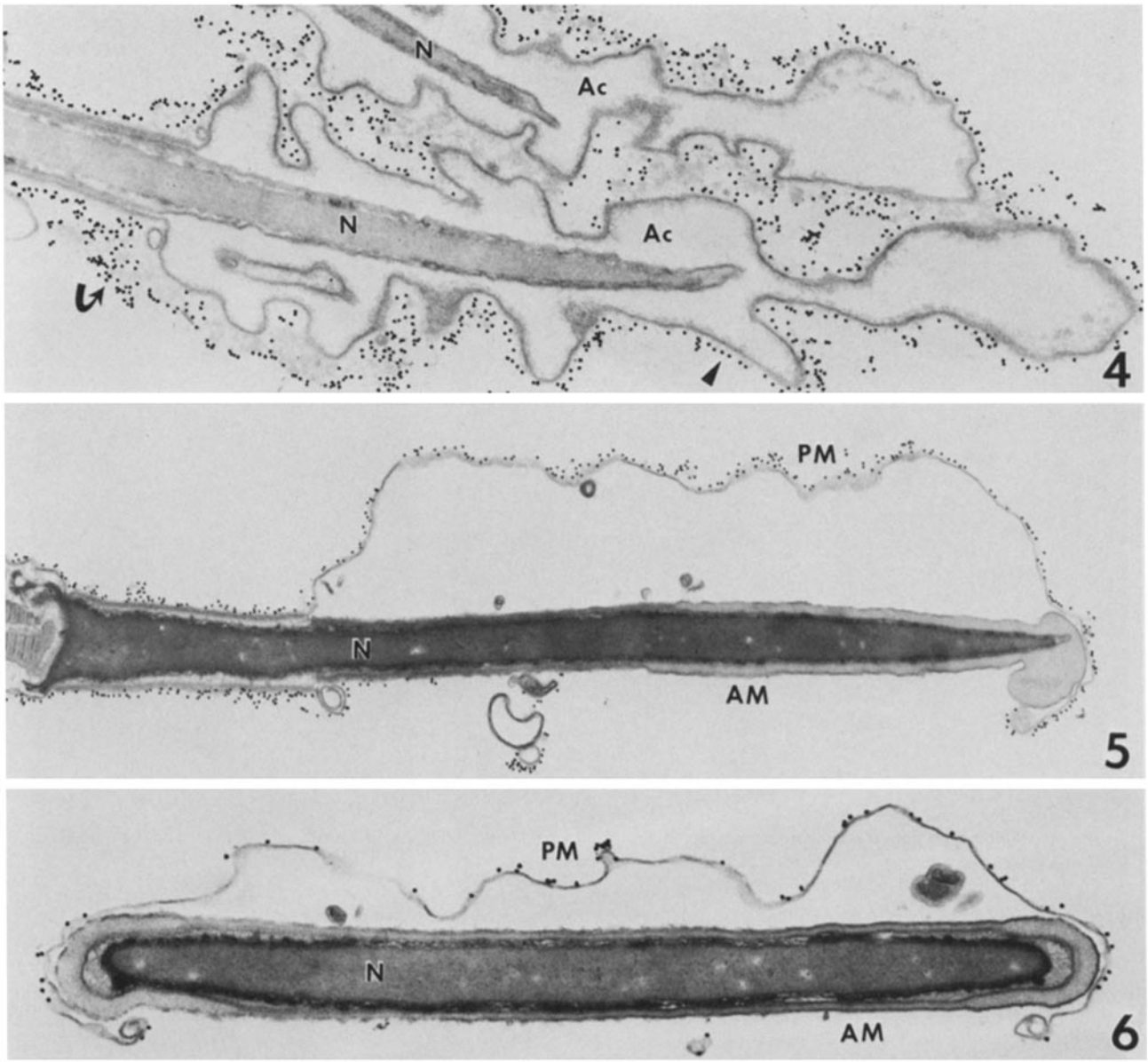
ANIONIC SITES: CF strongly marked the cytoplasmic surface of the acrosomal membrane. The CF particles were closely apposed to the acrosomal membrane profile; in contrast, a space of 8–11 nm separated the trilaminar profile of the plasma membrane (known to be paved by a fuzzy coat [3, 18]) and CF labeling (Fig. 7). Digestion of fixed cells by neuraminidase removed most of the binding sites for CF on the plasma membrane but did not decrease the density of the marker on the acrosomal membrane (Fig. 8).

DISCUSSION

In this study, we searched for the topology and relative density of glycocomponents on the acrosomal membrane of boar sperm using lectin–gold complexes to label residues of mannose (marked by Con A [31]) and sialic acid/*N*-acetyl-glucosamine (marked by WGA [32, 33]). The cells were submitted to methods of exposing the acrosomal surface (hypotonic shock and mechanical detachment of the plasma membrane from fixed sperm) and membrane halves (freeze-fracture) to labeling. Our results define the glycoconjugates of the acrosomal membrane according to four different parameters: (*a*) presence or absence on the side of the membrane (cytoplasmic) facing the plasmalemma; (*b*) partition with exoplasmic and protoplasmic leaflets split by freeze-fracture; (*c*) planar distribution along the membrane halves; and (*d*) comparative cytochemistry of acrosomal and plasma membranes.

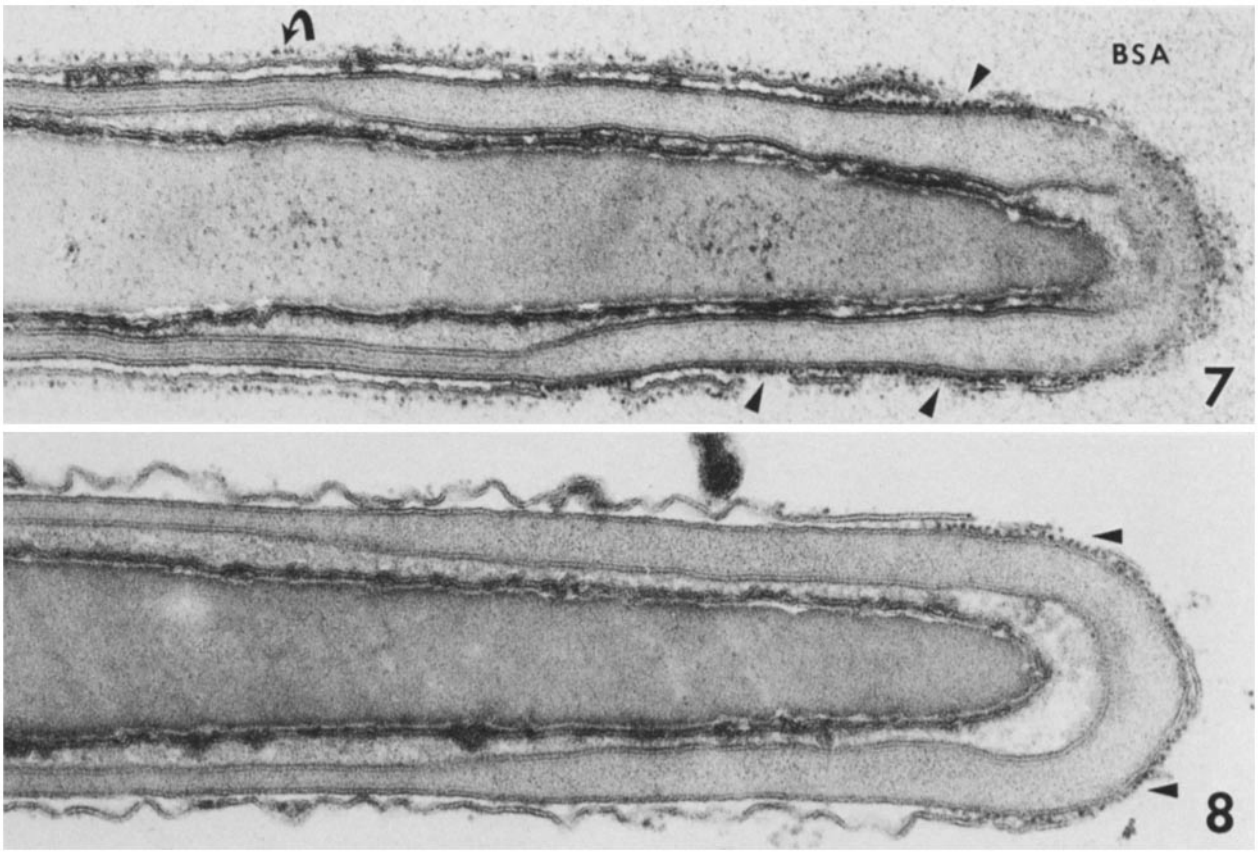
Cytoplasmic Surface of the Acrosome Lacks Glycoconjugates

Receptors for Con A were reported on the cytoplasmic side of the acrosomal membrane of hamster spermatozoa showing rupture of the cell surface (27). Similarly, in our initial labeling experiments done on sperm cells where disruption of the plasma membrane was either spontaneous or induced by



FIGURES 4–6 Lectin–gold labeling of the acrosomal membrane after removal of the plasma membrane before (Fig. 4) and after fixation (Figs. 5 and 6). In Fig. 4, the acrosome lumen (*Ac*) has decreased electron density and the WGA–gold marking is associated to a filamentous material adsorbed to (arrowheads) or in the vicinity of (arrow) the cytoplasmic surface of the acrosomal membrane. In Fig. 5 (Con A) and 6 (WGA), the acrosomal content and membrane are kept intact upon rupture of the plasma membrane induced in fixed sperm. No significant gold labeling is seen on the cytoplasmic surface of the acrosome (*AM*) whereas the exoplasmic side of the plasma membrane (*PM*) is well marked. *N*, nucleus. × 35,000; × 26,000; × 39,000.

hypotonic lysis, we found intense lectin marking associated with a fuzzy coat seen on the cytoplasmic surface of the acrosome. In those preparations, however, release of the acrosomal content had occurred, as it is evidenced by the low electron density of the vesicle lumen and the presence of a filamentous, lectin-labeled material in the vicinity of the disrupted cells. We reasoned that the label seen on the cytoplasmic surface of the acrosome might result from contamination of the membrane by components released from the vesicle lumen. Therefore, we devised a method to detach the plasma membrane from glutaraldehyde-fixed cells where protein cross-linking maintained the acrosome intact during mechanical rupture of the cell membrane. Here, the cytoplasmic surface of the acrosomal membrane was not labeled by either Con A or WGA. Moreover, in these preparations, the fuzzy coat seen in spontaneously or hypotonic-disrupted spermatozoa was no longer visible, and a dense layer of CF was seen closely apposed to the cytoplasmic side of the acrosomal membrane profile. These anionic sites were not affected by neuraminidase treatment, suggesting that sialic acid residues do not contribute to the negative charge of the cytoplasmic surface of the acrosomal membrane, in agreement with recent data obtained in the guinea pig sperm (50). We conclude that conventional methods to rupture the sperm surface, e.g., hypotonic shock, also induce release of components from the acrosomal lumen. Because adsorption of these elements to membrane surfaces resembles a fuzzy coat, hypotonic shock may lead to data on the structure and cytochemistry of the acrosomal membrane that is based on the interpretation of artifactual results.



FIGURES 7 and 8 Labeling of the acrosomal membrane by cationic ferritin (CF). In spermatozoa with intact acrosomes (Fig. 7), CF is closely apposed to the acrosomal membrane profile (arrowheads) but on the plasma membrane a space of 8–11 nm separates the labeling from the bilayer (arrow). Upon neuraminidase digestion (Fig. 8), CF density is maintained on the acrosomal surface (arrowheads) but is greatly reduced on the plasma membrane. BSA, bovine serum albumin. × 76,000; × 78,000.

Lectin Binding Sites Partition with the Exoplasmic Half of the Acrosomal Membrane

Boar sperm cells submitted to the fracture-label method showed partition of lectin–gold complexes with the exoplasmic half of the membrane. We showed (see references 37 and 49 for detailed discussion) that lectin marking of exoplasmic membrane halves appears to be due to reorientation, upon thawing, of the freeze-fractured exoplasmic monolayer of the membrane into interrupted bilayered structures. This process of reorientation involves membrane lipids: in *Acanthamoeba castellani*, where the plasma membrane is devoid of Con A-binding glycoproteins (51), we recently documented that numerous Con A-binding glycolipids are exclusively found over the exoplasmic membrane half (52). In addition to glycolipids, glycoproteins may also contribute to lectin labeling on exoplasmic membrane halves, as it was demonstrated for band 3 in freeze-fractured erythrocytes (53). Therefore, lectin marking on the exoplasmic half of the membrane cannot discriminate between glycoproteins and glycolipids. The labeling is consistent with the expression of oligosaccharides on the exoplasmic surface of intact membranes (28–30).

Con A-Binding Sites Are Denser on the Equatorial Segment

On fracture-label preparations, Con A-binding sites were accumulated on the equatorial segment of the acrosomal membrane. As different amounts of cholesterol were recently reported in the two portions of the acrosomal envelope (24), the regionalization may be related to distinct lipidic environment of the equatorial vs. distal segments of the membrane. Taken together, the different densities of cholesterol and of Con A receptors give chemical individuality to the equatorial segment of the membrane, an area previously defined by structural criteria (our results and references 2 and 3) and by its differentiated role during fertilization (see reference 17 for review).

Comparative Lectin Cytochemistry of Acrosomal and Plasma Membranes

The scarce labeling by WGA seen on freeze-fracture halves of the acrosomal membrane contrasts with dense marking of the plasma membrane by the lectin, as observed in the same freeze-fractured cells (Fig. 3; see also Figs. 7 and 9 in reference 40). In our previous fracture-label study on the sperm plasma membrane, we found topographical correlation between the densities of WGA receptors and large membrane particles (40). It is of interest to note that poor labeling of the acrosomal membrane by WGA is also accompanied by the paucity of the membrane in particles. The acrosomal membrane is derived from the Golgi apparatus (9–12), the site of insertion of WGA-binding sugars into glycoconjugates (54, 55). Previous cytochemical studies established that post-Golgi membranes (lysosomes, secretory granules, and the plasma membrane) generally have a high density of receptors for WGA (56, 57). On account of its poor labeling by the lectin, the acrosomal

membrane appears to be an exception to this rule. The marked difference in density of WGA-binding sites between acrosomal and plasma membranes may reflect the operation of intracellular sorting of distinct amounts of terminally glycosylated components to the two membrane systems.

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