MEMBRANE SPECIALIZATIONS OF GUINEA-PIG SPERM

by

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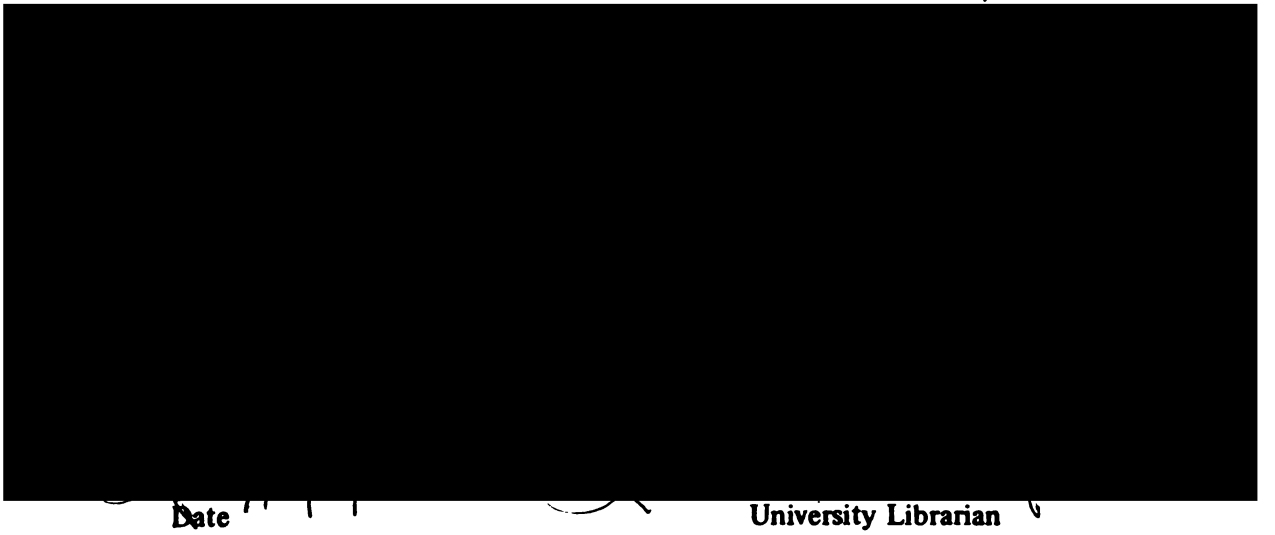

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MEMBRANE SPECIALIZATIONS OF GUINEA-PIG SPERM

ABSTRACT

To better understand the properties of sperm membrane differentiations, two specializations of guinea-pig sperm were examined. The zipper, a morphologically distinct, double row of transmembrane particles in the tail plasma membrane, appears to be both sperm specific and conserved through evolution. An *in situ* lectin binding study was performed to detect specific carbohydrates on the zipper. The zipper bound three of the nine gold- or ferritin-lectin conjugates examined (concanavalin A, *Ricinus commmunis* agglutinin I, wheatgerm agglutinin), suggesting the presence of complex N-linked oligosaccharides exposed on its surface. The lectin binding results were then used in conjunction with selective detergent solubilization to identify components in SDS gels which shared the same lectin-binding characteristics as zipper particles *in situ*.

The second membrane specialization studied was the cytoplasmic surface (P-surface) of the acrosomal membrane. The acrosome reaction, like many other examples of exocytosis, require an influx of extracellular Ca^{2+}. Studies with model liposomes suggest that Ca^{2+}, by binding to anionic sites, allows such close apposition between membranes that focal dehydration and fusion occurs. In this study, the cationic probes ruthenium red and cationic ferritin each demonstrated a dense concentration of available anionic sites on the P-surface of the acrosome. The ionic nature and relative strength of the interactions of cationic probes with the anionic sites was examined through binding-inhibition with competing salt solutions or incubation under acidic conditions. A variety of lytic

enzymes, including proteases of broad specificity, were unable to inhibit cationic ferritin binding to the P-surface of the acrosomal membrane. Extraction of fixed sperm with lipid solvents reduced the binding of the cationic probes, suggesting that a portion of the anionic sites are due to lipids. Upon the initiation of the acrosome reaction by the addition of Ca^{2+} to capacitated sperm, the hybrid plasma and acrosomal membrane vesicles no longer stained heavily with ruthenium red. This suggests that the influx of extracellular Ca^{2+} is responsible for both the elimination of the net negative charge on the P-surface of the acrosomal membrane (either directly or indirectly), and the fusion of the two membranes.

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INTRODUCTION

Sperm transmit the male's genetic information to the egg. To perform this function, mammalian sperm must swim through the female reproductive tract, penetrate the cummulus oophorus and zona pellucida surrounding the egg, and fuse with the ovum. Sperm have evolved a number of specializations which aid in performing these functions. Two of these membrane specializations are the subject of this dissertation.

The first specialization described resides in the sperm tail, that portion of the sperm which propels it forward. Within the plasma membrane of the tail is a morphologically distinct structure, the sperm "zipper." The zipper appears as an alternating double row of particles running longitudinally within that portion of the sperm tail referred to as the principal piece. While the function of the zipper remains unknown, zippers appear to perform a role unique and important to sperm function. They have not been reported on other cell types, yet they appear on sperm from mammals to true bugs and earthworms.

The first chapter describes the characteristics of the guinea-pig sperm zipper both morphologically (using a variety of ultrastructural techniques) and biochemically (using lectins, proteins which bind to specific carbohydrate moieties). This work should prove useful in the development of sperm zipper isolation procedures which may in turn lead to the determination of the function of this structure. Inhibitors of zipper function might prove effective as contraceptive agents.

The second membrane specialization studied resides in the sperm head. Within the head of sperm is a secretory granule called the acrosome which contains a variety of proteolytic and other hydrolytic enzymes.

These enzymes are released from the acrosome by the process of exocytosis (the sperm acrosome reaction). The release of lytic enzymes aids the sperm's penetration of the egg's coverings and also prepares the sperm membrane for fusion in the ovum.

Exocytosis, the fusing of a secretory granule with the plasma membrane, is a biological process common to most cell types. The acrosome reaction is a convenient model system in that the acrosome fuses with the plasma membrane over a large area, rather than in a limited domain as in some other cells. The fusion can be induced in vitro. Furthermore, the acrosome lies just below the plasma membrane, not buried within a cytoskeletal meshwork, making it accessible through relatively mild experimental manipulations.

Granular exocytosis in mammalian sperm, as in most other cells, requires an influx of extracellular Ca^{2+}. Work using liposomes to study membrane fusion indicates that less than mM concentrations of Ca^{2+} can induce the fusion of bilayers containing certain negatively charged lipids. Ca^{2+} induces fusion by binding to anionic sites on adjacent membranes allowing such close apposition of the two bilayers that focal dehydration and membrane fusion ensues. Thus, anionic sites on apposing membranes appear essential for Ca^{2+} induced fusion.

The second chapter describes the detection and initial characterization of a dense concentration of anionic sites on the surface of the acrosomal membrane which apposes the plasma membrane. The anionic sites were detected through the use of cationic probes which bind electrostatically to anionic sites. Following Ca^{2+} induced fusion of the plasma and acrosomal membranes, dense concentrations of anionic sites could no longer be detected on the hybrid membrane. It is hoped that the detection

and further characterization of the anionic sites on the acrosomal membrane will lead to a better understanding of the events which occur during the acrosome reaction and other fusigenic systems.

ABBREVIATIONS USED

a	acrosomal membrane
A	acrosome
Å	angstrom
A_2C	2-(2-methoxyethoxy)ethyl-8-(cis-2-n-octyl-cyclopropyl)-octanoate
BSA	bovine serum albumin
CF	cationic ferritin
ConA	concanavalin A
E-	ectoplasmic
ia	inner acrosomal membrane
K	1000 daltons
MCM	minimal culture medium
N	nucleus
p	plasma membrane
P-	protoplasmic
PBS	phosphate-buffered saline
PMSF	phenylmethylsulphonyl fluoride
RCA	*Ricinus communis* agglutinin
RR	ruthenium red
SDS	sodium dodecyl sulfate
WGA	wheatgerm agglutinin

CHAPTER I: ZIPPER PARTICLES: A LECTIN BINDING STUDY

ABSTRACT

Zipper particles are morphologically distinct transmembrane specializations of sperm tails. In freeze-fracture replicas of the guinea-pig sperm, they appear as interdigitating double rows of intramembranous particles running longitudinally within the plasma membrane of the principal piece. In thin section, zipper particles appear as an increase in electron density both above and below the bilayer. Zipper particles have been observed on a variety of both mammalian and non-mammalian species, suggesting that they have been conserved to serve an essential sperm function. As a first step towards biochemically characterizing guinea-pig zipper particles and towards developing a zipper isolation procedure, we performed an *in situ* lectin-binding study. Examination of nine gold- or ferritin-conjugated lectins revealed that three lectins, concanavalin A, *Ricinus communis* agglutinin I, and wheatgerm agglutinin, bound to zipper particles. These lectin binding results suggest the presence of N-linked oligosaccharides within zipper particles. The results of the lectin binding study were then used in conjunction with a detergent solubilization procedure to identify potential zipper components. Detergent solubilization involved two non-ionic detergents: digitonin, which solubilized most of the plasma membrane, but left approximately two-thirds of the zipper particles attached to the cytoskeleton, and Triton X-100, which solubilized the remaining zipper particles while leaving most other sperm structures intact. Within sodium dodecyl sulphate/polyacrylamide gels of the Triton X-100-soluble fraction, potential zipper components

with the same lectin-binding characteristics as *in situ* zipper particles were identified.

INTRODUCTION

Sperm are highly polarized cells specialized for the transmission of genetic information from one generation to the next. To perform this function, sperm must traverse the female reproductive tract, penetrate the investments of the egg, and fuse with the ovum. Within the sperm plasma membrane, multiple microdomains presumably aid in performing sperm functions. Although many of these microdomains are morphologically or antigenically unique (Friend & Fawcett, 1974; Friend, 1982; Myles, Primakoff & Bellvé, 1979; Tung, 1977), knowledge of their biochemical composition and function remains limited. One striking example of a morphologically defined microdomain is the "zipper."

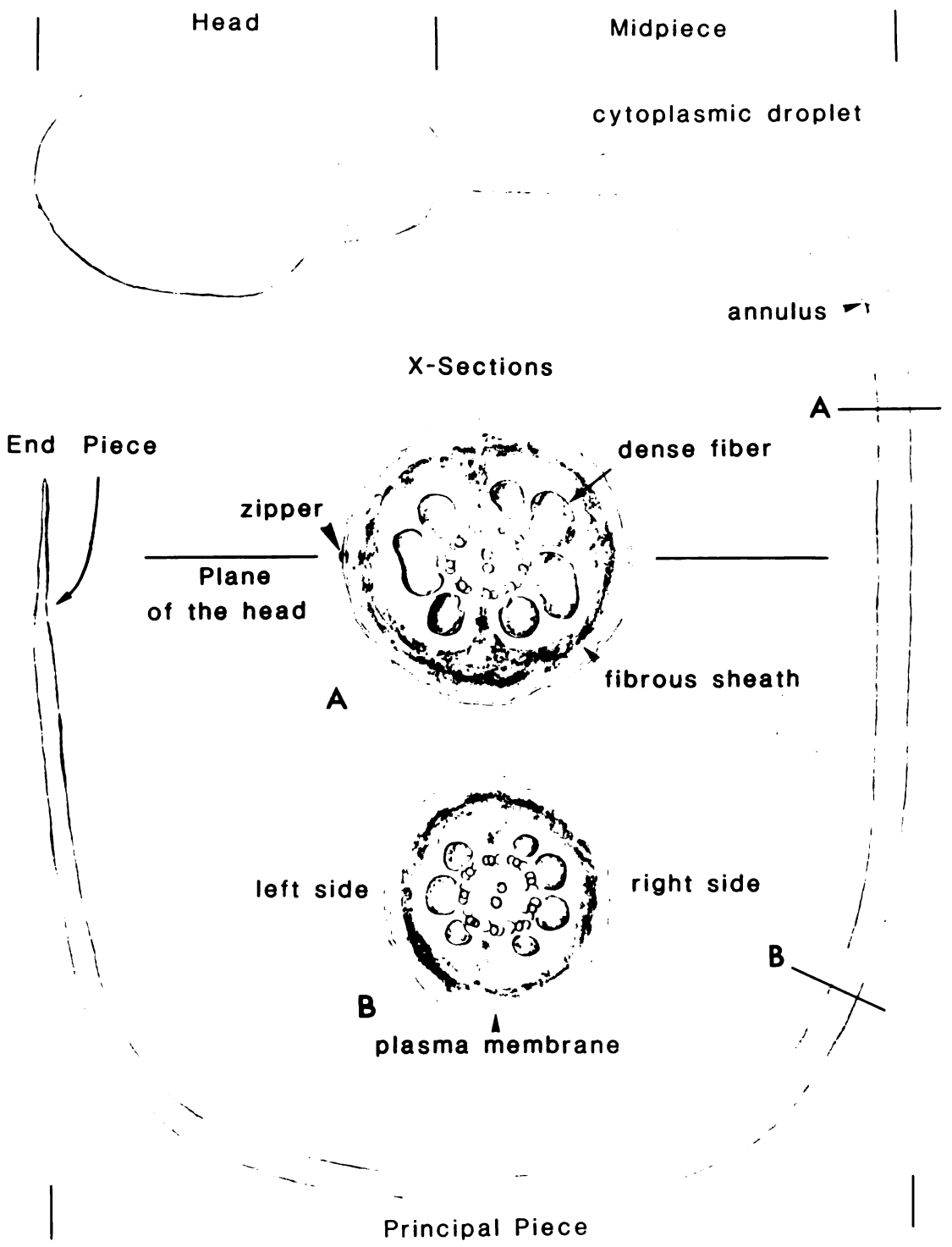
In freeze-fracture replicas of the guinea-pig sperm, the zipper appears as an alternating double row of P-face intramembranous particles running longitudinally within the tail plasma membrane (Friend & Fawcett, 1974; Koehler & Gaddum-Rosse, 1975). In thin sections across the principal piece of the tail, the zipper is seen opposite dense fiber number one as an increase in electron density bridging the cytoskeleton (fibrous sheath) below and protruding above the plane of the bilayer (see Figs. 1,5). In both surface replicas and freeze-fractured, deep-etched preparations, the zipper appears as an alternating double row of large particles protruding above the plane of the bilayer (Friend, Elias & Rudolph, 1979; Friend & Heuser, 1981). Thus, from a variety of ultrastructural techniques the guinea-pig sperm zipper appears to be a transmembrane specialization at a site of plasma membrane attachment to the cytoskeleton (Fawcett, 1975).

The zipper has been conserved through evolution, suggesting that it serves one or more essential sperm functions. Intramembranous zipper particles have been observed in the sperm of all mammalian species specifically examined (human, monkey, boar, opossum, rabbit, rat, mouse, and guinea-pig) (Friend & Fawcett, 1974; Stackpole & Devorkin, 1974; Koehler, 1977; Olson, et al. 1977; Suzuki, 1981; Koehler, 1983; Phillips, unpublished observations). However, zippers are not limited to mammalian sperm. Zippers and "zipper-lines" have been observed in sperm of the earthworm (Berstrom & Henley, 1973), the fruit fly (Friend, unpublished observation) and five true bugs (Hemiptera, heteroptera) (Dallai & Afzelius, 1982). Although most reports of mammalian intramembranous zipper particles have been made on either caudal epididymal or ejaculated sperm, zippers do not result from transit through the epididymis since they have also been observed in developing guinea-pig spermatids (Friend & Fawcett, 1974). Intramembranous zipper particles also appear unaltered in guinea-pig sperm exposed to a medium that allows capacitation (Friend & Rudolf, 1974; Koehler & Gaddam-Rosse, 1975).

We wanted both to characterize the zipper further and to work towards the development of a zipper isolation procedure. The development of a zipper isolation procedure could not rely on a plasma membrane preparation of mammalian sperm because the zipper tends to remain attached to the cytoskeleton (fibrous sheath) (Friend & Fawcett, 1974; Friend et al., 1979). An alternative approach was suggested by the work of Friend et al. (1979), who observed that the non-ionic detergent, digitonin, solubilizes most of the guinea-pig sperm plasma membrane, yet leaves the zipper attached to the underlying cytoskeleton. Subsequent treatment with Triton X-100 of digitonin-treated sperm solubilizes the

zipper from the cytoskeleton while leaving most other sperm structures intact. Preliminary attempts to identify zipper particles morphologically within the Triton-X-100-soluble fraction were unsuccessful. Thus, an alternative approach that did not rely exclusively on ultrastructural identification was needed. Because zipper morphology suggests that it is composed of protein and because most, if not all, plasma membrane proteins are glycosylated, we decided to undertake an *in situ* lectin-binding study, both to test the hypothesis that the zipper contains carbohydrate and to use this information to identify potential zipper components with the same lectin-binding characteristics within the Triton-X-100-soluble fraction.

Fig. 1. Sketch of guinea-pig sperm showing the relationship of the head to the components of the tail: the mid-piece, the principal piece, and the end piece. Cross-section A of the principal piece illustrates both the relationship of the zipper to the fibrous sheath and to the dense fibers while showing the orientation of the zipper to the plane of the head (Fawcett, 1968). Cross-section B illustrates the appearance of the principal piece when the zipper is no longer present.


Head
Midpiece
cytoplasmic droplet
annulus
X-Sections
A
End Piece
dense fiber
zipper
Plane
of the head
fibrous sheath
A
left side
right side
B
B
plasma membrane
Principal Piece

MATERIALS AND METHODS

Sperm collection

Sperm were obtained from large albino guinea-pigs purchased from two local suppliers (E-Z-H Co., Williams, CA; Simonson Co., Gilroy, CA). Anaesthetic was given by inhalation of ether or by intraperitoneal injection of 0.5 ml of Brevitol^{R} and Innovar-vet^{R}, followed by bleeding from the abdominal aorta and thoracotomy. Sperm were then removed from the vasa deferentes and caudae epididymides by gently squeezing cut sections into a drop of freshly gassed (95% air, 5% CO_2), calcium-free minimal culture medium (Ca^{2+}-free MCM) (Barros, 1974). The sperm were washed once by centrifugation and resuspended in fresh Ca^{2+}-free MCM. All procedures were performed at room temperature unless otherwise indicated.

Quick-Freezing

Sperm to be used in freeze-fracture experiments were quick-frozen using the methods outlined by Heuser (Heuser *et al.*, 1979; Friend and Heuser, 1981). Briefly, either fresh sperm, fixed sperm, or sperm fixed and exposed to lectin conjugates were concentrated to greater than 6 x 10^9 ml^{-1} by centrifugation at 1000 g for approximately 1 min. Fixed samples were placed in distilled water just before quick-freezing to reduce salt deposition during deep-etching. A drop of the concentrated sperm suspension was put directly on filter paper attached to an aluminum stage. (No lung was used as a cushion since a cell suspension rather than tissue was being frozen.) The stage was then quickly mounted on the freeze "Slammer" (no. E 7200, Polaron, Line Lexington, PA) and dropped

upon a copper block cooled by a stream of liquid helium at a temperature of 4° K. (The freeze Slammer, as obtained from Polaron, required replacement of its liquid helium transfer line (modelled after Heuser et al., 1979) to adjust to the liquid nitrogen Dewars, supplied by our local supplier.) The aluminum stage was rapidly removed and stored under liquid nitrogen until it was mounted on a pre-cooled freeze-fracture device (Balzers, Nashua NH). Once a vacuum of 2×10^{-6} Torr was attained, the sample was fractured at -110°C. Then the stage was warmed to -97°C for about 5 min to permit etching of the fracture surface, followed either by rotary or unidirectional shadowing with carbon/platinum and stabilization with carbon. Replicas were floated off into 10% sodium hypochlorite, left overnight, and then rinsed in distilled water and picked up on 300-mesh, Formvar-coated grids.

In situ lectin binding

Seven ferritin lectin conjugates were purchased in kit form from E-Y Laboratories (San Mateo, CA) (See Table 1 for the source of the plant lectin, abbreviation, electron-dense conjugate, and carbohydrate binding specificities.) In addition, to determine if Ricinus communis agglutinin I (RCA I) was binding to α- or β-D-galactose residues of the zipper, two α-D-galactose-specific lectin conjugates, ferritin-conjugated Bandeirea simplicifolia agglutinin I and colloidal gold-coated Maclura pomifera agglutinin, were obtained from E-Y Laboratories (gift of Albert Chu).

Experimental procedures for ferritin-lectin binding were generally modeled by those outlined by Nicolson et al. (1977). The lectin-binding experiments were generally performed with fixed sperm since reproducible results were obtained more consistently with fixed than with fresh sperm

(in agreement with the observations of Koehler, 1981). Sperm were fixed for 1-4 hr in dilute Karnovsky's (1965) fixative: 1% paraformaldehyde, 3% glutaraldehyde, in 0.1 M PO_4 buffer (pH 7.4). The sperm were then rinsed several times in phosphate-buffered saline (PBS) and exposed to 0.1 M glycine in PBS for 2 hr to react with any unreacted glutaraldehyde. Subsequently, the fixed sperm were washed in PBS or 0.1% bovine serum albumin (BSA, Pentax^R bovine albumin crystalized, no. 81-001-3, Miles Laboratories, Inc.) in PBS or, when concanavalin A (ConA)-ferritin was used, in 0.5 M NaCl, 0.1% NaN_3, 50 mM Tris-HCl, 0.5 mM Ca^{2+}, and 0.5 mM Mn^{2+} (buffer A; Burridge, 1978). Ferritin-lectin conjugates (supplied as 0.5 or 1 mg protein/ml) were generally added unaltered to 10-50($x10^6$) sperm/ml until a final concentration of 100 µg/ml was obtained. The mixture of sperm and ferritin-lectin conjugates was incubated for 15-30 min, during which time it was gently shaken periodically. The mixture was then diluted 1:5 with 0.1% BSA in PBS and washed twice by centrifugation at 1000 g for 1-2 min. The sperm were fixed again to cross-link bound ferritin-lectin conjugates to the sperm surface. (The presence of 0.1% BSA during the washes seemed to stabilize binding of ferritin-lectin conjugates to the sperm surface. This effect was inhibited by the presence of specific hapten sugars.) Fixed sperm were rinsed in buffer, post-fixed in buffered 1% OsO_4, and stained <u>en bloc</u> with uranyl acetate before being rapidly dehydrated in ethanol, cleared in propylene oxide, and embedded in Epon. Thin sections were cut and stained with uranyl acetate (30 min) and alkaline lead (5 min) to enhance contrast.

Control preparations were used for the lectins that demonstrated zipper-specific binding. For the controls, 0.1 M of the specific hapten

sugar was added during the incubation of sperm with ferritin-lectin conjugates: for ConA, α-methyl-D-mannoside was added; for RCA I, D-(+)-galactose; and for wheat-germ agglutinin (WGA), N-acetyl-D-glucosamine (sugars from Sigma, St. Louis, MO). Lectin-binding experiments were also performed with live sperm in either Ca^{2+}-free MCM or, when ConA-binding was being determined, in NaN_3-free buffer A. Due to the presence of NaN_3 in the ferritin-lectin conjugates, these conjugates were dialysed overnight, either in Ca^{2+}-free MCM or in NaN_3-free buffer A. Digitonin and Triton-X-100-extracted sperm (see below) were also fixed and exposed to ferritin-lectin conjugates.

Detergent solubilization

For selective detergent solubilization of guinea-pig sperm plasma membranes, we used the procedures outlined by Friend et al. (1979) and analyzed the detergent solubilization both morphologically and biochemically. Briefly, sperm were adjusted to a concentration of 150 ± 50 x 10^6/ml before being exposed sequentially to two non-ionic detergents, 0.1% digitonin (Hoffman-LaRoche, Basel, Switzerland) and 0.1% Triton X-100 (Beckman, Palo Alto, CA), in Ca^{2+}-free MCM to which 1.25 mM benzamidine (Sigma) was added to prevent excessive proteolysis caused by solubilized acrosin (Meizel & Mukeriji, 1976). The exposure to digitonin resulted in the loss of most of the plasma membrane, but left a majority of the zippers attached to the sperm cytoskeletons. Subsequent exposure of digitonin-treated sperm to Triton X-100 solubilized all of the zippers but left intact the sperm cytoskeleton, the axonemal complex, mitochondria, and the nuclear envelope and its contents (Friend, et al., 1979; Enders, unpublished observations). At each step during the

solubilization (see Fig. 2), samples of sperm and their detergent extracts were quickly frozen and stored at -20°C for biochemical analysis. Simultaneously, other samples of sperm were fixed and processed for electron microscopy. At the sperm concentration employed, two digitonin extractions before the Triton X-100 extraction were found to be most effective in solubilizing non-zipper plasma membrane components.

SDS/polyacrylamide gel electrophoresis

One-dimensional SDS/polyacrylamide gel electrophoresis was performed according to the procedures outlined by Laemmli (1970). Samples from the detergent solubilization were designated as indicated in Fig. 2: (1) epididymal plasma; (2) whole, washed sperm; (3a,b) digitonin supernatants; (4a,b) digitonin-treated sperm; (5) Triton supernatant; and (6) Triton-treated sperm. In some preparations, we added freshly prepared 100 mM phenylmethylsulphonyl fluoride (PMSF, a serine proteinase inhibitor) (Sigma) in ethanol to the sperm samples to obtain a final concentration of 1.0 mM PMSF. Epididymal plasma (1) and detergent supernatants (3a,b,5) were concentrated by precipitation in 5% trichloroacetic acid for 30 min on ice, and were then centrifuged in a microfuge and either resuspended in Laemmli sample buffer with the pH neutralized using NaOH (Pfeffer & Kelly, 1981), or washed three times in cold acetone before the addition of sample buffer (final concentrations: 1.0% β-mercaptoethanol, 0.125 M Tris (pH 6.8), 25% glycerol, 1% SDS). (The presence of non-ionic detergents, Triton X-100, and to a lesser extent, digitonin in the samples run on SDS/polyacrylamide gels generally resulted in abnormal "running" artifacts (streaking of lower molecular

weight components). This non-ionic detergent artifact could be eliminated by cold acetone washes of the trichloroacetic acid precipitate. Partial extraction of some of the low molecular weight components (<15,000) may have occurred during the acetone washes, though we did not quantify these data.) Samples were then sealed, boiled for 5 min, loaded onto 3% stacking gels on top of 7% to 18% linear polyacrylamide gradients and run for about 2-3/4 hr. Gels were fixed in 20% cold trichloroacetic acid for 1 hr, stained with Coomassie Blue, and then destained in 10% isopropanol, 10% methanol, 10% acetic acid, and 5% glycerol.

Protein concentration was determined by the protein assay of Lowry, et al.(1951) using BSA as a protein standard. All samples were hydrolyzed in 0.1 M NaOH and assayed in the presence of SDS.

Detection of specific carbohydrates in SDS/polyacrylamide gels

Specific carbohydrates were detected in SDS/polyacrylamide gels according to the methods outlined by Burridge (1978). Briefly, after the gels were destained, identical halves were split and equilibrated in buffer A or in buffer A plus 0.1 M hapten sugar. Lectins from either E-Y Laboratories or from Sigma, which bound to zippers, were iodinated with Na ^{125}I (Amersham, Arlington Heights, IL) using chloramine T (Hunter & Greenwood, 1962). Free iodine was separated from the labeled lectin by elution from a Sephadex G-25 column (Pharmacia, Uppsala, Sweden). Lectin (0.3-0.8 mg, 1-2 x 10^6 cpm.) was then applied to each gel half and left overnight. The radioactive lectin then was removed and the gels were rinsed in buffer A or in buffer A plus hapten sugar for 36 hr. Finally,

the gels were dried and exposed to X-ray film (X-Omat AR, Eastman Kodak, Rochester, NY) for 2-7 days.

Fig. 2. Diagram of the selective detergent solubilization of guinea-pig sperm. Samples at each step of the procedure are taken for both biochemical and ultrastructural analysis. Sperm were concentrated by centrifugation at 1000 g for 2 min.

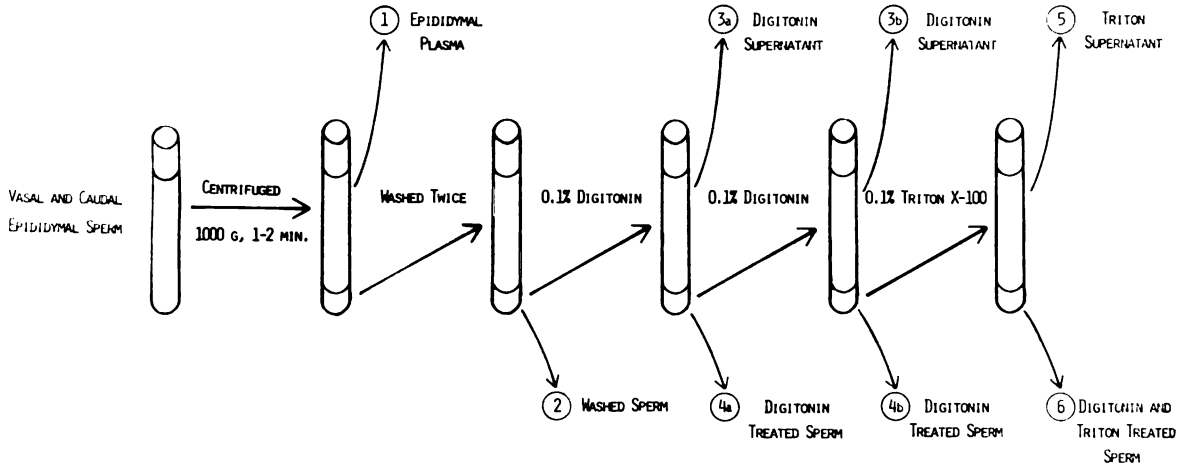

1 Epididymal Plasma
3a Digitonin Supernatant
3b Digitonin Supernatant
5 Triton Supernatant
Vasal and Caudal Epididymal Sperm
Centrifuged
1000 g, 1-2 min.
Washed Twice
0.1% Digitonin
0.1% Digitonin
0.1% Triton X-100
2 Washed Sperm
4a Digitonin Treated Sperm
4b Digitonin Treated Sperm
6 Digitonin and Triton Treated Sperm

Table 1. Lectin Conjugates Used

Source of lectin	Abbreviation-conjugate	Carbohydrate binding specificity
Arachis hypogaea (peanut)	PNA-ferritin	D-Gal-β-(1→3)-GalNAc
B. simplicifolia (I)	BSA-ferritin	α-D-Gal
Concanavalia ensiformis (jack bean)	ConA-ferritin	α-D-Glc, α-D-Man
Dolichos biflorus	DBA-ferritin	α-D-GalNAc, D-Gal
Glycine max (soybean)	SBA-ferritin	α-D-GalNAc, D-Gal
M. pomifera (osage orange)	MPA-gold	α-D-Gal
R. communis (I, 120 MW) (castor bean)	RCA I-ferritin	β-D-Gal, α-D-Gal
Triticum vulgaris (wheat-germ)	WGA-ferritin	β-(1→4)D-GlcNAc$)_{2 \text{ or } 3}$
Ulex europaeus (I) (gorse seed)	UEA-ferritin	α-L-fructose

RESULTS

Ultrastructural observations

In freeze-fractured, deep-etched replicas of guinea-pig sperm the zipper generally started within about 1 μm of the annulus (Fig. 3). However, in some sperm, it did not appear until 3 μm down from the annulus. This finding in freeze-fractured preparations explains why thin sections from this region often did not contain zippers in all sections. In occasional freeze-fractured, deep-etched preparations, extensive lengths of the principal piece plasma membrane were present. We have observed a nearly continuous zipper extending more than 21 μm and containing more than 1850 individual zipper particles. In thin sections, zippers were no longer visible at approximately the same cross-sectional level as that which the number 1 dense fiber becomes reduced in size and loses its bilobed character (see Fig. 1).

In freeze-fractured replicas, the guinea-pig zippers appeared as double rows of particles 85 Å in diameter protruding from the P-face (Fig. 4). In fortuitous fractures the dual row of intramembranous zipper particles is seen running opposite dense fiber number 1 (Fig. 4). This relationship of zipper to dense fibers was also apparent in thin sections across the principal piece (Fig. 5).

At higher magnification of E-surface replicas, the individual zipper particles appeared four- or five-sided and interdigitated with one another at their apices (Fig. 6). In some replicas the individual zipper particles appear to have substructure, suggesting the presence of subunits (Fig. 7). The center-to-center spacing of the particles along the length of the zipper was fairly constant at about 175 Å (Fig. 8).

If we assume that a uniform coat of carbon/platinum about 10 Å thick covers the zipper during the formation of replicas, then the guinea-pig zippers are about 260 Å wide. The base of each particle varied somewhat, from about 100 to 120 Å. The base to apex length is about 140 Å, slightly more than half the zipper width. These calculations, derived from freeze-fractured, deep-etched replicas, are in fairly good agreement with measurements of the zipper's appearance in thin section (Fig. 5). The electron density above the phospholipid bilayer was generally slightly wider than that of the axonemal complex microtubules, which are 250 Å in outer diameter. In addition, the electron density of the zipper extended above that of the rest of the phospholipid bilayer by 60 Å. In occasional freeze-fractured, deep-etched replicas, side images of zipper particles were included, and the particles appeared to protrude 70 Å from the phospholipid bilayer.

Particles from opposite sides of the zipper occasionally appeared fused at their apical ends, thus forming particles that appeared twice as large and oriented diagonally to the long axis of the zipper (Figs. 6,7,10). We interpret these images in part as artifacts of shadowing angle and/or the angle at which the particles are viewed, though some interaction between individual zipper particles must be responsible for the regular linear nature of the array they form. The appearance of a model of zipper particles viewed from different angles or lit from different angles helps to illustrate how we interpret the freeze-fracture images (Fig. 11,a-c).

Diagonally running patches of uncharacterized particles were often seen within the principal piece near the annulus (Figs. 3,10). These diagonally running patches apparently displace the normal location of the

zipper (Fig. 10) or delay the appearance of the zipper until further down the tail.

Occasionally, zippers were seen within freeze-fractured, deep-etched replicas of sperm that have patches of a screen-door-like mesh within the plasma membrane of their principal piece (Fig. 11). We interpret these patches as representing phospholipid in a gel state. Although gel-state lipids are seen on both sides of the zipper, the zipper appeared to prevent the spread of patches of gel-state lipids from one side of the zipper to the other.

Quick-frozen, deep-etched specimens of both human and murine sperm reveal zipper particles of similar dimension to those of guinea-pig sperm (Enders, unpublished observations). However, in human sperm the zippers are much shorter and more often randomly oriented along the principal piece. Murine sperm have multiple zippers running longitudinally within the plasma membrane of their principal piece.

Lectin binding

Of the nine lectin conjugates examined (listed in Table 1), three ferritin-lectin conjugates consistently bound to guinea-pig zipper particles: RCA I (Fig. 12), ConA (Fig. 16), and WGA (Fig. 18) (Table 2). Although all three of these ferritin-lectin conjugates bound to other sperm plasma-membrane components, they also reproducibly bound to zippers. None of the other lectins we examined consistently bound to zippers, although we observed occasional soybean agglutinin binding. The binding of all three ferritin-lectin conjugates noted above was inhibited by the addition of 0.1 M hapten sugar during incubation and washes, regardless of whether 0.1% BSA was present in the washes or not (ConA by

α-methyl-D-mannoside; RCA I by β-D-galactose; WGA by N-acetyl-D-glucosamine) (Figs. 13-15). Despite the development of small clumps in most of the ferritin-lectin conjugates, ferritin was reproducibly detected over zippers in thin sections cut across the tails of sperm in rouleaux (typical of cauda epididymal and vasal guinea-pig sperm). All three lectins bound to live sperm zippers (ConA, illustrated, Fig. 17), but binding to live sperm was less reproducible than to fixed sperm.

Morphological analysis of detergent solubilization

Digitonin treatment generally left the zipper morphologically intact (Fig. 19), and subsequent exposure to Triton X-100 removed it (Fig. 20). An indication of the extent and efficiency with which these detergents acted on the zipper was obtained by examining thin-sectioned material. Before treatment with detergents, zippers were visible on 81% ± 7%* (N = 500) of thin sections across the principal piece bearing dense fibers that still retain their bilobed character. Following two digitonin extractions, 54 ± 15%$^{+}$ (N = 250), of the zippers still adhered to the cytoskeleton. After exposure to Triton X-100, all zippers were removed (N = 250). Thus, about one-third of the zippers were removed by digitonin treatment, and the remaining two-thirds were completely solubilized by subsequent exposure to Triton X-100. Triton X-100 also solubilized other regions of the sperm which were often left attached to the cytoskeleton

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* and $^{+}$Mean ± standard deviation from 5 different experiments counting the first 100 (*) or 50 ($^{+}$) cross-sections of the principal piece in which the dense fibers retain their bilobed character.

after digitonin treatment. Some of these regions included portions of the inner acrosomal membrane, the annulus, and portions of the end-piece plasma membrane.

ConA-(data not shown), RCA I-(Fig. 21), and WGA-(Fig. 22) conjugated ferritin bound to the zippers that remained attached to the fibrous sheath after digitonin treatment. At times, ferritin-lectin conjugates were difficult to see owing to the formation of tubular plasma-membrane remnants typical of digitonin-treated sperm (Figs. 22,24, Elias, Georke, & Friend, 1978). The binding of ferritin-lectin conjugates was also seen in oblique sections of both untreated (Fig. 23) and digitonin-treated sperm (Fig. 24).

Occasionally, sperm with abnormal morphologies were observed. Several tails of these abnormal sperm often shared a single encircling plasma membrane. In those instances in which the cytoskeleton of the tail was attached to the plasma membrane, the site of adherence was where the zipper coursed opposite dense fiber number 1. These zippers were also labeled with ConA-ferritin (Fig. 25). This is further evidence that guinea-pig zippers are sites of plasma membrane attachment to the cytoskeleton.

Of all the lectins examined, RCA I bound most consistently to the zipper. This lectin recognizes β-D-galactose residues and, with a lower affinity, α-D-galactose residues (Goldstein & Hayes, 1978). To determine whether RCA I was binding α- or β-D-galactose residues, two α-D-galactose-binding lectins were examined. Neither Maclura pomifera agglutinin-gold (Fig. 9) in freeze-fractured, deep-etched samples, nor Bandeirae simplicifolia agglutinin-ferritin (data not shown) in thin

sections bound to zipper particles, suggesting that exposed α-D-galactose residues are not present.

Biochemical analysis of detergent solubilization

The two exposures of digitonin removed about 31% and 13% of the total sperm proteins, respectively (see Table 3). Subsequent exposure to Triton X-100 then removed about 11% of the total sperm proteins. Upon electrophoresis, most of the sperm proteins migrated with apparent molecular weights of between 130,000 and 10,000 (130-10K) (Fig. 26). The addition of 1.0 mM PMSF (1.25 mM benzamidine was already present) to the soluble fractions did not alter the Coomassie Blue staining pattern. Although there were quantitative differences between the first and second digitonin-soluble fractions (3a,b), the only qualitative differences were an occasional band in fraction 3b not apparent in fraction 3a. The digitonin-insoluble fractions (4a,b) appeared identical except for quantitative differences in some of the bands. Some components present in 4a and 4b were concentrated in the Triton-soluble fraction (5) and were lost from the Triton-insoluble fraction (6).

On the basis of our morphological analysis of the selective detergent solubilization described above, potential zipper components should fulfill the following criteria: (i) they should be concentrated within the Triton-soluble fraction (5); (ii) since Triton is extremely effective at removing zippers, they should be absent from the Triton-insoluble fraction (6); and (iii) they may be present within the digitonin-soluble (3a,b) and -insoluble (4a,b) fractions, but should be minor components. Five of the approximately 40 Coomassie Blue bands in the Triton-soluble fraction (5) matched these criteria. Their apparent

molecular weights were 110, 89, 34, 28, and 24(K) (Fig. 26). The 110 K M_r protein, however, was also present in all the early fractions. It seems unlikely that zipper particles, which form such a small part of the total sperm proteins, would be visible on gels of total sperm proteins.

Exposing the gels to the same radioactive lectins that bound to zipper particles allows the identification of components with the same carbohydrate residues as zipper particles. ^{125}I-ConA (Fig. 27) bound to four of the five potential zipper components: 110, 89, 34, and 24 K. ^{125}I-RCA I (Fig. 27) bound to the 110, 89, and 34 K proteins, but did not appear to bind to the 28 K or the 24 K proteins, though weak binding to the 24 K protein was apparent on other gels. ^{125}I-WGA (Fig. 27) bound to the 110. 89, 34, and 24 K proteins, but not to the 28 K components. Preliminary data (not shown) using galactose oxidase/sodium borohydrate surface labeling indicate that both the 89 and the 34 K proteins incorporate [^{3}H]$NaBH_4$, suggesting these polypeptides are exposed on the external surface of the sperm.

Fig. 3. E-surface rotary-shadowed replica of fixed sperm placed in water before quick-freezing. The zipper (large arrowhead) appears as a double row of alternating particles, starting less than 1 μm from the annulus (bracket). As yet, uncharacterized rows of diagonally running particles (small arrowheads) are often seen between the annulus and the start of the zipper. X 65,000.

Fig. 4. P-face unidirectional shadowed replica of fixed sperm that were quick-frozen. The zipper (arrowhead) appears as an alternating double row of particles running opposite dense fiber number 1 (labeled 1) (see Fig. 5 for identification of dense fibers). X 80,000.

Fig. 5. Thin section of tannic-acid-fixed guinea-pig sperm (4% tannic acid, 3% glutaraldehyde, in 0.05 M phosphate buffer). The zipper (arrowhead) appears as an increase in electron density both above and below the plane of the phospholipid bilayer opposite dense fiber number 1 (1). The numbers 1, 5, and 6 refer to the numerical identification of specific dense fibers. X 80,000.

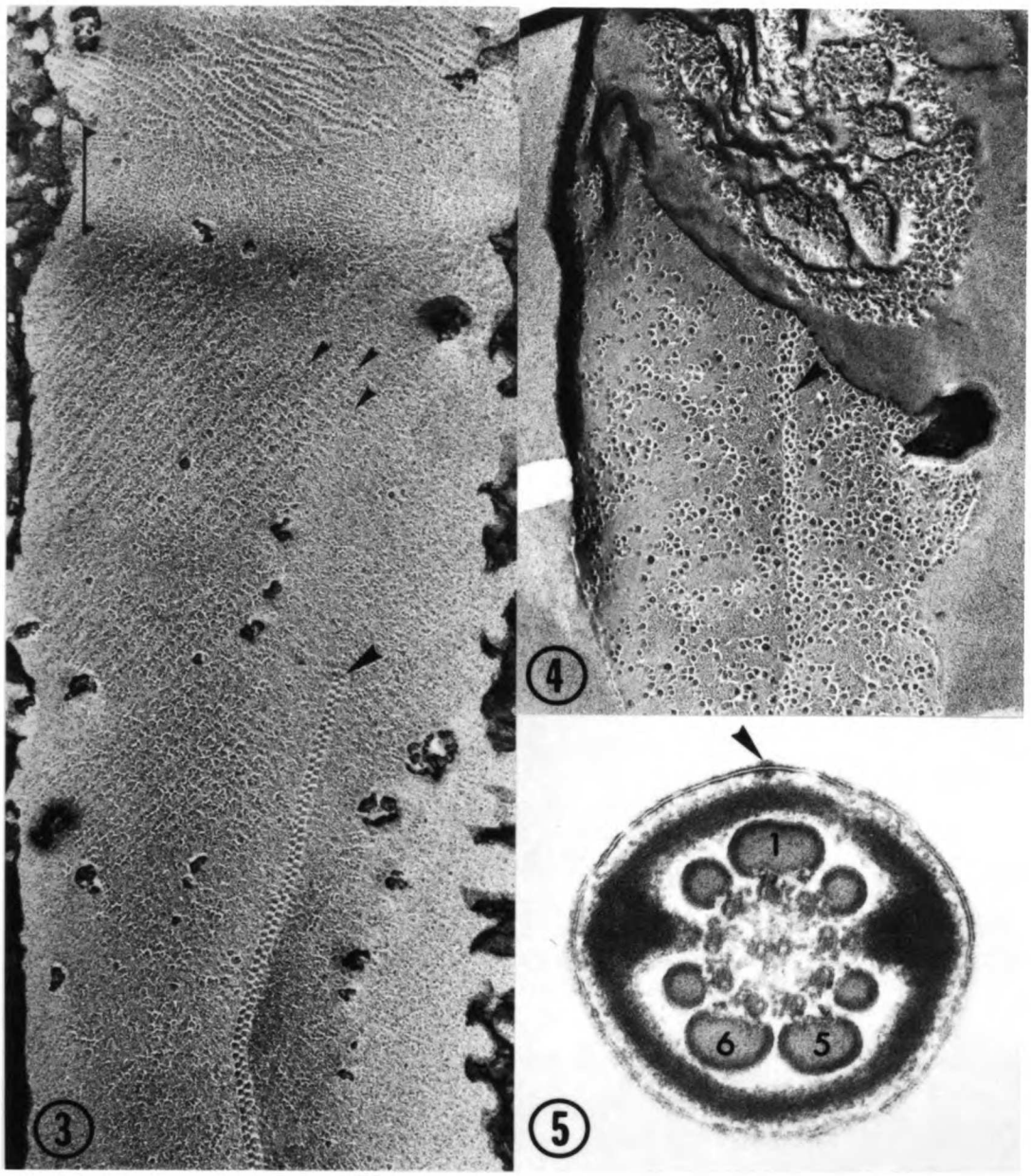
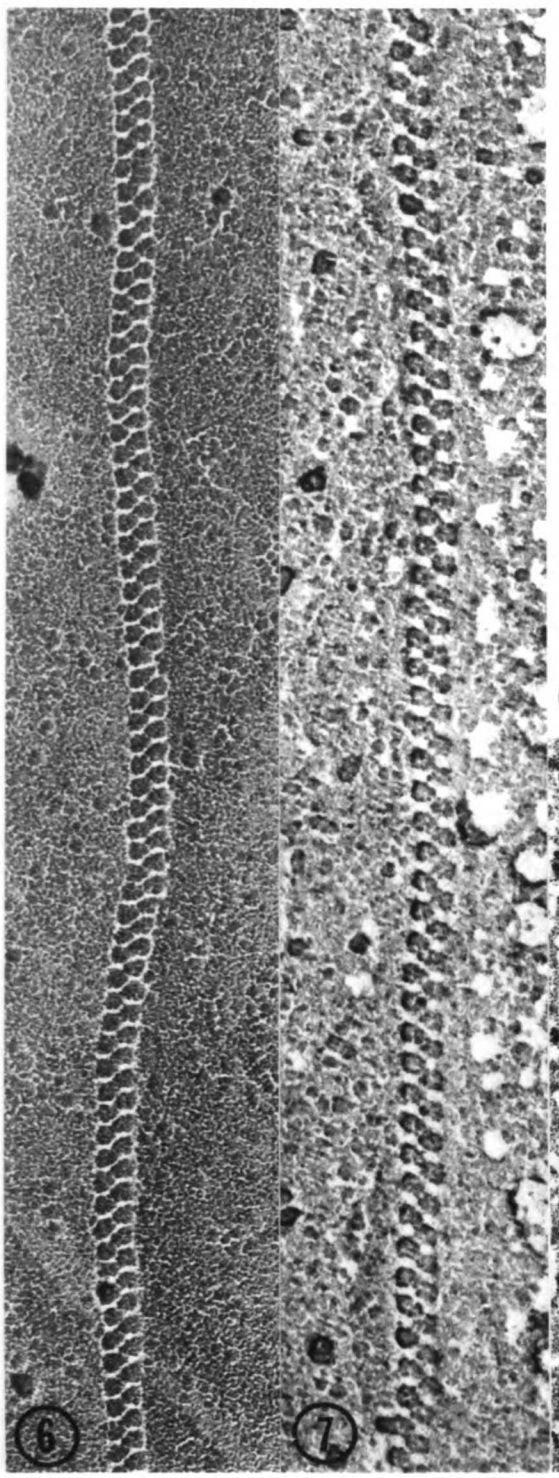

3
4
5
1
6
5

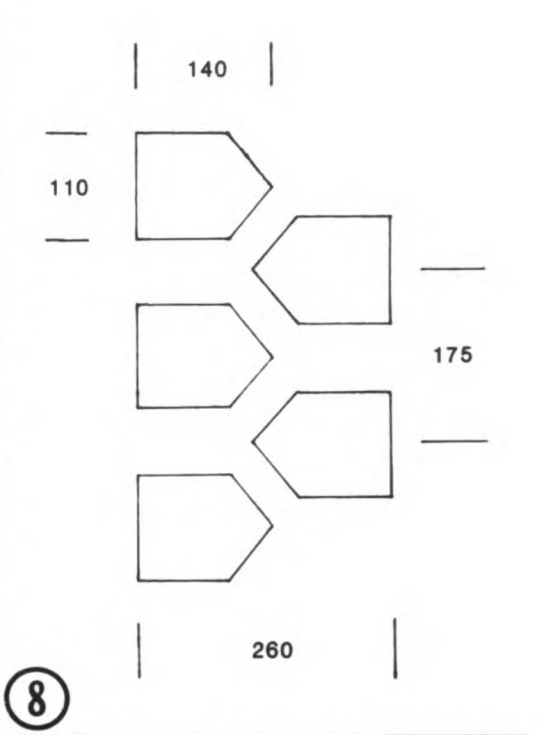
Fig. 6. E-surface rotary shadowed replica of fixed sperm placed in water before quick-freezing. The zipper appears as an alternating double row of five-sided particles, which interdigitate at their apices. X 170,000.

Fig. 7. E-surface rotary-shadowed replica of quick-frozen sperm. The images of the individual zipper particles suggest the presence of substructure, perhaps due to subunits. (This figure courtesy of Dr. Daniel Friend.) X 200,000.

Fig. 8. Schematic representation of the dimensions of the zipper and its particles. The center-to-center spacing of particles along the length of the zipper is about 175 Å. When the thickness of the carbon/platinum replica is taken into consideration the zipper is about 260 Å wide. The individual zipper particles have a base of about 110 ± 10 Å and a base-to-apex length of about 140 Å.

Fig. 9. E-surface unidirectional shadowed replica of fixed sperm that were exposed to M. pomifera-coated gold. Both specific gold particles with shadows (large arrowhead) and non-specific gold particles that do not have replica shadows (small arrowhead) are present. The gold particles do not appear to be selectively bound to the zipper particles. X 94,000.




140
110
175
260

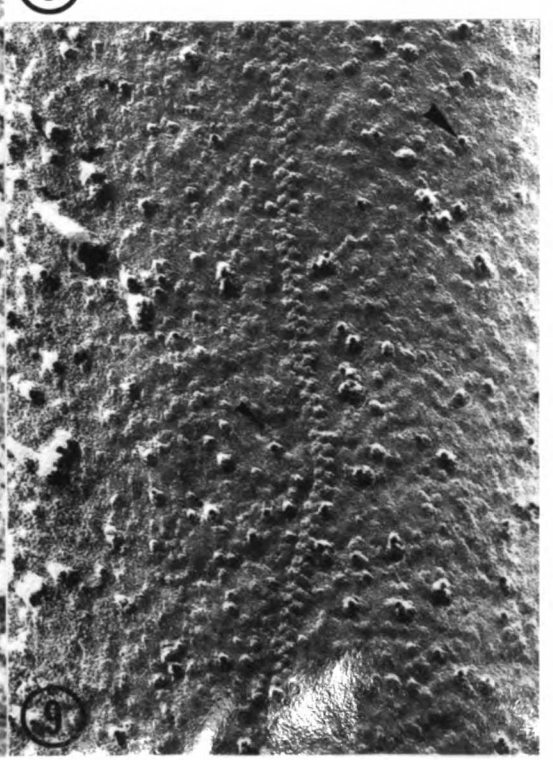


Fig. 10. E-surface rotary-shadowed replica of fixed sperm placed in water before quick-freezing. The normal course of the zipper appears altered by the presence of uncharacterized diagonally running particles (arrowheads) often found near the annulus. X 98,000.

Fig. 11. E-surface rotary-shadowed replica of fixed sperm placed in water before quick-freezing. The screen-door-like mesh may represent phospholipids in the gel state. Although the mesh is present on both sides of the zipper, the zipper appears to disrupt the spread of individual patches of gel state lipids from one side to the other. X 95,000.

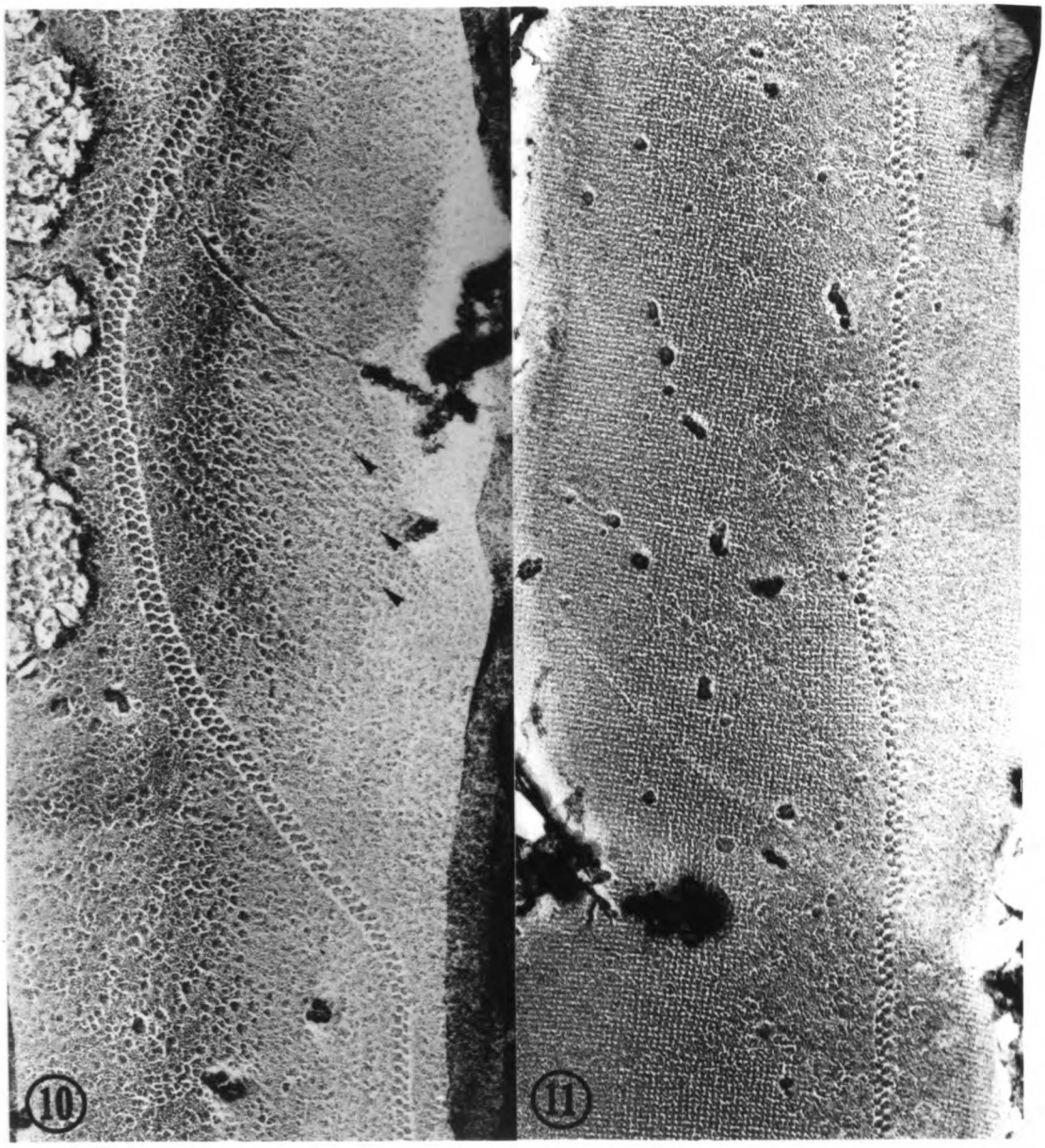
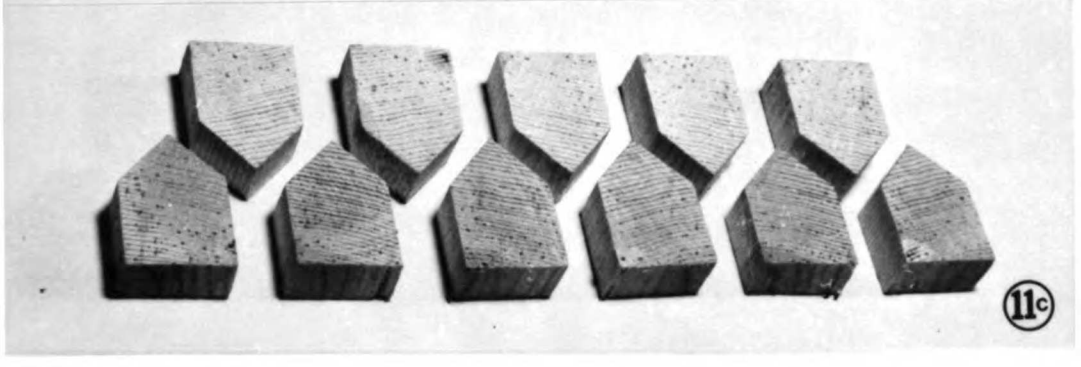

10
11

Fig. 11a-c. Photographs of a model representing zipper particles as they appear in freeze-fracture deep-etch replicas of the sperm surface. Fig. 11a. The model was photographed perpendicular to the plane of the zipper particles. Fig. 11b. The model was photographed perpendicular to the plane of the zipper particles, but lit from one direction, illustrating how unidirectional shadowing might result in 'fused' zipper particles which appear as large diagonally oriented particles. Fig. 11c. Illustrating how viewing the zipper model from a nonperpendicular angle may result in apparent 'fusing' of adjacent zipper particles.

11a

11b



11c

Fig. 12. Thin section of a rouleau of sperm that was fixed and exposed to RCA I-ferritin. Although small clumps of ferritin molecules are bound to other plasma membrane regions, ferritin is consistently bound to the zippers opposite dense fiber number 1 (arrowheads). X 64,000.

Figs. 13-15. Thin sections of fixed sperm that were exposed to ferritin-lectin conjugates in the presence of 0.1 M hapten sugar: Fig. 13, ConA and α-methyl-D-mannoside; Fig. 14, RCA I and D-(+)-galactose; Fig. 15, WGA and N-acetyl-D-glucosamine. No ferritin is bound to the zippers (arrowheads). X 80,000.

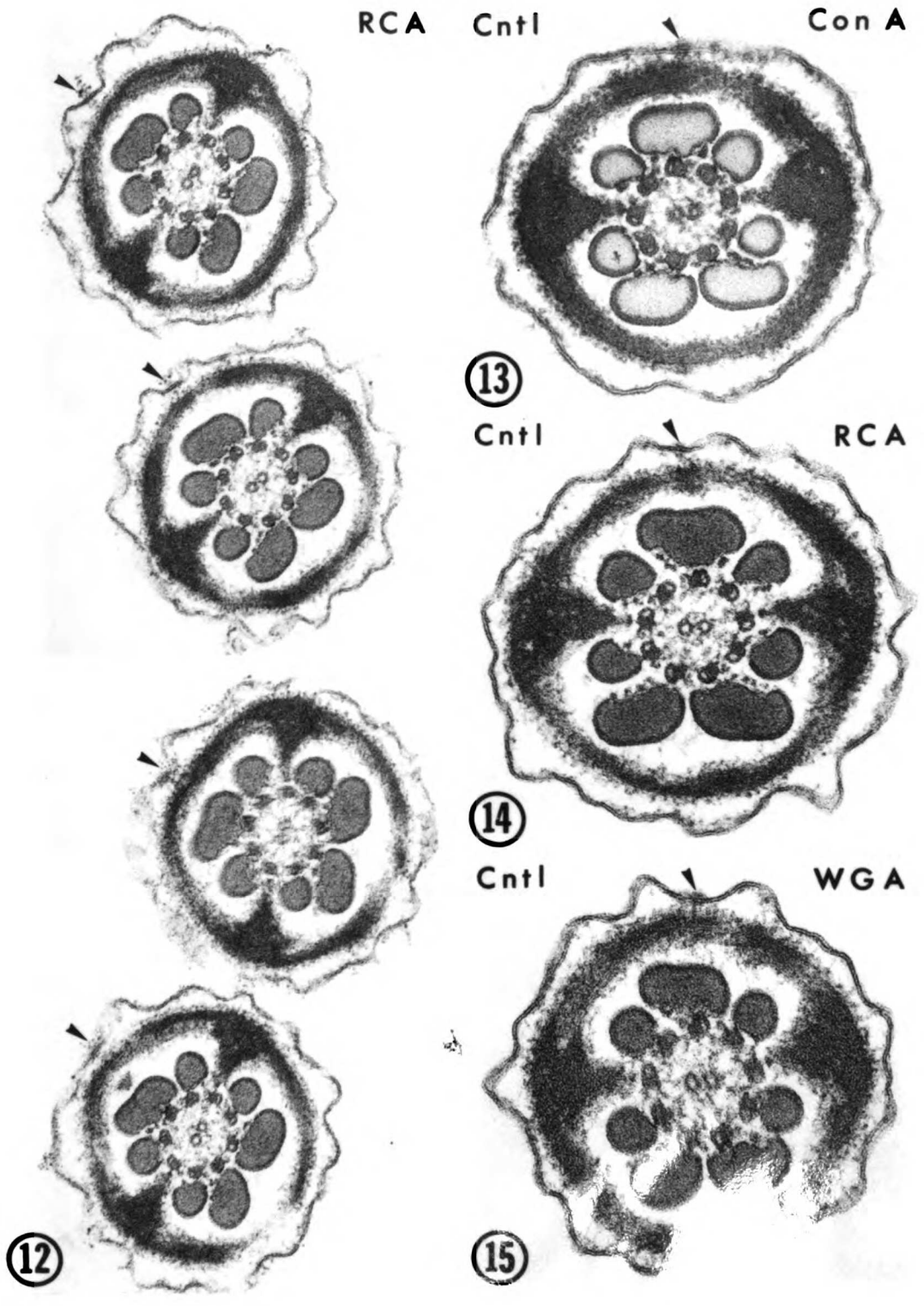
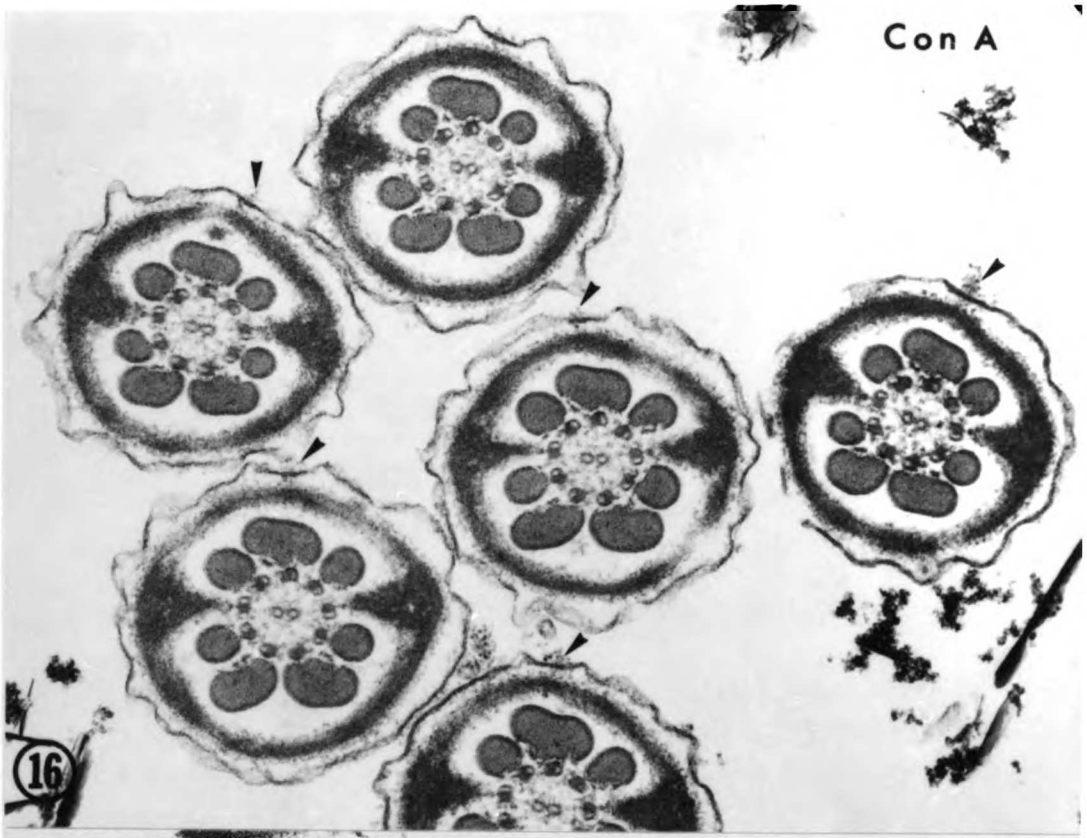

RCA
Cntl
Con A
13
Cntl
RCA
14
Cntl
WGA
15
12

Fig. 16. Thin section of a rouleau of fixed sperm exposed to ConA-ferritin. Although occasional ferritin molecules are bound to various regions of the plasma membrane, zippers are consistently labeled (arrowheads). X 55,000.

Fig. 17. Thin section of live sperm that were exposed to ConA-ferritin and then fixed. Ferritin molecules are bound to the zippers (arrowheads). X 68,000.


Con A
16

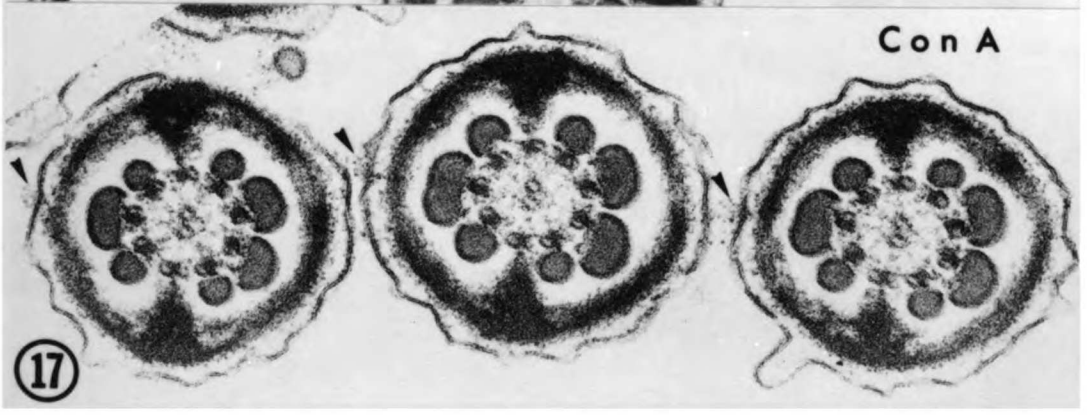

Con A
17

Fig. 18. Thin section of fixed sperm that were exposed to WGA-ferritin. Ferritin molecules are bound to the plasma membrane at the site of the zipper in three of the four sperm illustrated. The cross-section in the upper right-hand corner does not contain an identifiable zipper, suggesting that this section was taken near the annulus. X 55,000.

WGA

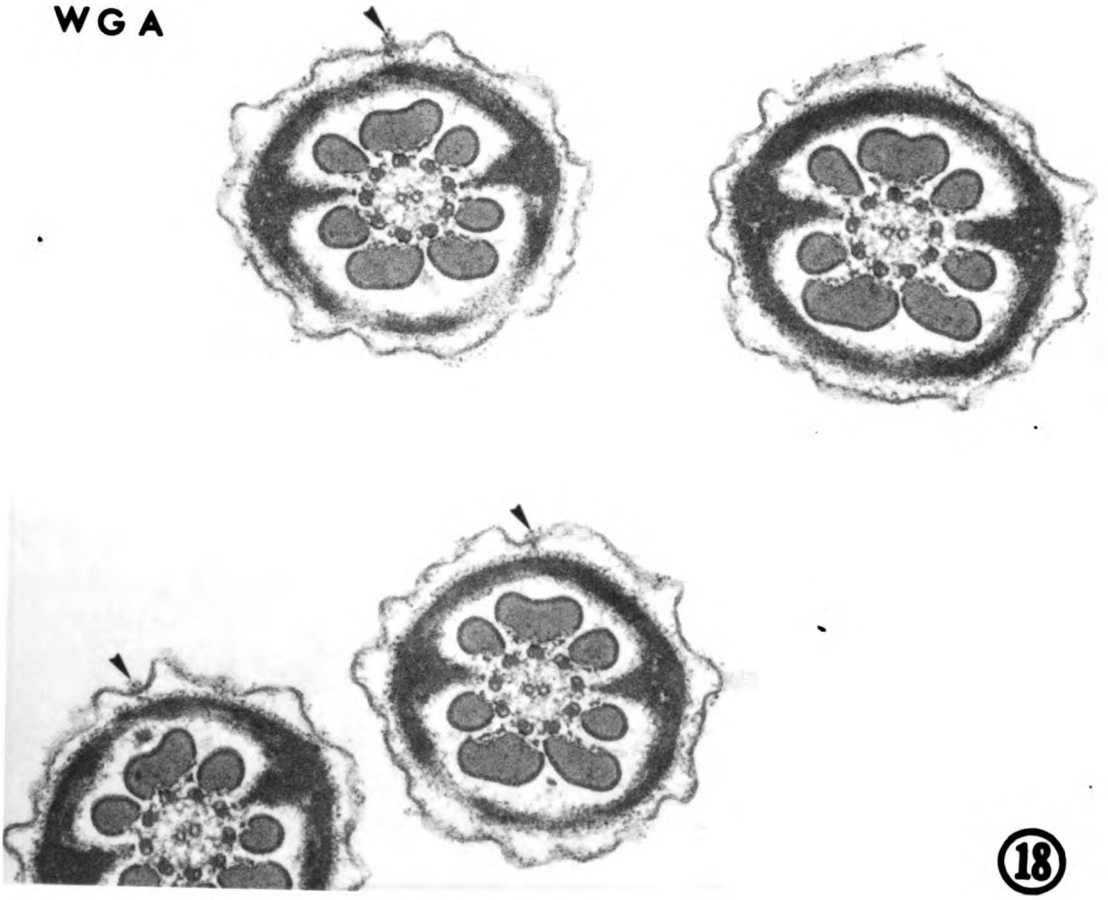


Fig. 19. Thin section of a digitonin-treated sperm. The zipper and a small portion of the bilayer are still attached to the fibrous sheath. Dig, digitonin treated. X 82,000.

Fig. 20. Thin section of a digitonin and Triton X-100-treated sperm. The zipper has been removed but the cytoskeletal components and the axonemal complex appears unaltered. Dig-Tri, digitonin and Triton X-100-treated sperm. X 80,000.

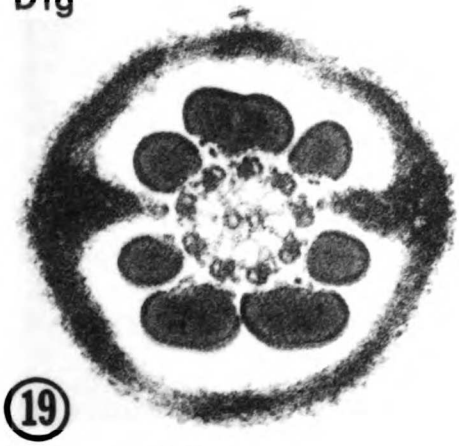
Fig. 21. Thin section of a digitonin-treated sperm that was fixed and exposed to RCA I-ferritin. Ferritin still binds to the zipper after digitonin treatment. X 85,000.

Fig. 22. Thin section of a digitonin-treated sperm that was fixed and exposed to WGA-ferritin. Ferritin is bound to the zipper, and also appears to be bound to a portion of the plasma membrane that has formed a tube-like structure adjacent to the zipper. X 76,000.

Fig. 23. An oblique thin section of a fixed sperm that was exposed to ConA-ferritin. Ferritin is bound to the zipper, which appears as a linear increase in electron density seen opposite dense fiber number 1. X 72,000.

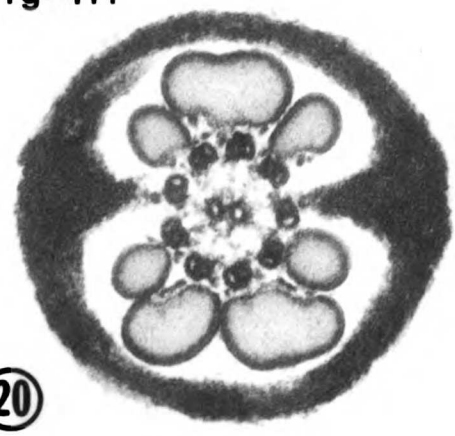
Fig. 24. An oblique thin section of a digitonin-treated sperm that was fixed and exposed to RCA I-ferritin. Ferritin is bound to the zipper, which is still attached to the fibrous sheath. Also present is a longitudinal section of the tube-like section of plasma membrane similar to that seen in Fig. 22, which is typical of digitonin-treated sperm. X 72,000.

Dig



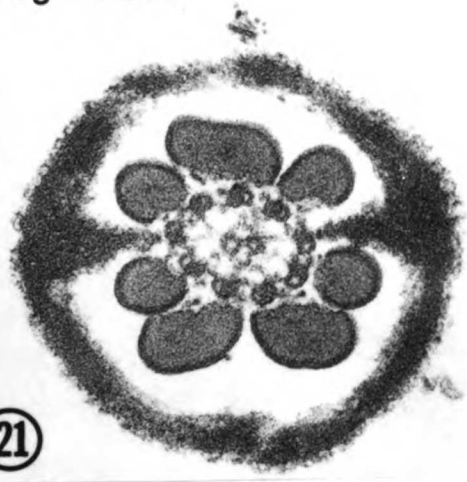
19

Dig-Tri



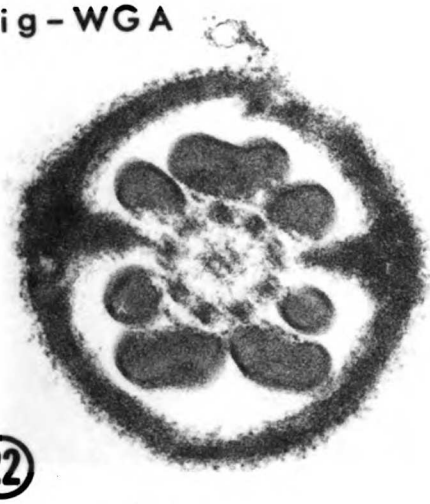
20

Dig-RCA



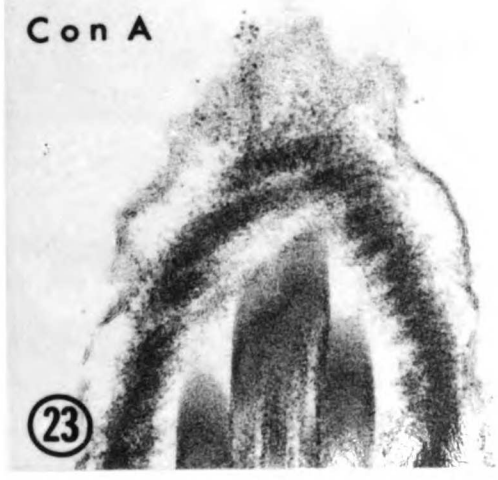
21

Dig-WGA



22

Con A



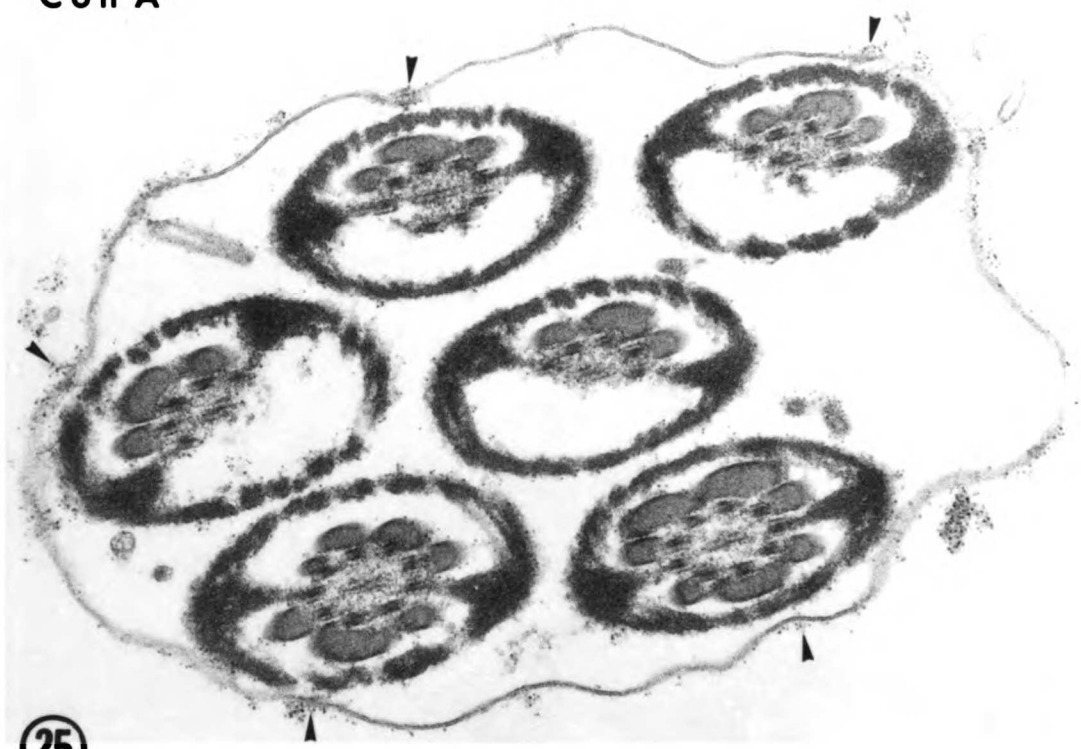
23

Dig-
RCA

24

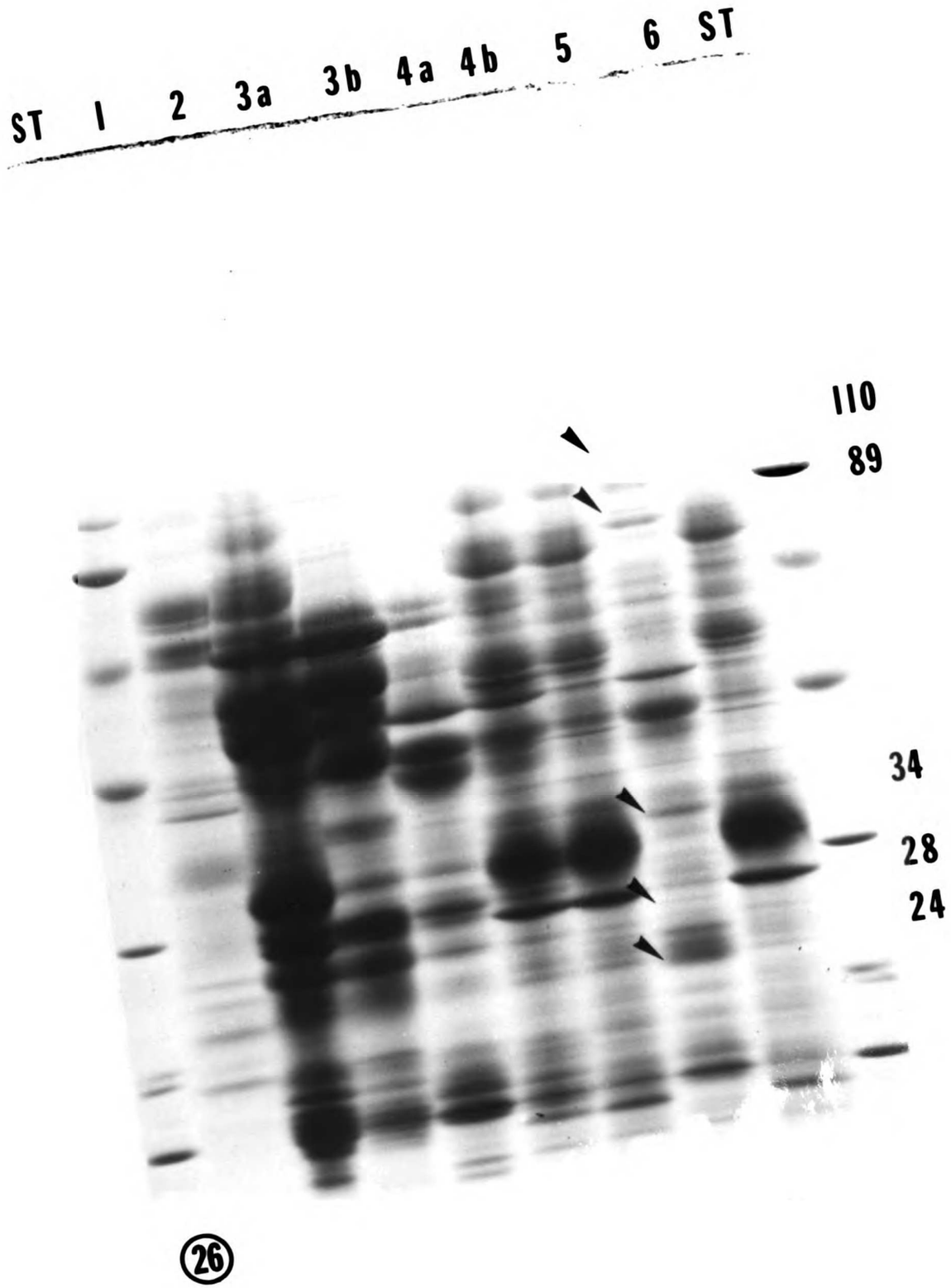
Fig. 25. Thin section of abnormal sperm that were fixed and exposed to ConA-ferritin. Most of the abnormal sperm cytoskeletons are attached to the single limiting plasma membrane at the site of their zipper, opposite dense fiber number 1. These zippers still bind ConA-ferritin (arrowheads). X 47,000.

Con A



25

Fig. 26. Coomassie Blue stain of a SDS/polyacrylamide gel of sperm that were subjected to selective detergent solubilization as outlined in Materials and Methods. The arrowheads indicate bands within the Triton-soluble fraction (5) which fulfill the criteria for potential zipper particles (see text). Each band has the molecular weight ($x10^{-3}$) indicated on the right. Lanes 1, epididymal plasma; 2, whole washed sperm; 3a,b, digitonin supernatants; 4a,b, digitonin-treated sperm; 5, Triton X-100 supernatant; 6, digitonin- and Triton X-100-treated sperm; ST, molecular weight standards ($x10^{-3}$): myosin, 200; β-galactosidase, 116; phosphorylase B, 92; bovine serum albumin, 66; ovalbumin, 45; carbonic anhydrase, 31; soybean trypsin inhibitor, 21.5; lysozyme, 14.5.



(26)

Fig. 27. Identification of polypeptide bands with the same lectin-binding characteristics as zipper particles. Lane I is a Coomassie Blue stain of the Triton X-100-soluble fraction (5; see Fig. 26). Lane II is the autoradiogram of lane I exposed to a [^{125}I]lectin: ConA, RCA, or WGA. Lane III is a control autoradiogram. On the same gel an identical Triton X-100-soluble fraction (5) was exposed to both [^{125}I]lectin and the specific hapten sugar. The 110 x 10^3 M_r polypeptide bound ConA and RCA; both the 89 and 34 (x10^3) M_r polypeptide bound all three lectins; the 28 x 10^3 M_r polypeptide bound none of the lectins (except perhaps for slight ConA binding); and the 24 x 10^3 M_r polypeptide bound ConA and WGA with slight RCA binding (unpublished results). The presence of high molecular weight components within the RCA autoradiogram (RCA lane II) is due to the presence of small quantities (not detectable by Coomassie Blue staining) of incompletely reduced proteins not generally present in other experiments.

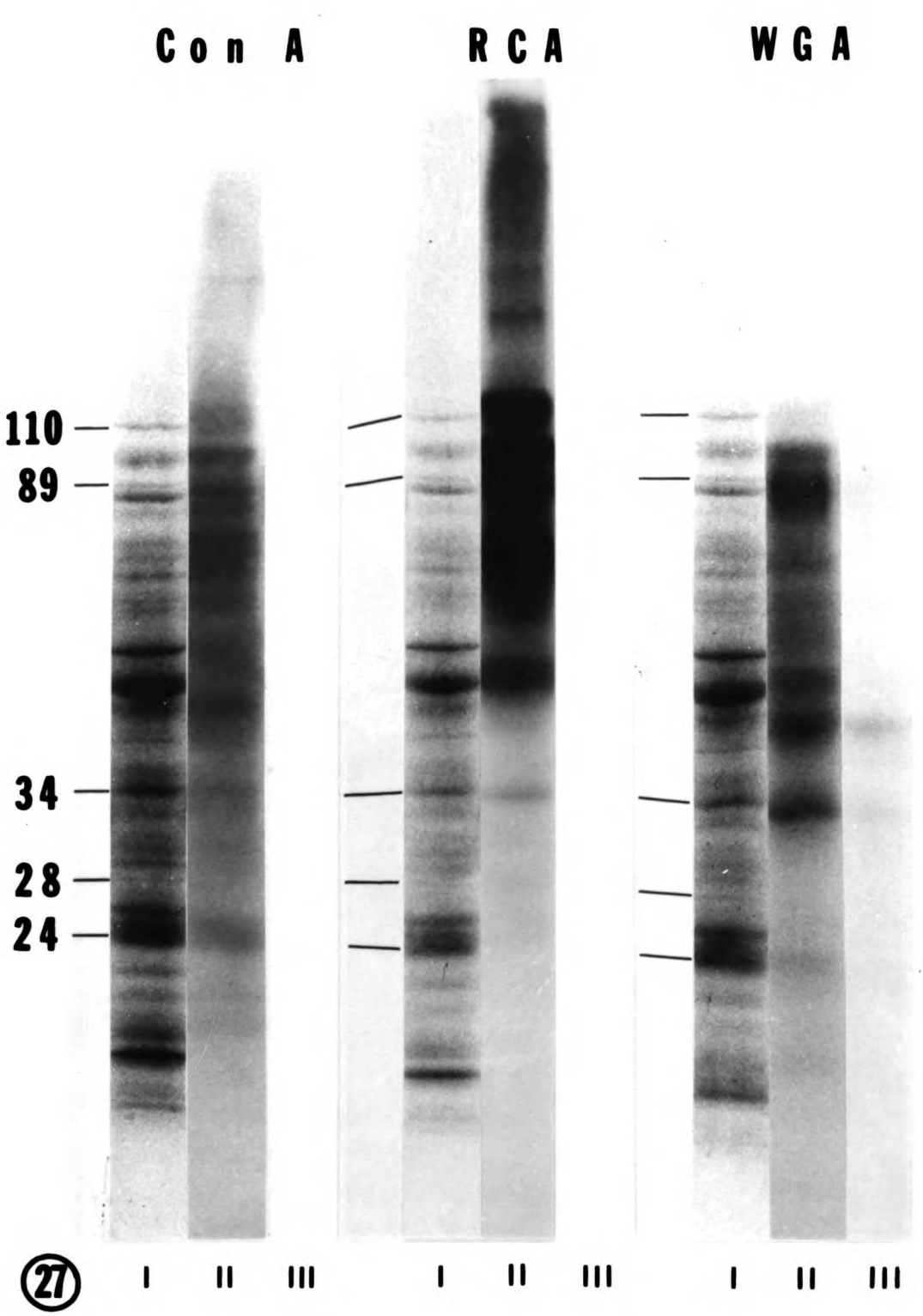

Con A
RCA
WGA
110
89
34
28
24
27
I
II
III
I
II
III
I
II
III

Table 2. Summary of *In Situ* Lectin Binding Results

Lectin	Carbohydrate Binding Specificity	Class of Oligosaccharides
ConA	α-D-Glc, α-D-Man	N-linked
RCA I	α- or β-D-Gal	N- or O-linked
WGA	$(\beta(1\rightarrow 4)$-D-GlcNAc$)_2$	N- or O-linked

None of the other lectin-conjugates examined bound consistently to the zipper.

Table 3. Effects of detergent solubilization of sperm proteins

Fractions	1 mg (%)	2 mg (%)	3 mg (%)	4 mg (%)	Average (%)
Digitonin-soluble (3a)	10.9 (30)	6.7 (32)	12.8 (33)	9.2 (27)	30.5 ± 2.7
Digitonin-soluble (3b)	5.3 (15)	2.8 (13)	5.6 (14)	3.6 (10)	13.0 ± 2.2
Triton-soluble (5)	3.6 (10)	2.7 (13)	4.3 (11)	3.9 (11)	11.3 ± 1.3
Total of non-soluble proteins (2, 4a, b, 6)	16.3 (45)	9.0 (42)	16.0 (41)	17.8 (51)	
Total sperm protein	36.1	21.2	38.7	34.5	

mg, mg protein/400-800 ($x10^6$) sperm was determined by the Lowry protein assay; %, % of total sperm proteins; average, mean ± standard deviation.

DISCUSSION

From the morphological appearance of the guinea-pig zippers, a number of conclusions about their probable biochemical composition can be made. First, the morphological data suggests that the zipper particles are composed of integral membrane protein(s). Zipper particles appear in P-face fractures as intramembranous particles, which are generally thought to result from the presence of integral membrane proteins (Pinto da Silva, Branton, & Douglas, 1971; Tillack, Scott, & Marchesi, 1972). Though recent work in acellular systems indicates that large intramembranous particles may be due to lipid (Miller, 1980; Hui & Stewart, 1981), zipper particles appear as punctate, 85 Å particles, suggesting proteinaceous rather than lipidic particles. The increase in electron density both above and below the membrane at the site of the zipper (analogous to desmosomes) also suggests the presence of protein or carbohydrate rather than lipid. Additionally, the finding that zipper-particle morphology is unaltered by exposure to either salt or EDTA (Enders, unpublished data) suggests the presence of integral rather than peripheral protein(s) (Singer & Nicolson, 1972).

Second, the large size of the individual zipper particles suggests the presence of more than one integral membrane protein. Guinea-pig zipper particles appear as approximately 110 Å x 140 Å x 60 Å particles protruding from the plane of the bilayer. If we estimate the molecular weight of the exposed portion of an individual zipper particle on the basis of a partial specific volume of 1.3 $Å^3$ per dalton (which has been used to calculate the volume of purple membrane protein and connexons of gap junctions; Unwin & Zamphghi, 1980), then a polypeptide

of greater than 500 K would be required. This calculation does not include that portion of the particle embedded within the bilayer or between the membrane and the cytoskeleton. No polypeptide of this magnitude has been seen concentrated within the Triton-X-100-soluble fraction in either reduced or unreduced gels (Enders, unpublished observations). Thus, it seems likely that the zipper particles are complexes of polypeptides.

The freeze-fracture results can be interpreted as supporting the notion that zipper particles are composed of several polypeptides. On the E-surface, zipper particles appeared to be approximately 110 Å x 140 Å, yet their corresponding intramembranous P-face particles were only 85 Å in diameter. The P-face particles also seemed less highly ordered and appeared slightly less frequently than E-surface particles. Recent work indicates that lectin binding sites may be pulled through the external half of the bilayer and onto the P-face during freeze-fracture (Pinto da Silva, Paskison, & Dwyer, 1981). A majority of the zipper particles seem to be pulled onto the P-face, with a small percentage of the particles being either fractured flush with the hydrophobic portion of the bilayer, or partitioned into the external half of the bilayer. Of those particles that are pulled onto the P-face, the reduction in particle size suggests that either a large plastic deformation of the particles occurs during fracturing (which is unlikely), or that only a portion of each zipper particle is pulled onto the P-face. It seems likely that only certain zipper polypeptide subunits partition onto the P-face.

Although zipper particles have a unique morphology, we found no unique lectin binding characteristics; the carbohydrate moieties detected

in our in situ lectin-binding study are common to many glycoproteins. However, the results of the lectin-binding study support our hypothesis that the zipper contains glycoproteins, since three lectins - ConA, WGA, and RCA I - bound to zippers. Concanavalin A, which has been extensively studied, generally binds to both terminal and non-terminal mannose and glucose residues (Goldstein & Hayes, 1978; Baenziger & Fiete, 1979). In mammalian species, mannose residues form the core of both simple and complex N-linked oligosaccharides (Kornfeld & Kornfeld, 1976), suggesting the presence of N-linked oligosaccharides in zipper particles. Results for WGA and RCA I support this possibility.

Wheat-germ agglutinin binds to two or three β-(1→4)-linked N-acetyl-D-glucosamine units, but may also interact with sialic acid (Greenaway & LeVine, 1973; Goldstein & Hayes, 1978). In our experiments, WGA-ferritin binding to the zipper was inhibited by the presence of the simple sugar, N-acetyl-D-glucosamine. However, it was difficult to eliminate completely the binding of ^{125}I-WGA to the SDS/polyacrylamide gels with this simple sugar. This inability to inhibit completely ^{125}I-WGA binding may be due to the greater affinity of WGA for complex sugars than for simple sugars. β-(1→4)-linked N-acetyl-D-glucosamine units are present in both O-linked and complex N-linked oligosaccharides.

R. communis agglutinin I binds preferentially to terminal galactose residues of the β rather than the α anomeric configuration. However, RCA I binding to a non-terminal galactose residue (an erythrocyte sialoglycoprotein) has also been reported (Adair & Kornfeld, 1974). Both terminal and penultimate galactose residues may be present in N- and O-linked oligosaccharides (Kornfeld & Kornfeld, 1976). To determine whether β- or α-D-galactose residues resided in zippers, we examined two α-D-galactose-

binding lectins, B. simplifolia, and M. pomifera. Neither lectin bound to zipper components, suggesting that the zippers contain only exposed β-D-galactose residues.

Even though our lectin-binding results do not exclude the possible presence of O-linked oligosaccharides, these findings could be consistent with the presence of only N-linked oligosaccharide chains of either the complex, or the complex and the high-mannose forms. It should be pointed out that the three different lectins may have been recognizing carbohydrate within the same olisaccharide (complex N-linked), within different oligosaccharides on the same molecule, or within different oligosaccharides on different molecules.

While zipper particles have only been reported on tails of sperm, linear rows of intramembrane particles are standard features of eucaryotic cilia and flagella. Single rows of intramembrane particles run longitudinally along the axis of a variety of cilia (Gilula & Satir, 1972; Sattler & Staehelin, 1974) and along the flagella of Chlamydomonas (Bergman, et al., 1975; Snell, 1976). Multiple rows of intramembrane particles have also been reported in the end piece plasma membrane of boar sperm (Suzuki & Nagano, 1980). These longitudinal arrays of intramembrane particles are thought to serve as sites of attachment of the axomenal complex to the plasma membrane, either directly or via intermediates.

A second example of linear rows of intramembrane particles common to both cilia and flagella is the ciliary necklace (Gilula and Satir, 1972). The ciliary necklace appears as wavy rows of intramembrane particles running circumferentially around the base of both cilia and flagella (Plattner et al., 1973; Sattler & Staehelin, 1974; Satir et al., 1976).

Recent work indicates the ciliary necklace of the quail oviduct binds ConA, WGA, and RCA I, as well as colloidal iron hydroxide (Sandoz et al., 1979). The ciliary necklace of the Discoglossus pictus (Anura, Amphibia) seminal vesicles also binds ConA and WGA (Chailley et al., 1981). The function of the ciliary necklace remains unknown, though it is thought to serve as an attachment site of the plasma membrane to underlying cytoskeletal components. Both the ciliary necklace and the longitudinal running intramembrane particles differ from the zipper particles of the guinea-pig sperm in that they do not have morphologically distinct portions extending above the phospholipid bilayer.

Although we have succeeded in identifying components within the Triton X-100-soluble fraction with the same lectin-binding properties as zipper particles, this approach towards isolating zipper particles is by no means ideal. The Triton-soluble fraction, which removes about 11% of the total sperm proteins, solubilizes not only the zipper, but also other regions of the sperm, some of which share some of the same lectin-binding characteristics (inner acrosomal membrane, annulus, and portions of the end-piece plasma membrane). Although isolation of zipper particles will probably rely on morphological confirmation at some point, the ability to assay for the same lectin-binding characteristics as in situ zipper particles should provide a useful assay criterion during the development of zipper isolation procedures. However, the exclusive use of lectin-affinity purification procedures would probably be of limited value because of the ubiquitous nature of the carbohydrates detected thus far in zipper particles. For example, serial use of lectin-affinity chromatography to isolate insulin receptor molecules, using three lectins with essentially the same binding characteristics as the zipper

particles, yielded only a minor increase in purity over single-lectin-affinity chromatography (Hedo, Harrison, & Roth, 1981).

Until the zipper has been isolated and subjected to experimental manipulation, proposals as to its function remain speculative. Whereas the zipper in guinea-pig sperm holds the plasma membrane to the fibrous sheath, this does not appear to be true to the same extent in mouse sperm (Enders, unpublished observations). If the zipper particles have some enzyme, channel, or receptor function it is possible that lectin binding to peripheral oligosaccharides might not affect function (Hedo et al., 1981). The binding of lectins to zipper particles of live guinea-pig sperm did not have any obvious adverse effect on motility that could be distinguished from the normal lectin agglutination described by Talbot & Franklin (1978).

The results described in here should assist in the further characterization of zipper particles, and in the development of isolation procedures for these structures. Zipper isolation should allow the production of zipper-specific antisera. The binding of zipper-specific antisera may permit the assessment of zipper function in vivo through the inhibition of its normal function. Perhaps then such questions as why zipper particles have been conserved through evolution and why they form lengthy linear arrays may be explored.

FUTURE DIRECTIONS

Because zipper particles do not possess unique lectin binding characteristics which allow their isolation using exclusively lectin affinity chromatography procedures, future efforts toward zipper particle isolation should rely on other zipper particle properties.

While zipper particle attachment to the sperm cytoskeleton has already been utilized during selective detergent solubilization, further use of this zipper property can be made by isolating guinea-pig sperm tails from other sperm structures. Preliminary work indicates that 10 to 20% of zipper particles remain attached to the tail cytoskeleton following Polytron disruption and separation by centrifugation on sucrose and Percoll gradients (unillustrated results). The isolation of a sperm tail fraction prior to detergent solubilization resulted in a 5 to 10 fold further purification beyond the techniques described in this chapter.

Zipper particle exposure on the external surface of sperm tails allows experimental manipulation not possible for cytoplasmic components. Preliminary work indicates that zipper particles are resistant to mild proteolysis, but subtilopeptidase A appears to be effective at reducing some of the normal zipper electron density which extends above the phospholipid bilayer (unillustrated observations). Assays for the loss of lectin binding sites following subtilopeptidase A treatment should be performed so that potential zipper components could be identified by both a decrease in apparent molecular weight and loss of lectin binding sites.

Ultimately, isolation of zipper particles will likely rely on morphological confirmation of solubilized zipper particles. Preliminary

attempts to identify zipper particles within the solubilized Triton X-100 fraction proved fruitless. Further efforts towards the positive identification of zipper particles within soluble fractions need to be made, for this would allow manipulation of soluble zipper particles. Future efforts should include the substitution of a nonionic detergent with a high critical micelle concentration such as β-D-ocytlglucoside, which would allow reconstitution of zipper particles into phospholipid bilayers (Helenius et al., 1977). Reconstitution of zipper particles would probably allow easier recognition in negative stain, surface replicas, or thin sections. The reconstitution of zipper particles might also allow the use of sperm tails as nucleation sites for reconstitution, thus acting as a "natural" affinity column. It would be interesting to determine if zipper particles reassociated opposite dense fiber number 1.

Once a soluble assay for zipper particles has been developed, then there are several other zipper particle properties which may be utilized in the development of zipper isolation procedures. First, since nonionic detergents generally do not disrupt protein-protein interactions (though apparently they disrupted zipper-cytoskeleton interactions), the large size of the zipper particles may be a useful property to exploit. Preliminary work using sucrose gradients in Triton X-100 did not result in the concentration of what was considered potential zipper components. The use of alternative nonionic detergents and other procedures such as gel filtration may prove more useful.

A second zipper particle property which should be utilized in the development of zipper isolation procedures is the amphipathic nature of the zipper particles. The retention of membrane proteins on alkyl-derivitized agarose has proved useful in the isolation of membrane

components including adenylate cyclase (Homcy et al, 1977). These procedures involve the mixing of detergent solubilized membrane components near the detergent's critical micelle concentration with alkyl-agarose. The alkyl-agarose matrix is then washed with salt to remove hydrophilic components. Components with hydrophobic domains will be retained by the matrix. Subsequently, the hydrophobic contents may be resolubilized from the matrix by adding detergent.

CHAPTER 2: ANIONIC SITES OF THE ACROSOMAL MEMBRANE: DETECTION USING TWO CATIONIC PROBES

ABSTRACT

The sperm acrosome reaction is an example of exocytosis, accomplished through the fusion of the acrosomal and plasma membranes. As in other examples of exocytosis, the acrosome reaction is initiated by an influx of Ca^{2+}. Model systems (liposomes) suggest that Ca^{2+} binding to anionic sites cause apposition, then dehydration, and fusion of the adjacent layers.

In this study we used ruthenium red (RR) and cationic ferritin (CF) to detect anionic sites on the cytoplasmic surface of the guinea-pig sperm acrosomal membrane. The addition of RR to both fixative and OsO_4 resulted in heavy electron-dense deposits on the external surface of the plasma membrane, and on the cytoplasmic surface of the outer acrosomal membrane, with less extensive labeling of the cytoplasmic surface of the plasma membrane. The addition of 0.4-0.6 M NaCl to the RR containing solutions eliminated most of the RR staining of the plasma membrane while an electron dense deposit on the cytoplasmic surface of the acrosomal membrane remained. The added NaCl also increased the separation of the plasma membrane from the acrosomal membrane. Extraction of fixed sperm with chloroform:methanol:water (1:2:0.8) prior to RR staining dramatically reduced the RR deposition on both the plasma membrane and acrosomal membranes.

To allow CF access to the acrosomal membrane, unfixed sperm were mechanically disrupted under hypotonic conditions. Upon exposure to CF, dense, uniform labeling of the cytoplasmic surface of the acrosomal

membrane resulted. The addition of NaCl during CF/sperm incubation inhibited much of the CF binding to the plasma membrane, but was less effective at inhibiting CF binding to the cytoplasmic surface of the acrosomal membrane. Disrupted sperm were exposed to a variety of lytic enzymes to gain insight into the biochemical nature of the anionic sites. A variety of proteolytic enzymes inhibited much of the CF binding to the plasma membrane, but had no effect on CF labeling of the cytoplasmic surface of the acrosomal membrane. This work thus suggests that the dense concentration of anionic sites on the cytoplasmic surface of the acrosomal membrane may be due in part to anionic lipids.

Upon initiation of the acrosome reaction by the addition of Ca^{2+} to capacitated sperm, the hybrid vesicles formed from the fusion of the plasma and acrosomal membranes no longer stained heavily with RR, suggesting that the influx of extracellular Ca^{2+} neutralized (either directly or indirectly) the dense concentration of anionic sites in the cytoplasmic surface of the acrosomal membrane, allowing membrane fusion.

INTRODUCTION

Membrane asymmetry is a universal biological phenomenon which includes all components of the bilayer: proteins, lipids, and carbohydrates (Hirano et al., 1972; Rothman & Lenard, 1977; Lodish & Rothman, 1979; Op den Kamp, 1979). Each membrane surface is specialized to serve different cellular functions. One such cellular function is exocytosis, the fusion of membrane-bound secretory granules with the plasma membrane. The sperm acrosome reaction is a dramatic example of exocytosis. The sperm acrosome is a Golgi derived organelle which contains a variety of hydrolytic enzymes (Stambaugh, 1978), some of which (e.g., proacrosin) are stored as inactive precursors (Meizel & Huang-Yang, 1973; Meizel & Mukerji, 1975). Thus, in many ways, the acrosome is analogous to a large primary lysosome or secretory granule (Friend, 1977). Following capacitation, the acrosomal membrane fuses with the overlying plasma membrane, releasing the acrosomal contents which aid the sperm's penetration of the egg's investments (See Fig. i).

Membrane fusion of the acrosome reaction involves the apposition and eventual fusion of the plasma with the acrosomal membrane (Barros et al., 1967; Russell et al., 1979). The initial apposition occurs at the cytoplasmic surfaces (P-surface, for protoplasmic (Branton et al., 1975)) of the two membranes (See Fig. i). Fusion of the two membranes requires extracellular Ca^{2+} (Barros, 1974; Yanagimachi & Usui, 1974; Singh et al., 1980) and evidence from several sources suggests that it is an influx of this extracellular Ca^{2+} which initiates the fusion of the acrosomal and plasma membranes (see Yanagimachi, 1981). Ca^{2+} is also required for membrane fusion in a wide variety of other biological systems (see Lucy, 1978).

Models for the mechanism of Ca^{2+} induced membrane fusion have been developed in acellular systems using phospholipid vesicles. Ca^{2+} has been shown to induce fusion of liposomes by bridging acidic phospholipids on adjacent vesicles thereby allowing such close apposition of the bilayers that dehydration of the intervening aqueous space occurs, leading to destabilization and fusion (Papahadjopoulus et al., 1974; 1976). Thus, work from model systems indicates that anionic sites which bind Ca^{2+} may also be necessary for fusion of cellular membranes.

Much of the previous work on capacitation and the acrosome reaction has studied changes that occur within the overlying plasma membrane (Koehler, 1981; Friend, 1982a; O'Rand, 1982). Comparatively little is known about the properties of the acrosomal membrane, though there is evidence that the fixed acrosomal membrane of sperm may bind Ca^{2+} (Roomans, 1975; Friend, 1977).

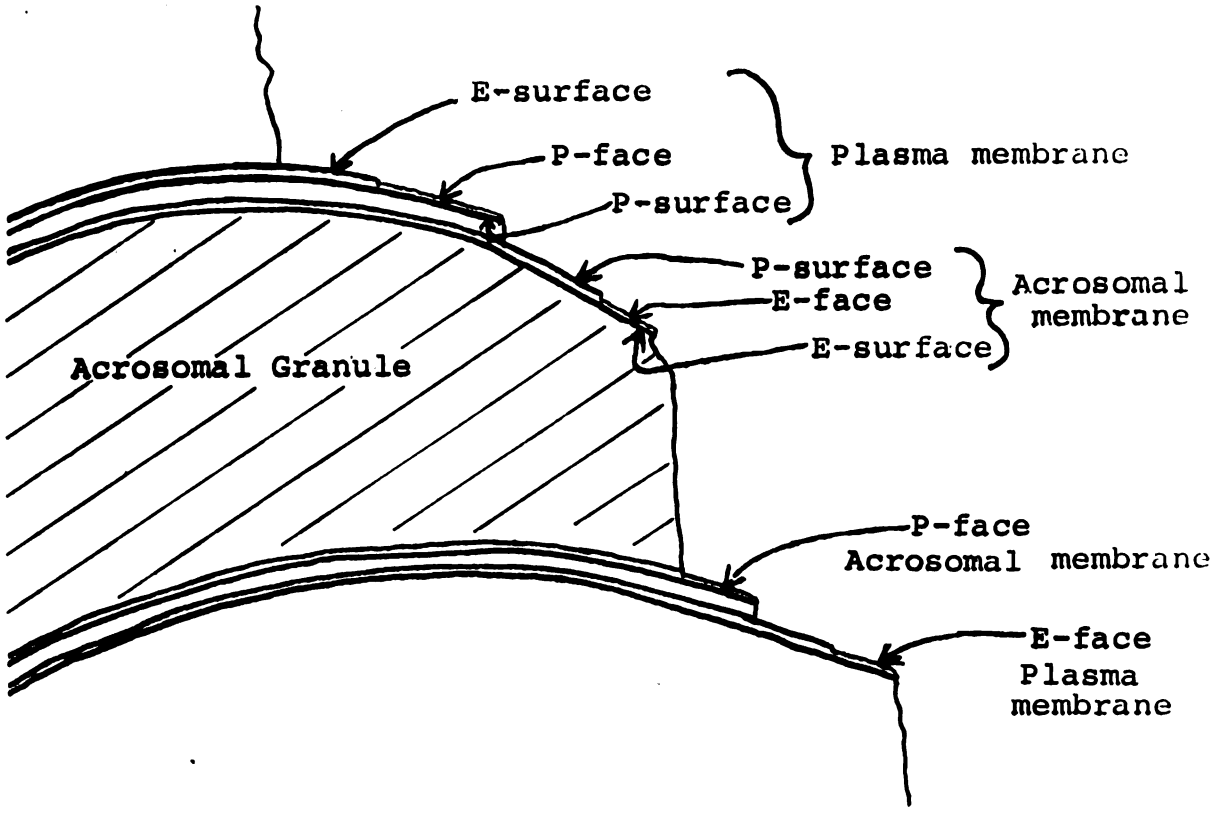
In this paper we describe the detection of a dense concentration of anionic sites on the cytoplasmic surface of the acrosomal membrane using two different cationic probes, ruthenium red (RR), and cationic ferritin (CF). The ionic nature of the interaction of the cationic probes with the acrosomal membrane was demonstrated by altering the conditions under which the probes were used. These alterations included the addition of salts to act as competing electrolytes, and exposure to increasingly acidic conditions to eliminate the charge of the anionic sites. In the course of these experiments, insight into how the fixed acrosomal and plasma membranes may be held apposed to each other was gained. We also exposed sperm to a variety of experimental treatments designed to discern the biochemical character of the anionic sites. The experimental manipulations included exposure of fixed sperm to lipid solvents, and

digestion of unfixed sperm with a variety of lytic enzymes. The cationic probes were used to monitor the effects of the treatments on the anionic sites. In addition we examined the changes that occurred after the acrosomal and plasma membranes fuse with each other in the acrosome reaction.

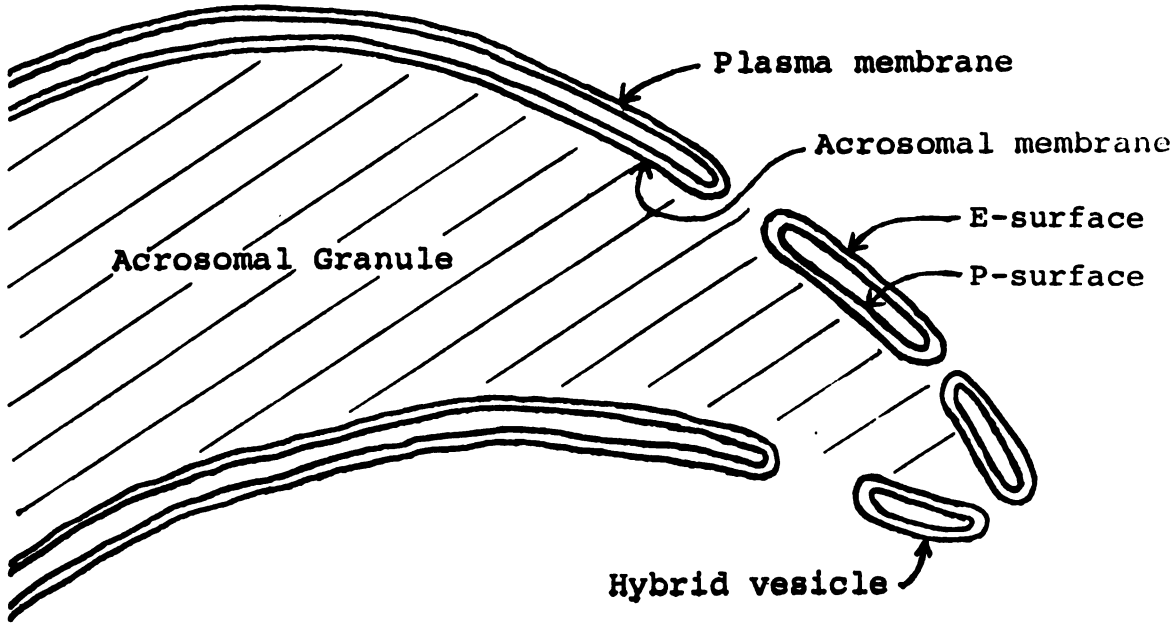
Characterizing the cytoplasmic (P-) surface of the outer acrosomal membrane contributes to our understanding of both membrane fusion events and cytoplasmic membrane properties. Knowledge of the unique properties of cytoplasmic membranes should help unravel the mechanisms governing maintenance of membrane asymmetry and intracellular membrane trafficking.

Fig. i. Sketch of the tip of the head of a guinea-pig sperm showing the relationship of the plasma membrane to the acrosomal membrane. The top sketch illustrates the E-(ectoplasmic) and P-(protoplasmic) surfaces on the adjacent membranes, while the bottom sketch shows the vesiculation that occurs during the acrosome reaction that allows the release of the acrosomal contents.

SURFACES AND FACES OF THE HEAD OF THE SPERM



THE ACROSOME REACTION



MATERIALS AND METHODS

Sperm Collection

Sperm were collected from mature albino guinea pigs (∿700 gm) purchased from Simonson, Gilroy, CA. The guinea pigs were killed with ether-anesthetized and the sperm removed from the vasa deferentes and cauda epididymies by gently squeezing cut sections into freshly gassed (95% air, 5% CO_2, resulting pH 7.6 ± 0.1) calcium-free minimal culture medium (Ca^{2+}-free MCM, Barros, 1974). The sperm were then washed by centrifugation at 1000 g for 1 min and resuspended in fresh Ca^{2+}-free MCM. All procedures were performed at room temperature unless otherwise indicated.

Ruthenium Red (RR) Staining

The RR staining procedures were modeled after the procedures of Luft (1971a), and Charonis and Wissig (1983). Sperm were initially fixed for 1-4 hrs in Karnovsky's aldehyde fixative [1% paraformaldehyde, 3% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4) with 2 mM $CaCl_2$ generally added]. The fixed sperm were then exposed overnight at 4°C to a secondary fixative of fresh Karnovsky's to which 0.1% ruthenium red (Aldrich Chemical Co., Milwaukee, WI) was added and the pH adjusted to pH 5.8 ± 0.1 (Luft, 1971a; Charonis and Wissig, 1983). Sperm fixed overnight in the secondary fixative were postfixed for 3 hrs in 1% OsO_4 in 0.1 M Na cacodylate buffer (pH 5.8) to which 0.05% RR was added. Sperm were then processed routinely for thin section electron microscopy which included *en bloc* staining with uranyl acetate at 37°C for about 1 hr, rapid dehydrating in graded ethanols, clearing in propylene oxide,

and embedding in Epon. Thin sections, either unstained or stained with uranyl acetate and lead citrate where examined on a Siemens 1A electron microscope at 80KV.

Alterations in conditions of ruthenium red (RR) staining

Salts

A series of experiments were designed to confirm the ionic nature and determine relative strength of the interactions of RR with the anionic sites on the sperm membranes. Varying concentrations of salt (0 to 2.6 M NaCl; KCl and $CaCl_2$ were also tested) were added to both the RR containing secondary fixative and the OsO_4 to act as competing electrolytes for the anionic sites (Charonis and Wissig, 1983).

To be certain that the staining was due to the presence of RR, sperm were exposed to secondary fixative and OsO_4 with and without added NaCl but omitting RR.

Osmolarity

Exposure of sperm to increasing salt concentrations resulted in an increase in the frequency with which the overlying plasma membrane separated away from the outer acrosomal membrane. To determine if the increased separation was simply due to the increased osmolarity of the RR containing secondary fixative and OsO_4, 1.3 M sucrose was substituted for 0.65 NaCl in the secondary fixatives and OsO_4.

To confirm that the RR staining of the outer acrosomal membrane was not simply due to 'trapping' of the stain between the plasma membrane and outer acrosomal membrane, sperm in MCM were diluted in an equal volume of distilled H_2O which causes the plasma membrane to separate from the

acrosomal membrane. These sperm were the fixed and exposed to RR as described above.

Salt-Desalt

Because increasing concentrations of salt inhibited the RR binding we wanted to confirm that the high salt conditions were not extracting anionic sites, thereby inhibiting RR deposition. Sperm were exposed to both RR and high NaCl (2.6 M) overnight (pre-incubation). The next day the sperm were washed three times on 0.1 M Na cacodylate buffer to remove the salt and then reexposed to RR containing fixatives and OsO_4 as described above.

Primary fixative

In an attempt to evaluate potential effects of the primary fixative, two fixatives other than Karnovsky's primary fixative were employed. A tannic acid fixative containing 4% tannic acid and 2% glutaraldehyde, and Nakane's fixative (McLean and Nakane, 1974), which is designed to cross link carbohydrate residues, were used as primary fixatives. After 1-4 hrs in the primary fixative, the sperm were then placed in Karnovsky's as a secondary fixative with RR added and processed as described above.

pH

To determine if acidic pH could neutralize the anionic sites and thus prevent RR deposition, HCl was added to both the RR containing secondary fixative and OsO_4 until pH's of approximately 5, 3 and 2 were obtained. At pH 3 and 2, RR is converted to ruthenium brown (Luft, 1971a). Because the pH of both solutions was unstable between 5 and 3, and we did not wish to induce the variable of another buffer, the results within this pH range were disregarded.

Cationic ferritin (CF) labeling

To allow CF access to the acrosomal membrane, the plasma membrane was disrupted. Sperm in Ca^{2+}-free MCM were diluted in an equal volume of distilled H_2O, and the plasma membrane was broken either by sonication, or brief treatment with a Polytron (Brinkman model #PT 10-35, setting 5-6 for 10-30 sec, with sperm suspension cooled on ice). The disrupted sperm were centrifuged and resuspended twice in fresh Ca^{2+}-free MCM, or in Ca^{2+}-free phosphate buffered saline (PBS). Ca^{2+}-free media (more preceisely, no added calcium, Yanagimachi, 1982) was used throughout because Ca^{2+} stimulates the fusion of the acrosomal and plasma membranes. The washed sperm (10-40 x 10^6) were then exposed to CF (either Miles Laboratories Inc., Elkhart, IN, cat #91-114-1, or Sigma Chemical Co., Stl Louis, MO, cat #F 7879) at concentrations of 0.5 to 1.0 mg/ml for 15 min. Sperm were then washed twice in either Ca^{2+}-free MCM or Ca^{2+}-free PBS to remove unbound CF prior to fixation in Karnovsky's fixative, postfixation in 1% OsO_4 in Palade's buffer, and processed routinely for thin section electron microscopy as described above.

Alterations in the conditions of cationic ferritin (CF) labeling

Salt

To examine the relative strength and confirm the ionic nature of the interaction of CF with the anionic sites on the sperm, varying concentrations of NaCl (0-0.9 M) were added to the sperm immediately prior to the 15 min incubation with the CF. The same concentration of NaCl was maintained in the washes. The sperm were then fixed as described above.

Pre-fixation

To determine if CF binding was affected by pre-fixation, Polytron-disrupted sperm were washed once and fixed for 1 hr in Karnovsky's fixative. These pre-fixed sperm were washed with two changes of 0.1 M glycine in Ca^{2+}-free PBS, and left in a third change for 1 hr to interact with unreacted aldehyde groups. The pre-fixed sperm were then placed in fresh Ca^{2+}-free PBS and exposed to varying concentrations of NaCl (0-0.6 M) and CF.

Salt - Desalt

Since high salt concentrations inhibited the CF binding to the acrosomal membrane of unfixed sperm, and are known to extract peripheral membrane proteins, controls were designed to determine whether CF binding sites were extracted in high salt concentrations. Polytron-disrupted sperm were pre-incubated in 0.6 M NaCl in Ca^{2+}-free MCM for 15 min, then washed twice in Ca^{2+}-free PBS to remove the salt, and exposed to CF and varying NaCl concentrations. Following 15 min of incubation, the sperm were washed in the appropriate buffer and fixed.

pH

The effect of increasingly acidic pH on the distribution of CF binding was also examined. These experiments were carried out on unfixed Polytron-disrupted sperm either by the addition of HCl to Ca^{2+}-free MCM, or through the use of 0.1 M citrate/phosphate buffer to achieve pH's of 6, 5, 4, and 3. CF was then added to the sperm in the acidic buffers. After 15 min the sperm were washed twice in the appropriate acidic buffer prior to fixation.

In addition, to determine whether the acidic buffer which inhibited CF binding (pH 3) was removing peripheral membrane component(s) responsible for CF binding, unfixed Polytron-disrupted sperm were pre-incubated in 0.1 M citrate/phosphate buffer (pH 3) for 15 min, then washed twice in PBS and exposed to CF at both varying salt concentrations and varying pH's.

Freeze-fracture, deep-etching

Sperm were processed for freeze-fracture, deep-etching according to the procedures outlined by Enders et al. (1983).

Lytic enzymatic digestion

To characterize the biochemical nature of the anionic sites on the P-surface of the outer acrosomal membrane, Polytron-disrupted sperm were exposed to a variety of lytic enzymes. All lytic enzymes were obtained from Sigma Chemical Co. (See Table 1 for more information). Polytron-disrupted sperm were washed once in Ca^{2+}-free MCM, divided into equal aliquots (10-40 x 10^6), washed a second time in either Ca^{2+}-free MCM, pH-adjusted Ca^{2+}-free MCM, or an alternative buffer (See Table 1) before incubation with the lytic enzymes. All the polytron-disrupted sperm were incubated with the lytic enzymes for 30 min in 3 ml total volume, with occasional mixing at the temperature indicated in table I. After digestion, the sperm were washed twice and exposed to CF, or NaCl and CF in Ca^{2+}-free MCM or PBS for 15 min.

To assure that the lytic enzymes were functioning in the anticipated manner, the lytic supernatants were analyzed by SDS/polyacrylamide gel electrophoresis. Following the 30 min incubation of the

Polytron-disrupted sperm with the lytic enzymes, the sperm were centrifuged (1000 g for 1 min) and the supernatants were collected and frozen for further analysis. The frozen supernatants were thawed, 1 mM PMSF added, and microfuged for 1 min to remove insoluble material (mostly tail fragments). The cleared supernatants were then precipitated with trichloroacetic acid, resuspended in sample buffer and electrophoresed according to the procedures outlined in Enders et al. (1983). The gels were fixed and silver stained according to the procedures of Merrill et al. (1981).

Lipid solvent treatment

To determine the effect of lipid solvents on the anionic sites on the P-surface of the outer acrosomal membrane, fixed sperm were extracted with lipid solvents prior to either RR staining or CF labeling. Sperm fixed in Karnovsky's fixative were extracted overnight in chloroform:methanol:water (1:2:0.8) with at least one solvent change prior to rehydration and exposure of RR containing secondary fixative and OsO_4 as described previously. Additionally, Polytron-disrupted sperm were fixed and extracted with either chloroform:methanol:water, or acetone, rehydrated and exposed to either RR or CF as described above.

Induction of the acrosome reaction

Vasal and caudal epididymal sperm were collected and washed twice in Ca^{2+}-free Tyrode's medium (Fleming & Yanagimachi, 1981), (omitting oxaloacetic acid and antibiotics and substituting Miles bovine albumin, crystallized # 81-00-1-3 for Sigma, fraction V BSA). So that a large number of sperm could be examined undergoing the acrosome reaction, the

membrane-lipid mobility agent A_2C (2-(2-Methyoxyethoxy)ethyl-8-(cis-2-n-octyl-cyclo-propyl)-octanoate) was used to promote capacitation prior to intiation of acrosome reaction upon the addition of Ca^{2+} (Fleming et al., 1982). Sperm were incubated at 37°C for 1 hr in Ca^{2+}-free Tyrode's medium to which 2.5 x 10^{-4} ml A_2C/ml (Sigma Chemical Co., #M-9010) was added. After 1 hr, the sperm sample was divided and the acrosome reaction was initiated in one of the samples by adding 1 M $CaCl_2$ to obtain a final concentration of 2.0 mM Ca^{2+}. No Ca^{2+} was added to the other sample. Ten min later, both sperm samples were fixed in Karnovsky's fixative, and exposed to RR containing secondary fixative and OsO_4 as described above.

Table 1. Enzymes and conditions used to digest Polytron-disrupted sperm prior to cationic ferritin (CF) exposure

Enzymes	Sigma Cat. #	Media or Buffer	pH	Temp. (°C)	Time (min)	Concentration or Units of Activity
Trypsin (type III)	T 8253	MCM	7.6	37	30	1 mg/ml
Pronase E(I) (protease type V)	P 5005	MCM	7.6	37	30	1.7 mg/ml
Pronase E(II) (protease type XIV)	P 5147	MCM	7.6	25 and 37	30	1.7 mg/ml 1 mg/ml
Subtilopeptidase A (protease type VII)	P 5380	MCM	7.6	37	30	1.7 mg/ml
Proteinase K (protease type XI)	P 0390	MCM	7.6	37	30	1.7 mg/ml and 1 mg/ml
Neuraminidase	N 2876	MCM	5.5	37	30	1.3 gm/ml
Chondroitinase ABC	C 3509	MCM	7.6	37	30	1.7 U/ ml
Hyaluronidase	H 3506	MCM	5.5	37	30	300 U/ ml
Arylsulfatase (type IV)	S 8504	Na Acetate 0.1 M	5.0	37	30	1.7 gm/ml
Arylsulfatase (type H-I)	S 9626	Na Acetate 0.1 M	5.0	37	30	1.7 gm/ml
β-glucuronidase	G 8132	Citrate/PO_4 0.1 M	4.0	37	30	1.7 mg/ml and 2.0 mg/ml

RESULTS

Ruthenium Red (RR) Staining

When RR was present in both the secondary fixative and OsO_4 a uniform electron dense deposit was observed on the E-surface of the plasma membrane and between the plasma membrane and the outer* acrosomal membrane (Figs. 1,2).

Effect of NaCl. When increasing concentrations of NaCl were added to the RR containing secondary fixative and OsO_4 the resulting electron dense deposits on the plasma membrane and the outer acrosomal membranes gradually decreased. Though there was some variability within a given thin section, the plasma membrane staining generally decreased markedly as added salt increased from 0.4 to 0.6 M (Figs. 3,4). At these concentrations of added salt, the staining of the acrosomal membrane also decreased, but less dramatically. Increasing the added NaCl concentrations from 0.9 to 2.6 M continued to reduce the electron dense deposits on the P-surface of the outer acrosomal membrane (Figs. 5,6), although even at 2.6 M added NaCl an electron dense deposit was seen on the P-surface of many acrosomes (Fig. 6). These and other results are summarized in Table 2.

When sperm were exposed to identical conditions, without RR added to the secondary fixative and OsO_4, no extensive staining of either the plasma or the outer acrosomal membrane occurred (Fig. 7). The acrosomal P-surface was slightly more electron dense than the other membrane sur-

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*The "outer acrosomal membrane" is that portion of the acrosomal membrane adjacent to the plasma membrane, as opposed to the "inner acrosomal membrane," which is acrosomal membrane adjacent to the nuclear envelope.

faces (plasma membrane P- and E-surfaces and acrosomal E-surface). The acrosomal P-surface electron density was accentuated by overnight incubation in the secondary fixative even in the absence of RR, when compared to sperm fixed by routine procedures which do not include a second aldehyde fixative.

In addition to inhibiting the RR staining, the increasing concentrations of NaCl more frequently caused a separation of the plasma membrane from the adjacent outer acrosomal membrane (Figs. 3-6). When the two membranes did separate, the RR-induced electron density on the P-surface of the acrosomal membrane was the greatest, followed in decreasing order by the E-surface of the plasma membrane and the P-surface of the plasma membrane (Fig. 3). When RR was not added to the secondary fixative or OsO_4, the plasma membrane tended to separate from the outer acrosomal membrane. Addition of NaCl to the secondary fixative and OsO_4 enhanced this separation. The separation of the plasma membrane from the underlying outer acrosomal membrane stopped abruptly at the equatorial segment (not illustrated).

Effect of Osmolarity. To determine whether the separation of the plasma membrane from the outer acrosomal membrane was simply due to the increasing osmolarity of the RR containing secondary fixative and OsO_4, 1.3 M sucrose was substituted for NaCl. While the 1.3 M sucrose inhibited the RR staining somewhat, it did not cause an increase in the separation of the plasma membrane from the acrosomal membrane, and did not inhibit the RR staining as effectively as 0.65 M NaCl (Fig. 10).

Most experiments were performed with NaCl, though KCl, and $CaCl_2$ produced results similar to NaCl. The RR staining does not appear to be a simple trapping effect. When sperm were placed into hypotonic media

prior to fixation, the plasma membrane separates from the outer acrosomal membrane. No changes in the relative strength of the RR deposition on the two membranes were seen (not illustrated).

Effect of Salt - Desalt. Sperm which were pre-incubated in high salt concentrations (2.6 M, which inhibits RR deposition) and then desalted and restained with RR had a similar appearance to sperm prepared without such pre-incubation (Fig. 9). While the high salt preincubation both reduced the overall RR staining somewhat and caused an increase in the plasma membrane buckling, it did not change the relative distribution of RR deposits on the membrane surfaces.

Effect of Primary Fixative. When tannic acid was included in the primary fixative, subsequent RR staining was enhanced (Fig. 8). Increasing concentrations of NaCl up to 2.6 M had very little effect on the appearance of the sperm, neither inhibiting the RR electron deposition, nor causing an increased separation of the plasma membrane from the outer acrosomal membrane. When, on occasion, the plasma membrane separated from the acrosomal membrane, the electron dense staining on the P-surface of the acrosomal membrane was much thicker than on the P-surface of the plasma membrane (not illustrated).

The use of Nakane's fixative as the primary fixative did not dramatically affect subsequent RR deposition. The quality of fixation was reduced when compared to Karnovsky's fixative and often the less-electron-dense portion of the acrosome was not fixed, disrupting both acrosomal and plasma membranes. Increasing salt concentrations in the secondary fixative and OsO_4 both reduced the RR electron dense staining and tended to cause the loss or separation of the plasma membrane from the acrosomal membrane (not illustrated).

Effect of pH. Increasing acidity yielded results similar to increasing salt concentrations. A pH of 5 reduced RR deposition somewhat, though the results were variable (not illustrated). The electron dense staining seen at pH's 2 and 3 was decreased dramatically compared to that seen at pH 5.8, and buckling of the overlying plasma membrane was observed (Fig. 11).

Cationic Ferritin (CF) Binding

When sperm in Ca^{2+}-free MCM were diluted with distilled water, sonicated, and exposed to CF, the CF bound to the plasma membrane in a patchy distribution similar to the pattern of anionic sites seen by investigators using colloidal iron hydroxide (Copper and Bedford, 1971; Yanagimachi et al., 1972). However, when the plasma membrane was stripped off, exposing the outer acrosomal membrane, a very dense uniform deposit of CF was observed (Fig. 12). The CF binding was so extensive and uniform it was often easier to visualize in unstained preparations (Fig. 13). Because brief sonication resulted in the disruption of few sperm plasma membranes and none of the acrosomal membranes, Polytron-treated sperm were generally used throughout most of this study. Polytron treatment broke open most of the acrosomal granules, which offered the advantage that CF then had access to the E-surface of the acrosomal membrane. Some of the CF binding results are summarized in Table 3.

When sperm were exposed to native ferritin, very little ferritin bound, though occasional binding to the outer acrosomal membrane was observed (Fig. 14).

Effect of NaCl. The addition of 0.2 M NaCl to both the sperm while they were incubated with CF and to the subsequent washes, inhibited CF binding to most of the P-surface of the plasma membrane, but had little or no effect on CF binding to the P-surface of the outer acrosomal membrane (Fig. 15). Occasionally, CF particles were seen on the E-surface of the plasma membrane. In sperm with broken acrosomal membranes, CF bound extensively to the acrosomal contents, but generally did not bind to the E-surface of the acrosomal membrane. The addition of 0.4 M NaCl almost completely eliminated CF binding to the E-surface of the plasma membrane, while reducing CF binding to the acrosomal contents and to the P-surface of the acrosomal membrane (Fig. 16).

CF binding to all surfaces was essentially eliminated by the addition of 0.6 (Fig. 17) to 0.9 M NaCl (not illustrated). These salt concentrations were also increasingly effective at extracting acrosomal contents, making positive identification of acrosomal and plasma membranes more difficult. There were no detected differences between experiments performed in Ca^{2+}-free MCM and Ca^{2+}-free PBS.

Effect of Prefixation. To determine if CF binding resulted in the redistribution of anionic sites, Polytron-treated sperm were prefixed and then exposed to CF and varying concentrations of competing NaCl. In general, the results with prefixed and unfixed sperm were similar, but the following differences were noted. Overall CF binding was slightly enhanced in prefixed sperm with higher salt concentrations required to inhibit CF binding to prefixed sperm (Figs. 18,19). For example, 0.4 M NaCl gave results on prefixed sperm similar to the effects of 0.2 M NaCl on unfixed sperm (compare Fig. 19 with 15). The acrosomal contents were less extensively extracted when sperm were fixed prior to salt exposure.

In the prefixed sperm, acrosomal contents more often adhered to the E-surface of the acrosomal membrane. Also, the CF binding to the acrosomal contents was less extensive, presumably due to the decreased permeability of CF through the cross-linked contents.

Effects of Salt - Desalt. To eliminate the possibility that decreased CF binding was due to the salt extraction of anionic sites, unfixed sperm were exposed to 0.6 M NaCl, then desalted and exposed to CF. While the acrosomal contents were extracted, the binding of CF to the membrane surfaces was generally unaltered. CF still bound extensively to the P-surface of the outer acrosomal membrane, and 0.2 M NaCl was able to inhibit CF binding to most of the E- and P-surfaces of the plasma membrane (Fig. 20).

Effect of pH. When the pH of the media in which the sperm and CF were incubated was adjusted to increasingly acidic levels, the binding of CF was reduced. A pH of 5 (Fig. 21) had little effect on CF binding to any of the membrane surfaces, compared to sperm incubated in Ca^{2+}-free MCM (pH 7.6) and Ca^{2+}-free PBS (pH 7.4). At pH 4, the CF binding was reduced on all membrane surfaces, including the P-surface of the acrosomal membrane (Fig. 22). If CF was incubated with sperm at pH 3, nearly all CF binding was eliminated (Fig. 23). Only minor differences were seen between experiments using Ca^{2+}-free MCM adjusted to the desired pH with HCl versus those performed in phosphate/citrate buffer. There was slightly greater overall CF binding at each pH with the phosphate/citrate buffer system and more clumping of CF was seen.

To test if the exposure to acidic pH was irreversibly removing anionic sites, sperm were pre-incubated at pH 3 for 15 min, then washed with PBS and exposed to CF at different pH's or salt concentrations.

Increased extraction of acrosomal contents was seen with these sperm, but the distribution of CF binding was unaltered from the corresponding previous experiments (Fig. 24).

Periodicity of the Cationic Probes and Freeze-Fracture, Deep-Etch Results

In occasional thin sections of both RR-treated and CF exposed sperm, a periodicity to the RR staining and CF binding on the P-surface of the outer acrosomal membranes was seen (Figs. 13, 25-27). In freeze-fracture, deep-etch replicas of guinea-pig sperm, the E-face of the outer acrosomal membrane reveals linear rows of intramembrane particles. While often there was no corresponding linear arrangement evident on the P-surface of many replicas (Fig. 28), slight indications of a linear arrangement of the membrane components was seen in thin replicas. The center-to-center spacing of the linear row of E-face intramembrane particles was 15.2 nm ± 0.9,[+] while the center-to-center spacing of the linear rows of CF was 14.0 nm ± 2.1.[+] However, further experimentation will be required to determine if there is an exact correspondence between the E-face intramembrane particles and the P-surface anionic sites.

Effects of Lytic Enzyme Digestion

None of the lytic enzymes examined under the conditions used inhibited CF binding to the P-surface of the outer acrosomal membrane. Neuraminidase, chondroitinase ABC, hyaluronidase, β-glucuronidase, and the arylsulfatases did not alter sperm structure or cause any detected chang-

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[+]mean ± standard deviation, N=11, from 3 or 4 negatives with each measurement being the mean of 10 consecutive center-to-center spacings.

es in CF binding to either the plasma or acrosomal membranes (Figs. 29,30). Recent work indicates that neuraminidase only removed about 20% of the sialic acid residues on the surface of the rat caudal epididymal sperm (Toowicharanont and Chulavatnatol, 1983).

The proteolytic enzymes (trypsin, Pronase $E_{(I \& II)}$, proteinase K, and subtilopeptidase A) all altered sperm ultrastructure, but had little effect on CF labeling of the P-surface of the outer acrosomal membrane (Figs. 31-34). Morphologic evidence that the proteolytic enzymes were functioning included increased head-tail separation, dispersion of the acrosomal contents, and loss of the axoneminal complex components (microtubules, dyncin arms, etc.). CF binding to the E-surface of the plasma membrane was often reduced, especially following pronase $E_{(I \& II)}$, proteinase K, and subtilopeptidase A treatments. However, none of the proteolytic enzymes completely inhibited plasma membrane labeling.

Analysis of the lytic supernatants on SDS/polyacrylamide gels revealed that soluble sperm components were reduced in both quantity and apparent molecular weight by the proteolytic enzymes (Fig. 35). The soluble sperm components were unaltered by the other lytic enzymes or the alternative buffers used.

Effects of Lipid Solvent Treatment

Lipid solvent treatment reduced the RR deposition on both the plasma and acrosomal membranes. Following lipid extraction, distinct bilayers were generally not visible, making definitive identification of the plasma and acrosomal membrane difficult. In intact sperm where the plasma membrane separates from the acrosomal membrane, most of the electron density remained associated with the separated plasma membrane,

with little electron density associated with the outer acrosomal membrane (Figs. 36,37). The reduction in the electron density staining of the P-surface of the outer acrosomal membrane also occurred in Polytron-disrupted sperm (Fig. 38). Acetone extraction was less effective than chloroform:methanol:water at reducing RR deposits (Fig. 39). Though there was some variability within a given thin section, reduced RR deposition following lipid solvent treatment was always evident.

The effects of the lipid solvents on subsequent CF binding were more difficult to interpret. In general, the CF binding was reduced, but not as dramatically as the RR deposition (Fig. 40). Interpretation of the CF binding results were complicated by the extensive CF binding to the acrosomal contents.

Ruthenium Red (RR) Staining of Acrosome Reacted Sperm

Sperm which were incubated in A_2C containing Ca^{2+}-free Tyrode's and stained with RR appeared essentially identical to sperm incubated in Ca^{2+}-free MCM. Ten minutes after the addition of Ca^{2+} most sperm had undergone the acrosome reaction. When the plasma and acrosomal membranes fused to form hybrid vesicles, the RR staining properties changed noticeably. While there was a light RR deposition on the E-surface of the hybrid vesicles, no heavy RR deposition was seen inside on the P-surface. This reduction of P-surface RR staining does not appear to be due simply to the small radius of curvature of the hybrid vesicles, since in areas where the vesicles appeared flattened against non-acrosome reacted sperm, there is also a reduction in RR deposition (Figs. 41, 42). Within partially acrosome-reacted sperm, regions of adjacent acrosomal

and plasma membranes often appear with decreased RR deposition (Fig. 42), and increased distance between the two membranes. In contrast, in non-acrosome reacted sperm, there continues to be heavy RR deposition on the P-surface of the outer acrosomal membrane (Figs. 41,42).

Often within acrosome reacted sperm, portions of the plasma and acrosomal membranes remain apposed to each other, rather than vesiculated. When these two membranes remain apposed, the acrosomal membrane often displays a distinct periodicity on its E-surface when RR stained (Figs. 43,44). The periodicity of the staining was 15.4 ± 1.9 nm.[+] This value is similar to that obtained for the distance between rows of acrosomal membrane intramembrane particles. Occasionally, periodicity was also seen on the opposing membrane.

Fig. 1 and 2. Thin sections across a rouleau of guinea-pig sperm stained with RR. Fig. 1: An extensive, uniform electron dense deposit is present on the E-surface of the plasma membrane, and between the plasma membrane and the acrosomal membrane. A, acrosome; N, nucleus; bar, 100 nm. X 37,700. Fig. 2: Higher magnification of two adjacent sperm, similar to those in Fig. 1. The small arrowheads point to the acrosomal membranes and the longer arrowheads point to the plasma membranes. a, acrosomal membrane; p, plasma membrane. bar, 100 nm. X 71,050.

Fig. 3-5. Thin sections of sperm exposed to RR containing varying concentrations of competing NaCl. Fig. 3: 0.4 M NaCl; there is only a slight reduction in the RR deposition on the E-surface of the plasma membrane and on the P-surface of the acrosomal membrane compared to no NaCl (Fig. 2). The plasma membrane has separated from the underlying acrosomal membrane. bar, 100 nm. X 71,050. Fig. 4: 0.6 M NaCl; the electron dense deposits on the plasma and acrosomal membranes are reduced compared to 0.4 M NaCl (Fig. 3). bar, 100 nm. X 71,050. Fig. 5: 0.9 M NaCl; the electron dense deposit on the plasma membrane is further reduced compared to 0.6 M NaCl (Fig. 4), while the P-surface of the acrosomal membrane retains an electron dense deposit. Note the separation of the plasma membrane from the acrosomal membrane. bar, 100 nm. X 71,050.

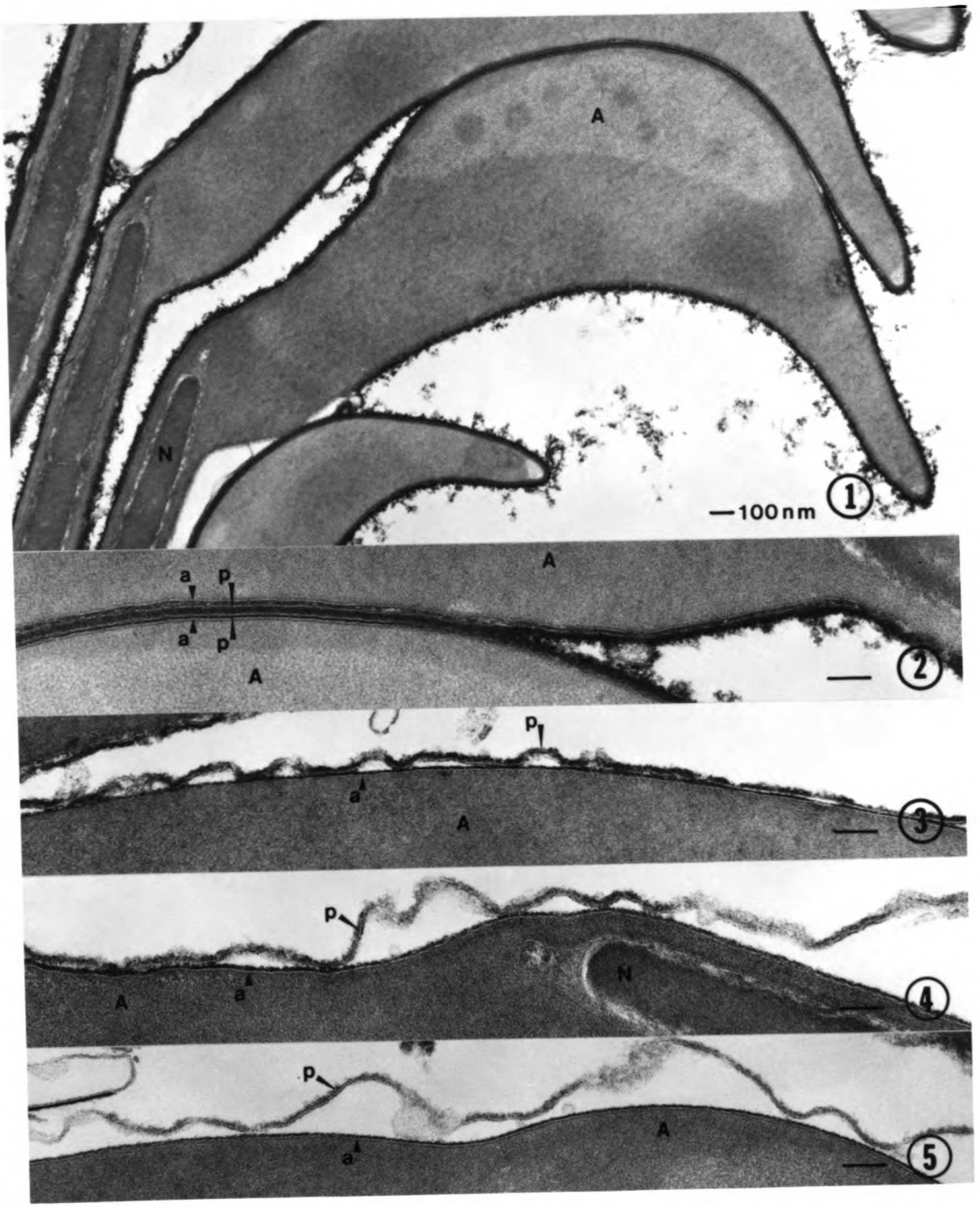
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Fig. 6. Sperm exposed to RR containing 2.6 M NaCl. This high salt concentration inhibits much of the RR deposition, and results in separation of the plasma membrane from the acrosomal membrane. bar, 100 nm. X 37,700.

Fig. 7. Control for RR staining. Sperm exposed to primary and secondary aldehyde fixatives and OsO_4, none of which contained RR. There is no heavy electron dense deposits on the membranes, but the P-surface of the acrosomal membrane has the greatest electron density. There is slight separation of the plasma membrane from the acrosomal membrane. bar, 100 nm. X 71,050.

Fig. 8. The RR staining of sperm fixed with tannic acid containing primary fixative. A highly uniform, electron dense deposit is present on the plasma membrane and between the plasma and the acrosomal membranes. bar, 100 nm. X 71,050.

Fig. 9. The RR staining of sperm which were exposed to 2.6 M NaCl, then desalted and stained with RR. The RR staining is slightly reduced compared to sperm which were never exposed to high salt (Fig. 2). Separation of the plasma membrane from the acrosomal membrane is observed. bar, 100 nm. X 71,050.

Fig. 10. Sperm exposed to RR containing 1.3 M sucrose. Sucrose decreases the RR depositions slightly compared to sperm exposed only to RR (Fig. 2). The addition of sucrose did not stimulate the separation of the plasma membrane from the acrosomal membrane. bar, 100 nm. X 71,050.

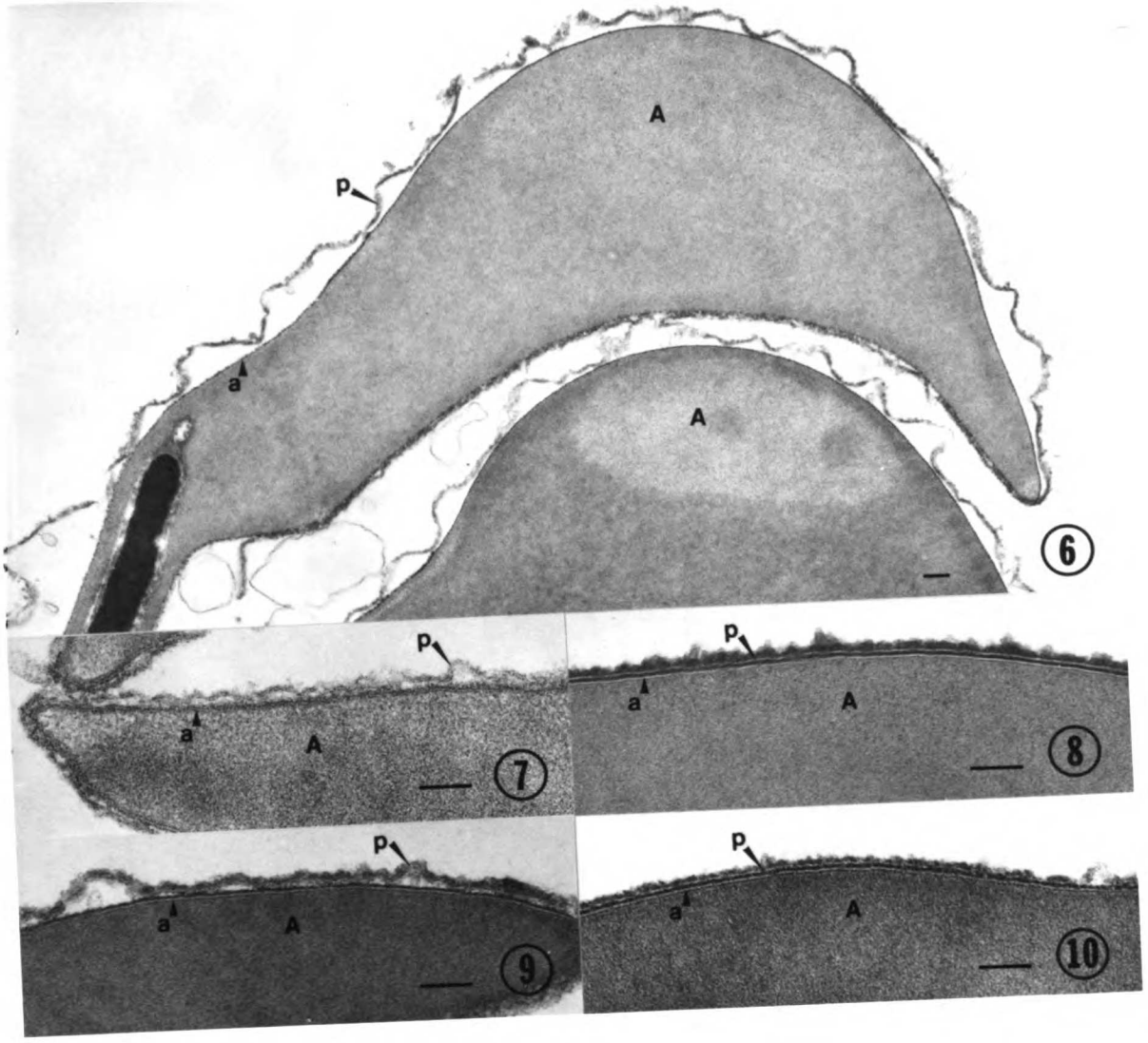
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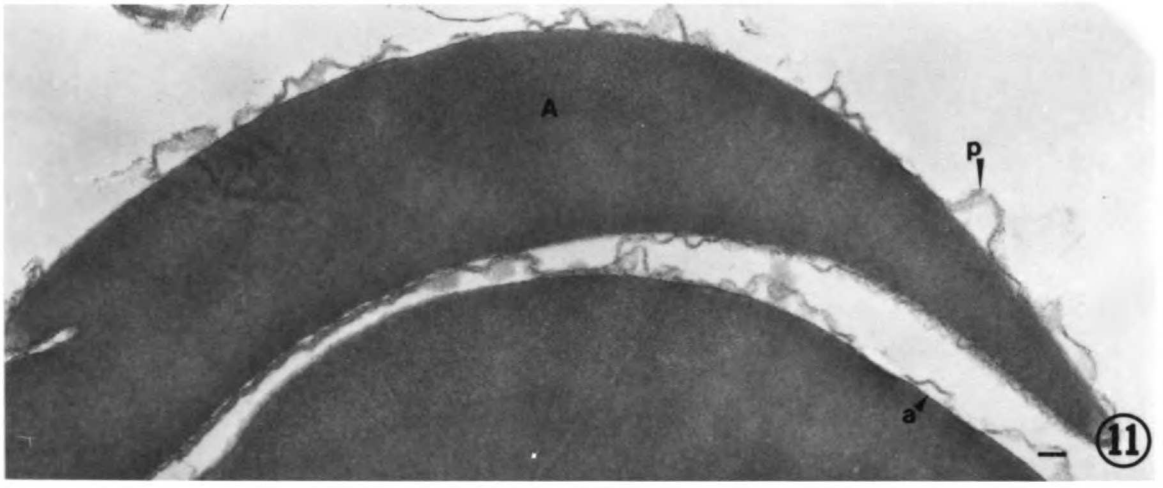
Fig. 11. Sperm exposed to RR at pH 2 (RR converts to ruthenium brown). There is only a slight electron dense deposition on the P-surface of the acrosomal membrane. The plasma membrane is separated from the acrosomal membrane. bar, 100 nm. X 37,700.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Figs. 12 and 13. Sperm in which the plasma membrane was removed by mechanical disruption. The sperm were then exposed to CF. Fig. 12: A very dense, uniform layer of CF is bound to the acrosomal membrane. Remnants of the plasma membrane are less heavily labeled. bar, 100 nm. X 71,050. Fig. 13: Dense, uniform labeling of the acrosomal membrane with CF. Note the linear arrays of CF particles as the acrosomal membrane becomes tangential to the plane of the section. Unstained thin section. bar, 100 nm. X 71,050.

Fig. 14. Disrupted sperm exposed to native ferritin. Occasional ferritin particles are bound to the acrosomal membrane. bar, 100 nm. X 71,050.

Fig. 15. Polytron-disrupted sperm exposed to 0.2 M NaCl and CF. CF is uniformly bound to the P-surface of the acrosomal membrane, while CF is infrequently bound to the E-surface of the acrosomal membrane. CF has access to the E-surface of this membrane as demonstrated by the extensive CF binding to the acrosomal contents. The remnants of the plasma membrane bind few CF particles. bar, 100 nm. X 71,050.

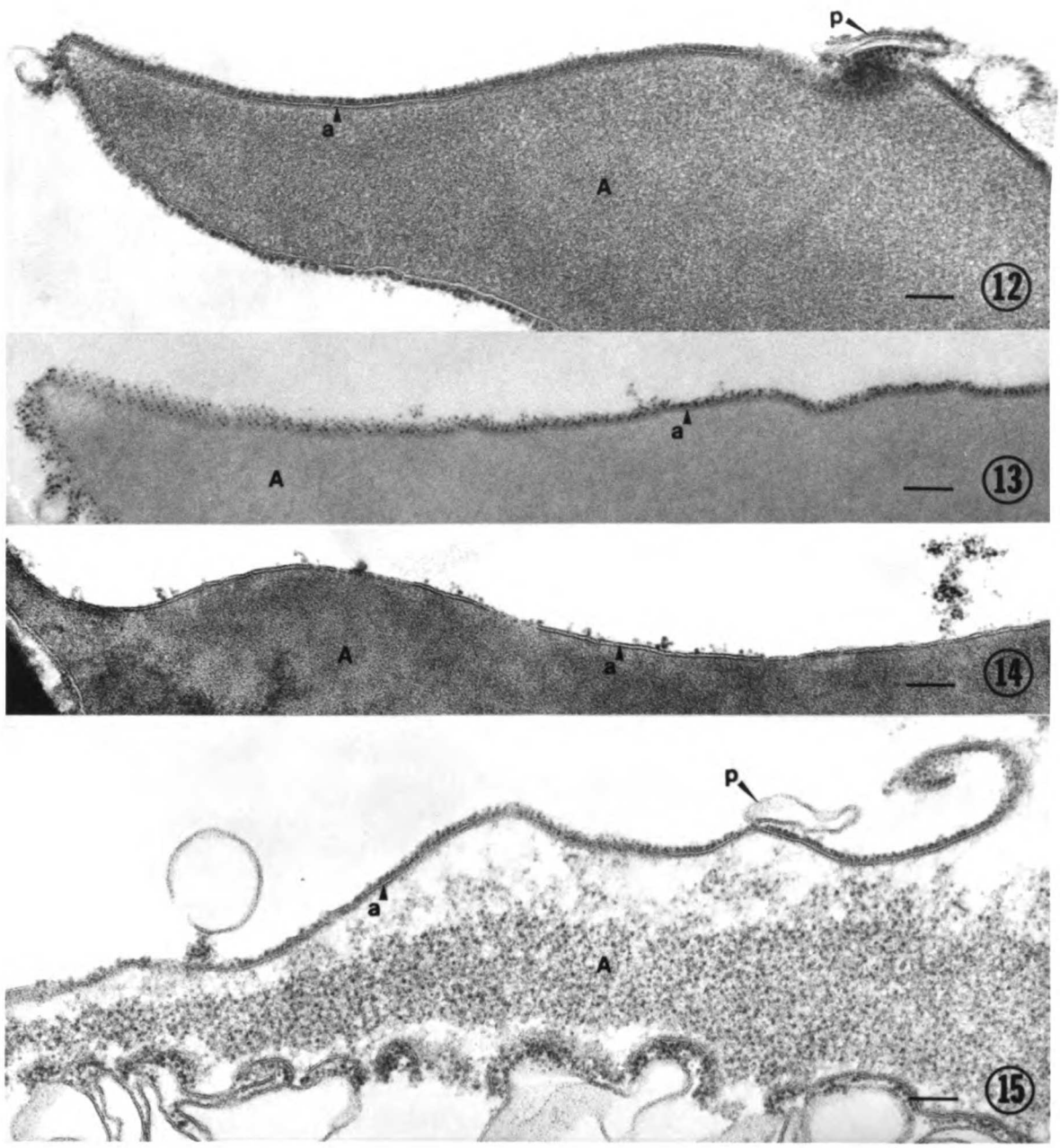
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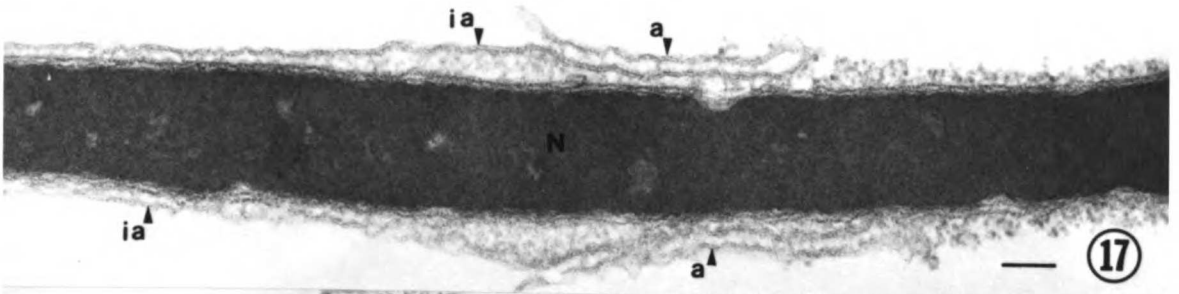
Fig. 16. Polytron-disrupted sperm exposed to 0.4 M NaCl and CF. The CF binding to the P-surface of the acrosomal membrane is reduced compared to 0.2 M NaCl (Fig. 15). There is also an increased extraction of the acrosomal contents. Unstained thin section. bar, 100 nm. X 71,050.

Fig. 17. Polytron-disrupted sperm exposed to 0.6 M NaCl. A thin section across the equatorial portion of the sperm head. The acrosomal contents have been extracted and the inner acrosomal membrane (ia) is seen on either side of the nucleus. Only a few CF particles bind to the P-surface of the outer acrosomal membrane. CF also binds to the exposed nuclear envelope and adjacent cytoplasm. bar, 100 nm. X 71,050.

Fig. 18. Polytron-disrupted sperm prefixed prior to exposure to 0.2 M NaCl and CF. The CF is uniformly bound to the P-surface of the acrosomal membrane with fewer CF particles binding to the E-surface of the plasma membrane. The CF particles bind to less of the acrosomal contents compared to unfixed sperm (Fig. 15). bar, 100 nm. X 71,050.

Fig. 19. Prefixed sperm exposed to 0.4 M NaCl and CF. CF is bound uniformly to the P-surface of the acrosomal membrane, yet does not appear to bind to the P-surface of a plasma membrane remnant. bar, 100 nm. X 71,050.

16



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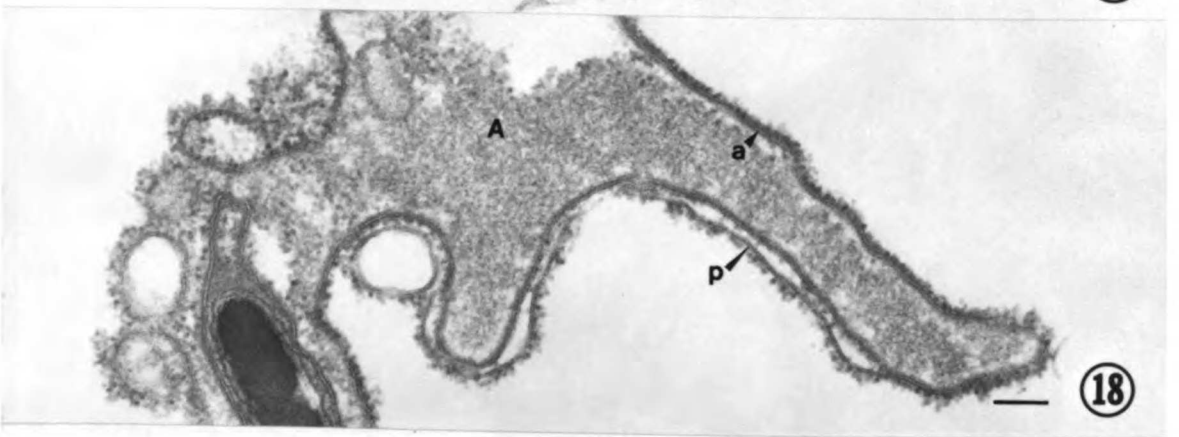

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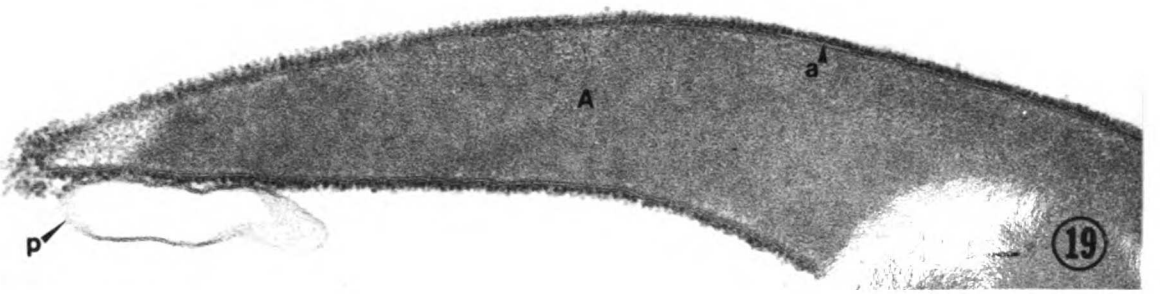

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Fig. 20. Unfixed sperm which was pre-treated with 0.6 M NaCl then desalted and exposed to 0.2 M NaCl and CF. Most of the acrosomal contents have been extracted. There is extensive CF binding on the P-surface of the outer acrosomal membrane, but little binding to the E-surface of the outer acrosomal membrane, and P-surface of the plasma membrane. The E-surface of the inner acrosomal membrane also is not labeled by CF. bar, 100 nm. X 71,050.

Figs. 21-23. Disrupted sperm exposed to CF at varying pH's: pH 5, Fig. 21; pH 4, Fig. 22; pH 3, Fig. 23. Note the decrease in CF binding to both the E-surface of the plasma membrane and the P-surface of the acrosomal membrane occurring with increasingly acidic pH. bar, 100 nm. X 71,050.

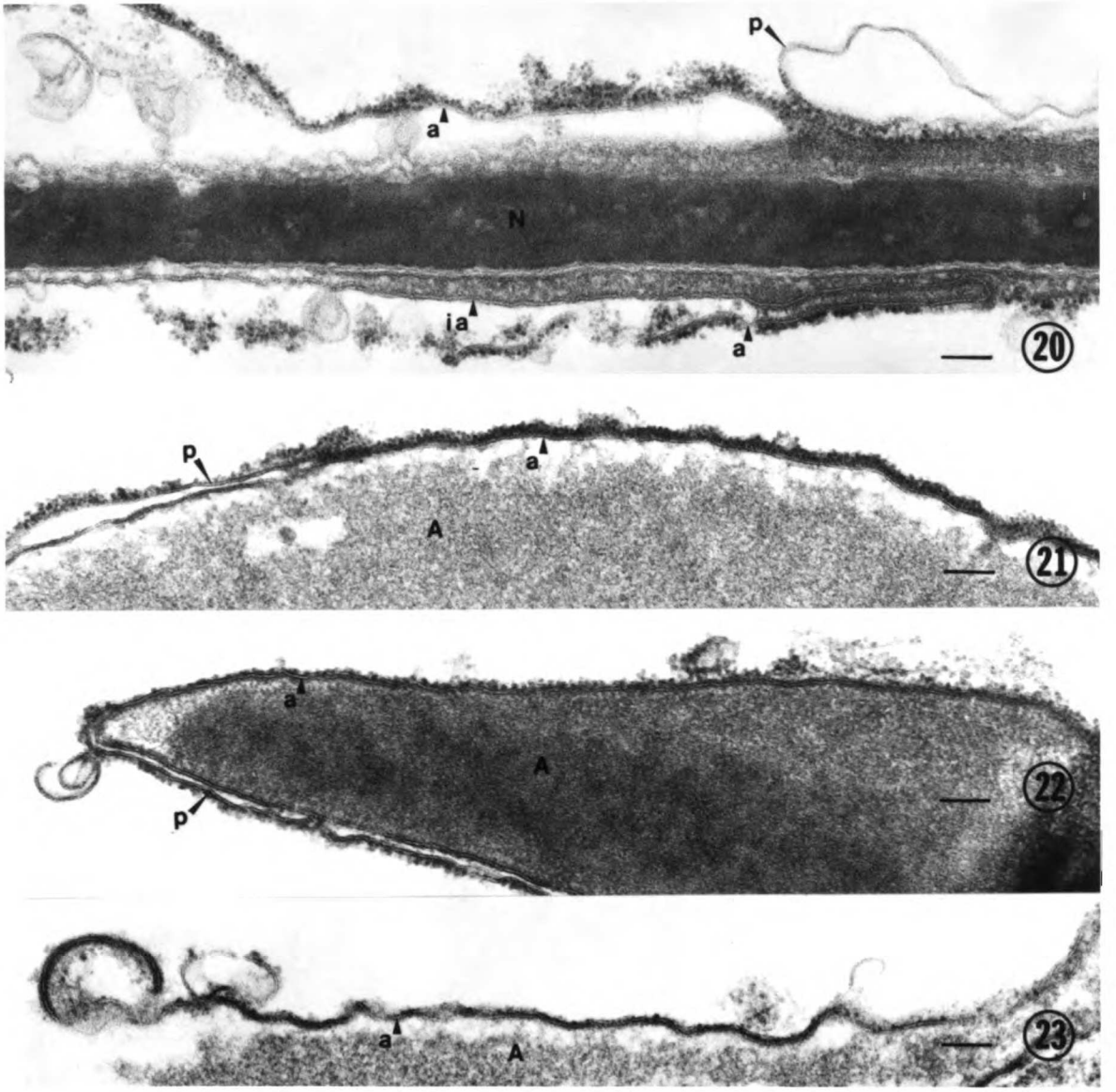
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Fig. 24. Sperm pre-incubated at pH 3 for 15 min, then washed and exposed to CF at pH 7.4 in PBS. Heavy CF binding is present on the P-surface of the outer acrosomal membrane. A patchier distribution of CF binding is present on the P-surface of the plasma membrane. Occasional CF particles are seen on the E-surface of the inner acrosomal membrane. bar, 100 nm. X 71,050.

Figs. 25 and 26. Thin sections of sperm stained with RR. Note the periodicity (arrows) that appears in the RR staining, which is especially noticeable in tangential section (Fig. 25). bar, 100 nm. X 71,050. Fig. 26: bar, 100 nm. X 99,900.

Fig. 27. Thin section tangential to the tip of the acrosome of guinea-pig sperm which was exposed to CF. The linear periodicity (arrows) of the bound CF is evident. bar, 100 nm. X 99,900.

Fig. 28. Freeze-fracture, deep-etch replica of both the E-face and P-surface of the acrosomal membrane. The intramembrane particles in the E-face of the membrane appears in linear arrays, while the P-surface appears less ordered. EF, E-face; PS, P-surface. bar, 100 nm. X 99,900.

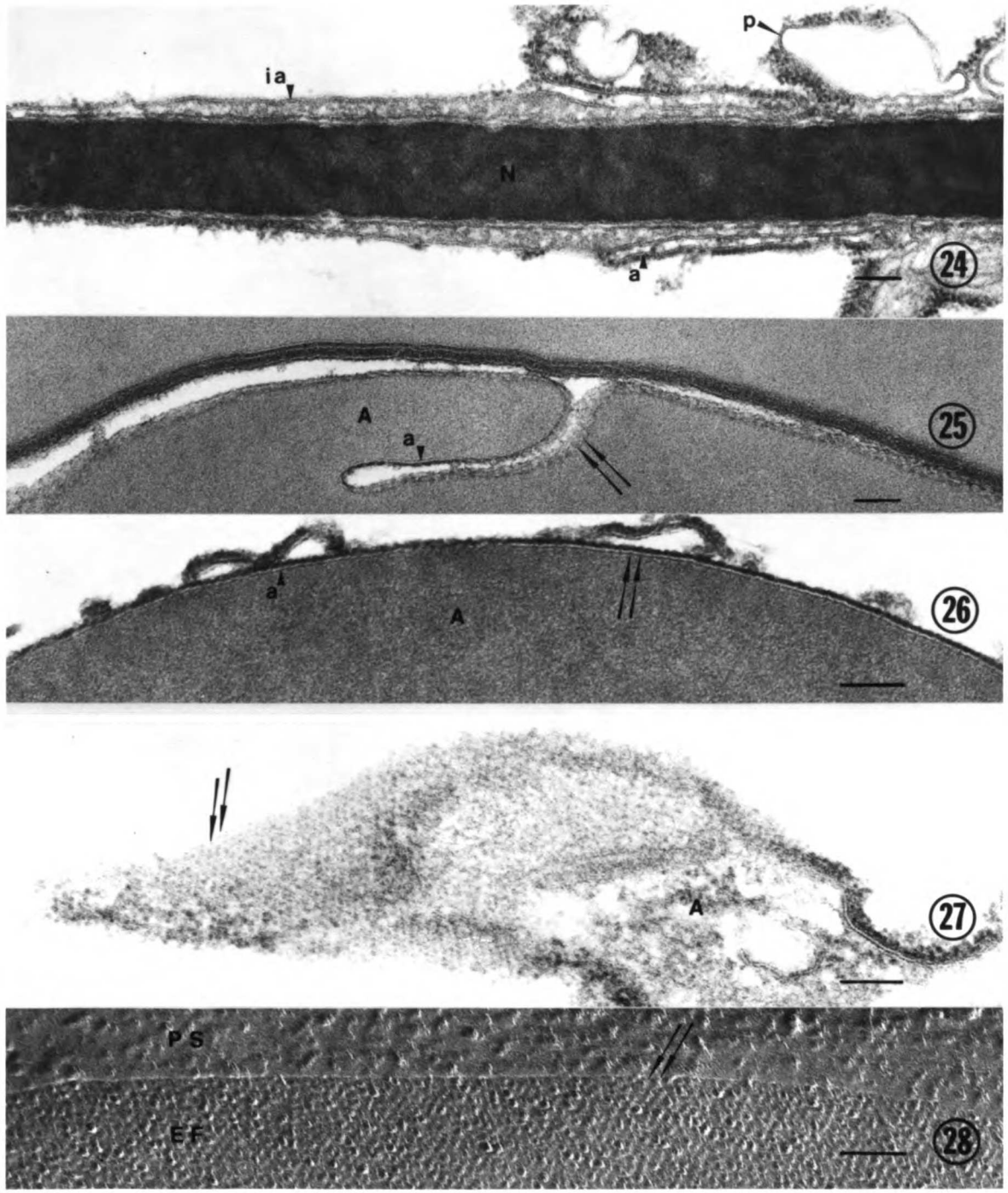
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Fig. 29. Polytron-disrupted sperm exposed to hyaluronidase for 30 min prior to CF labeling. CF is seen bound to the E-surface of the acrosomal membrane and to the dispersed acrosomal contents. The linear periodicity of the CF labeling is evident in tangential views of the outer acrosomal membrane (arrows). X 71,050.

Fig. 30. Polytron-disrupted sperm exposed to chondroitinase ABC for 30 min prior to CF labeling. CF is bound to the outer acrosomal membrane. X 71,050.

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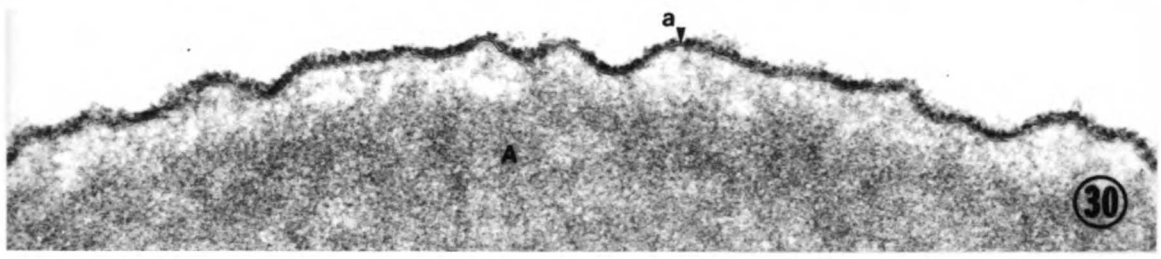

30

Figs. 31 and 32. Polytron-disrupted sperm exposed to pronase $E_{(II)}$ for 30 min prior to CF labeling. CF binding to the E-surface of the plasma membrane is sparse (Fig. 31) while binding to the acrosomal membrane remains dense and uniform (Fig. 32). Note that the components of the axonemal complex have been digested (star, Fig. 32). X 71,050.

Fig. 33. Polytron-disrupted sperm exposed to proteinase K for 30 min prior to CF labeling. Dense, uniform CF labeling of the P-surface of the acrosomal membrane is present, and the linear periodicity of the labeling is evident in tangential views of the membrane (arrows). X 71,050.

Fig. 34. Polytron-disrupted sperm exposed to subtilopeptidase A for 30 min prior to CF labeling. CF labels the P-surface of the outer acrosomal membrane. X 71,050.

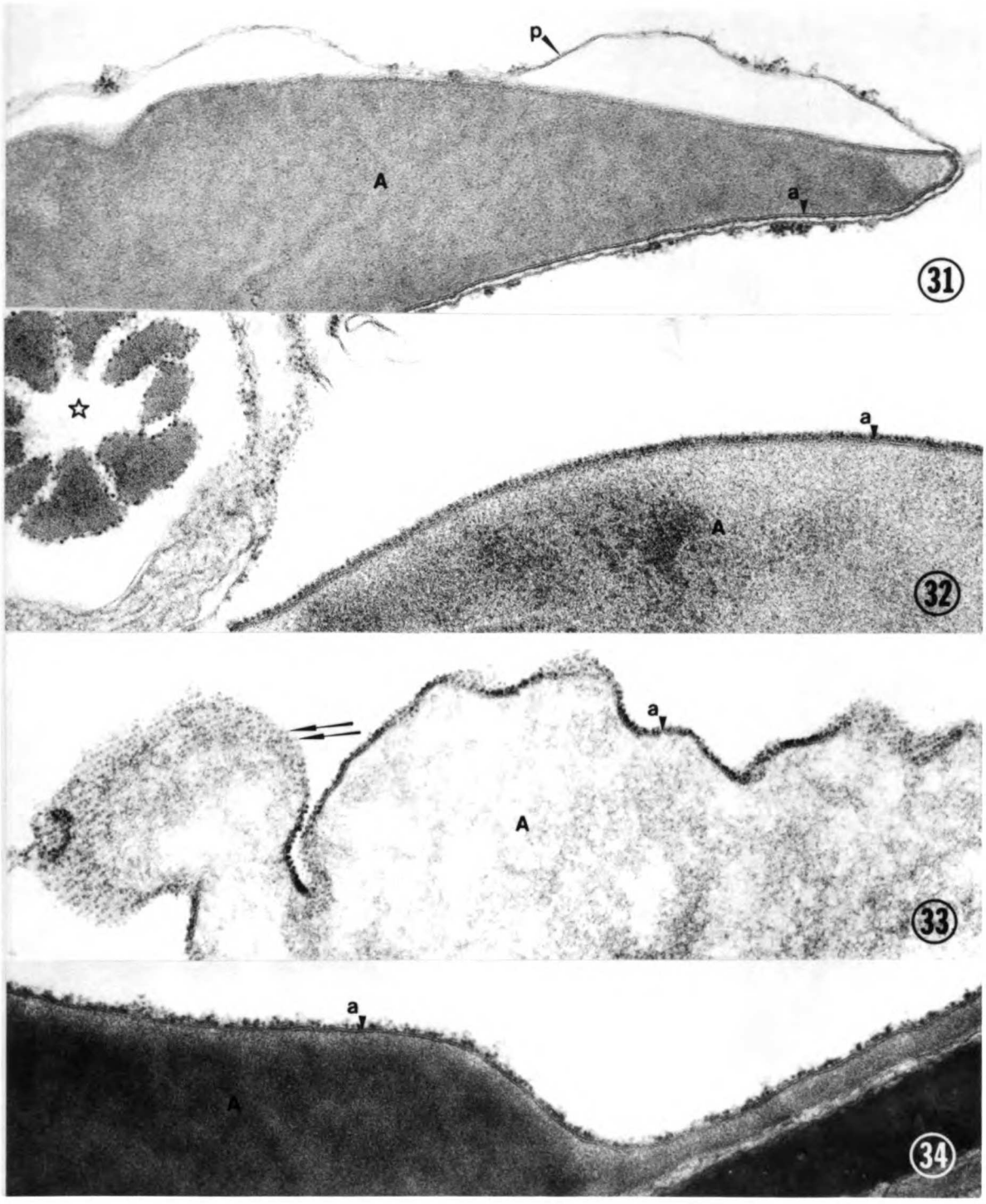
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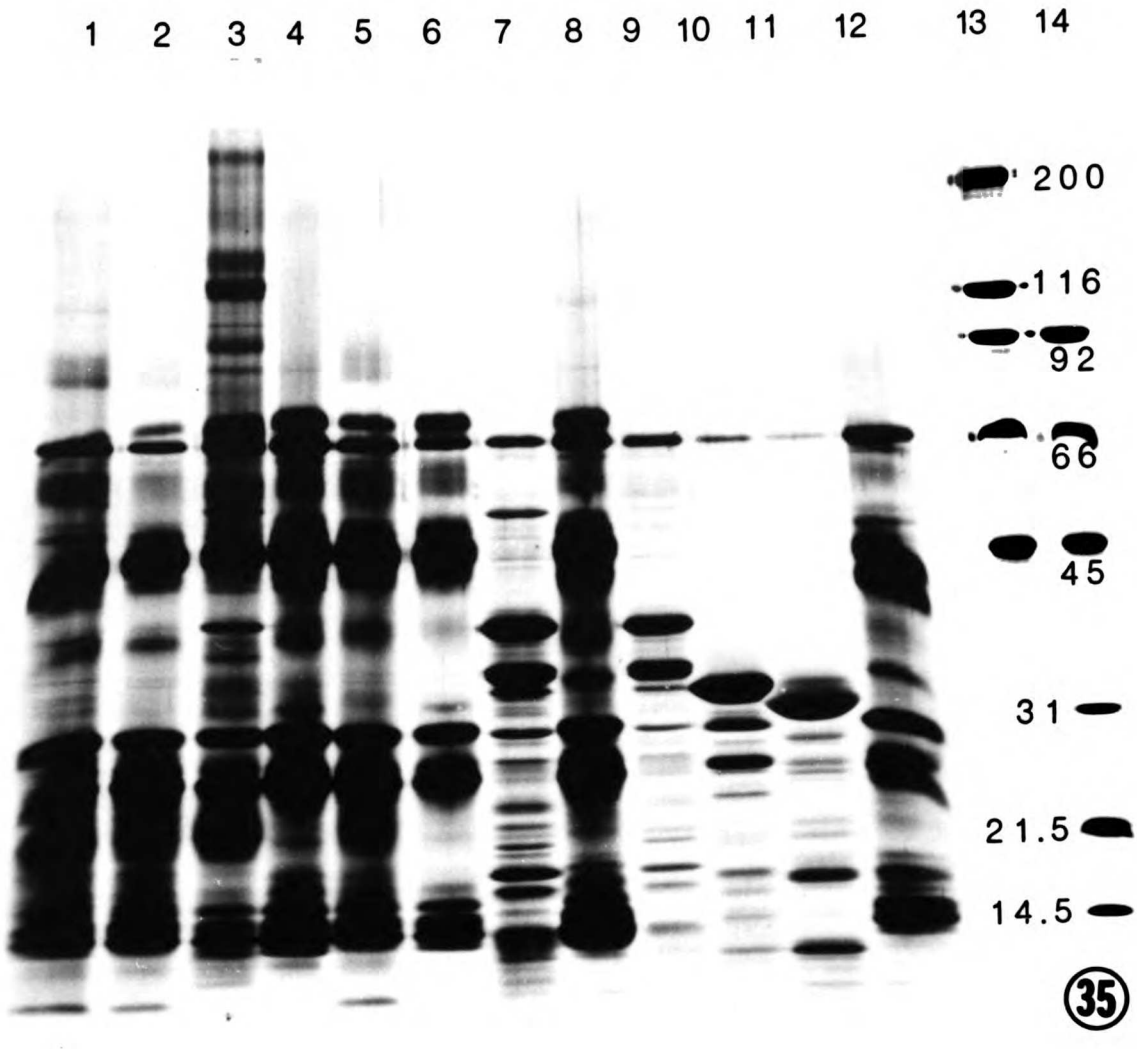
Fig. 35. SDS/polyacrylamide gel of the supernatants from Polytron-disrupted sperm incubated with lytic enzymes. Results from two separate experiments (lanes 1-8, and lanes 9-12) are shown. Within both experiments each lane represents the supernatant of an equal quantity of sperm exposed to the following:

lane 1: aryl-sulfatase (IV), (pH 5)
lane 2: aryl-sulfatase (IV) and sulfatase inhibitor (Sigma, p-nitrocatechol sulfate, Cat # N 7251), (pH 5)
lane 3: aryl-sulfatase (H-I), (pH 5)
lane 4: no enzyme added, pH 5 control for lanes 1-3 (Na acetate buffer)
lane 5: β-glucuronidase, (pH 4)
lane 6: no enzyme added, (pH 4), control for lane 5 (Na acetate buffer)
lane 7: pronase $E_{(II)}$, (pH 7.6)
lane 8: no enzyme added, pH 7.6 control for lane 7 (Ca^{2+}-free MCM)
lane 9: pronase $E_{(I)}$ (pH 7.6)
lane 10: proteinase K (pH 7.6)
lane 11: subtilopeptidase A (pH 7.6)
lane 12: no enzyme added, pH 7.6 control for lanes 9-11 (Ca^{2+}-free MCM)
lane 13: Biorad high molecular weight standards (in kilodaltons)
lane 14: Biorad low molecular weight standards (in kilodaltons)

The results show that the addition of aryl-sulfatases and β-glucuronidase did not alter the electrophoretic mobility of the soluble sperm components (compare lanes 1,2,3 with lane 4; lane 5 with lane 6). It is also evident that the pH at which the sperm were incubated did not alter the electrophoretic pattern (compare lanes 4,6, and 8). The proteolytic enzymes were effective at reducing the numer of high molecular weight components (compare lane 7 with lane 8, lanes 9,10,11 with lane 12).


1 2 3 4 5 6 7 8 9 10 11 12 13 14
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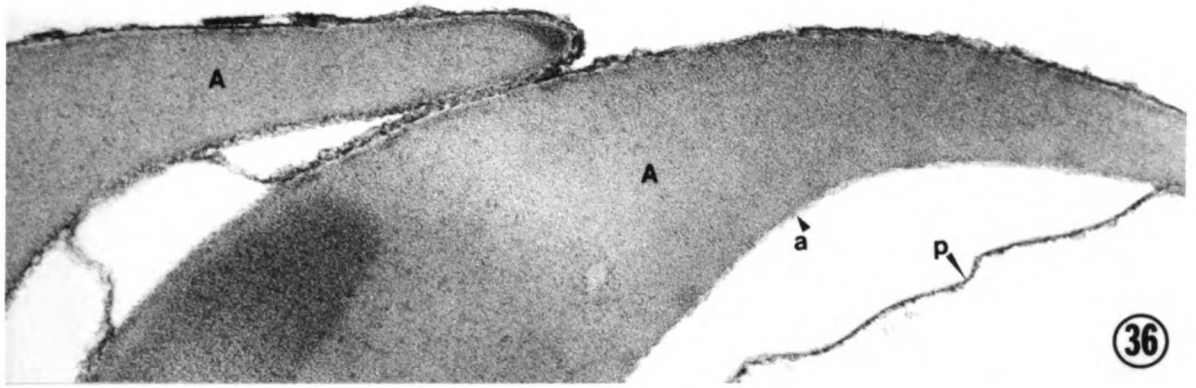
35

Figs. 36 and 37. Fixed sperm that were extracted with chloroform:methanol:water (1:2:0.8) prior to RR staining. The RR staining is reduced compared to sperm which were never exposed to lipid solvents (see Fig. 2). Due to the lipid solvent treatment, distinct bilayers are not visible. When the plasma membrane separates from the acrosomal membrane much of the RR staining remains associated with the plasma membrane. X 71,050.



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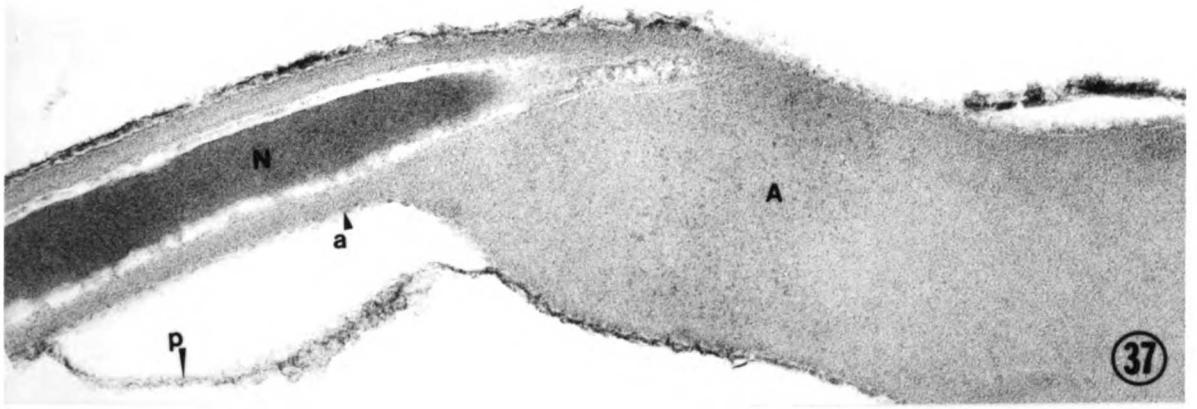

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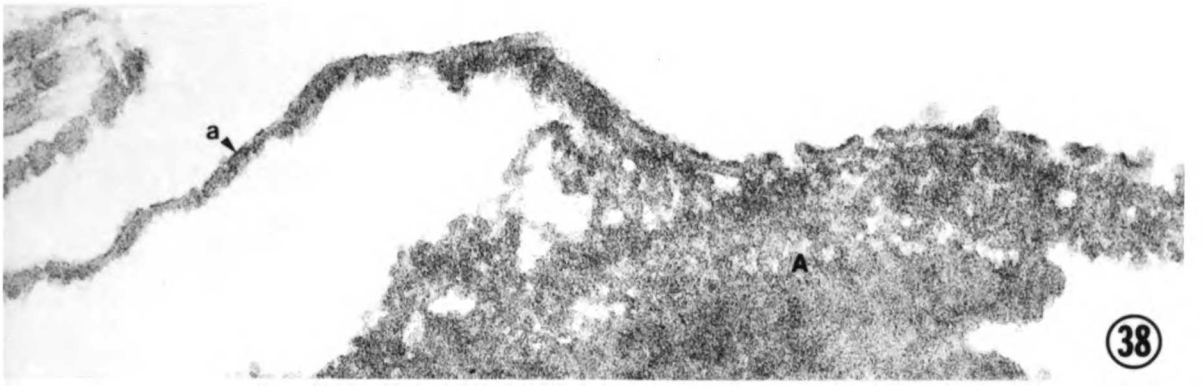
Fig. 38. Polytron-disrupted sperm that were fixed and extracted with chloroform:methanol:water prior to RR staining. Remnants of the acrosomal membrane stain lightly with RR. X 71,050.

Fig. 39. Polytron-disrupted sperm that were fixed and extracted with acetone prior to RR staining. Acetone extraction was less effective than chloroform:methanol:water at inhibiting RR staining (See Fig. 38). X 71,050.

Fig. 40. Polytron-disrupted sperm which were fixed and extracted with chloroform:methanol:water prior to CF labeling. CF binds to both the extracted acrosomal membrane and exposed acrosomal contents. X 71,050.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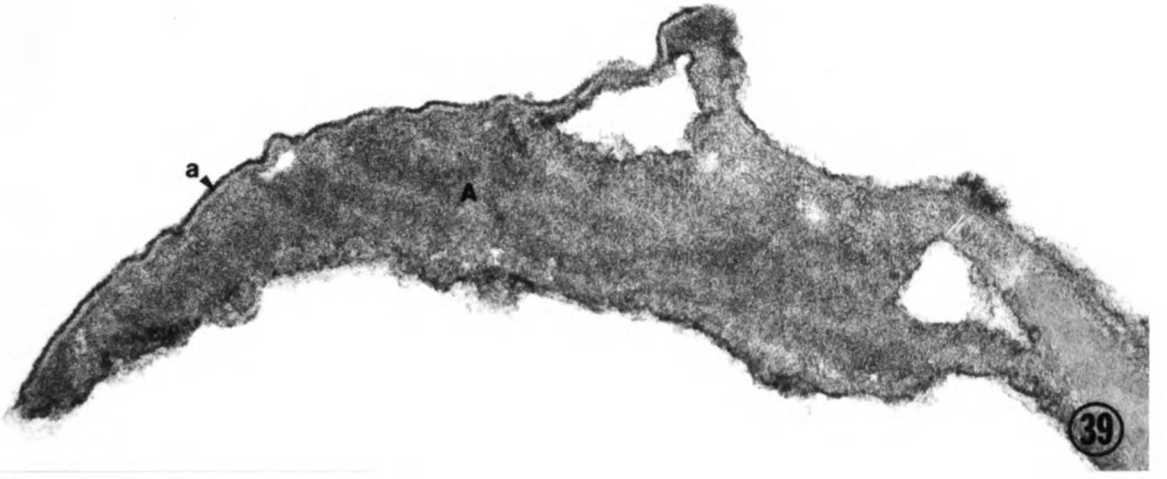
38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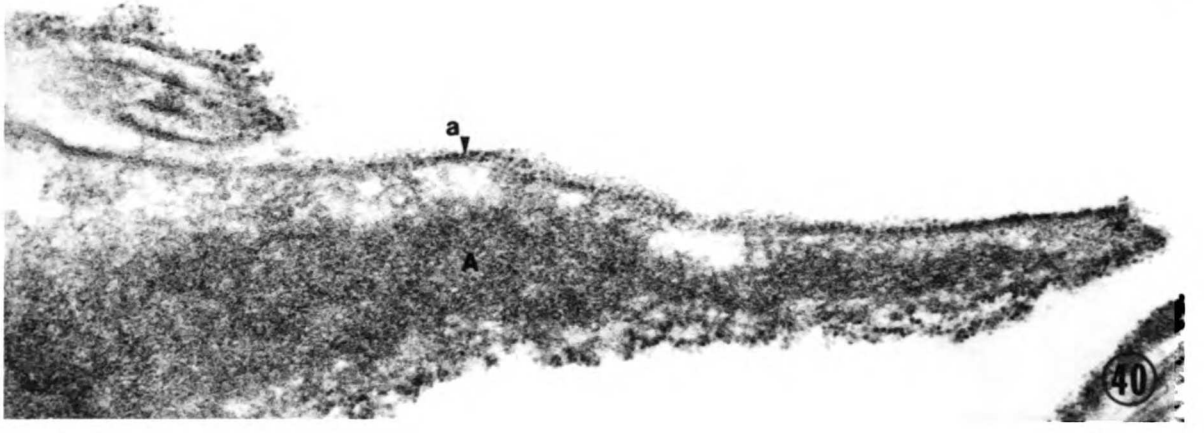
39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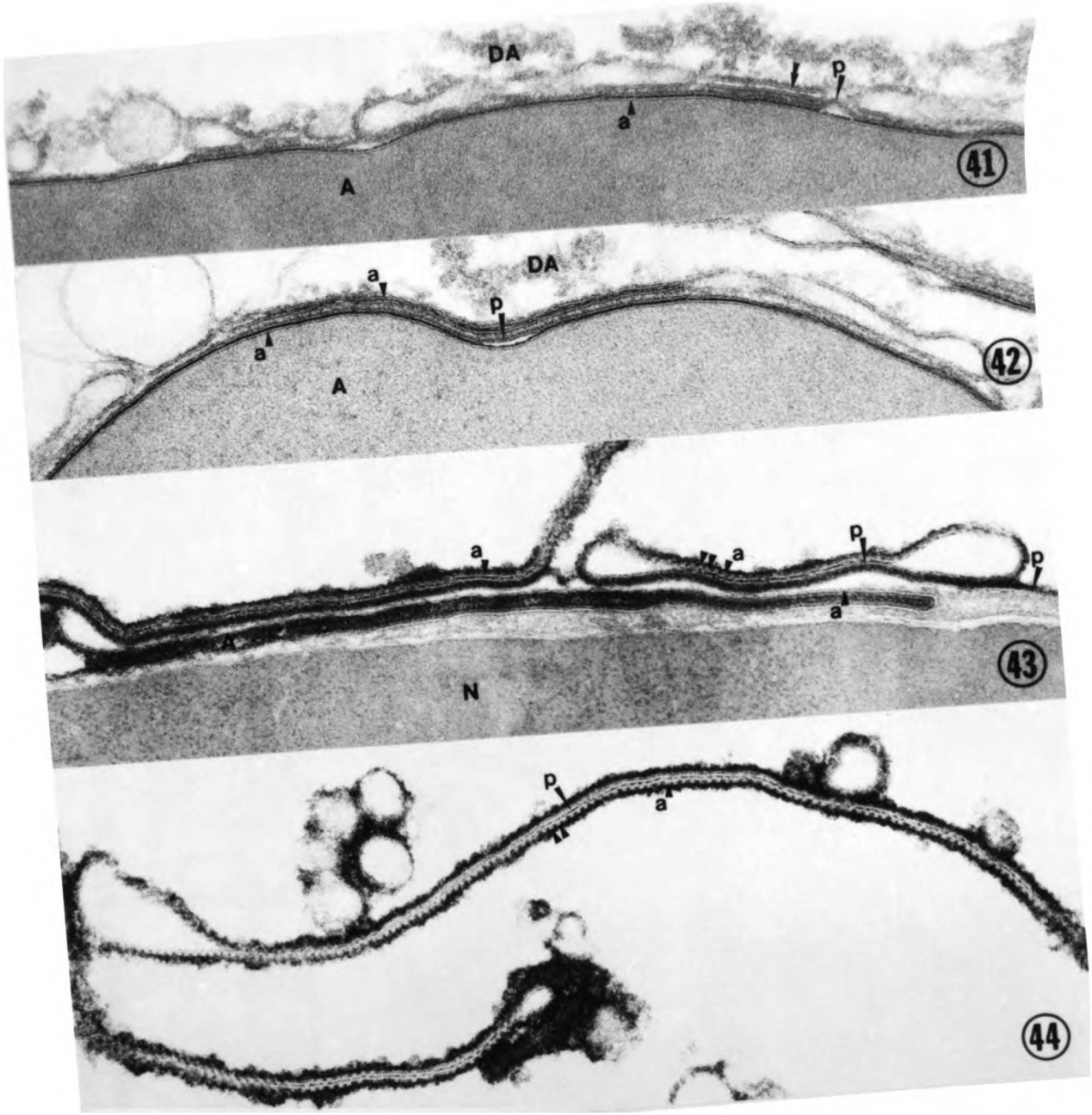
40

Figs. 41-44. Thin sections of sperm capacitated in A_2C and exposed to 2 mM Ca^{2+}. X 71,050. Figs. 41 and 42: In the non-acrosome reacted sperm, a heavy electron dense deposit is present between the acrosomal and plasma membranes. When the plasma membrane separates from the outer acrosomal membrane (at the P-arrowheads), the heavy electron dense staining remains with the acrosomal membrane. In adjacent acrosome-reacted sperm, the plasma and acrosomal membranes have fused at multiple foci and formed vesicles. The fused vesicles do not have a heavy electron dense deposit on their inner surfaces. Even when the fused vesicles are flattened against the non-acrosome reacted sperm (arrow, Fig. 29), they do not have the heavy electron dense deposit on their newly created inner surface that is present between the membranes of non-acrosome reacted sperm. DA, dispersed acrosomal material from acrosome reacted sperm. Fig. 43: Thin section across the equatorial region of a sperm which has undergone a partial acrosome reaction, with remnants from an adjacent sperm which has not vesiculated. The acrosomal contents and the intercellular space stain with RR. In the sperm which has undergone a partial acrosome reaction there is light staining between the plasma and acrosomal membranes. Within the remnants of an adjacent sperm, when the plasma membrane and acrosomal membranes remain apposed to each other, the RR staining appears between the apposed membranes. A periodic electron dense and electron lucent staining is present on the E-surface of the acrosomal membrane (arrowheads) in some regions. Fig. 44: Remnants of acrosome reacted sperm which have not vesiculated. Note the electron dense and electron lucent regions on the E-surface of the acrosomal membrane. The plasma membrane generally remains uniformly stained. Occasionally, alternating electron dense and lucent staining is seen on both of the apposing membranes.


DA
a
p
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41
DA
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A
42
a
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N
43
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a
44

Table 2

Summary of the effects of alterations in the conditions of ruthenium red staining

Membrane and Surface		Conditions of exposure									
		RR	0.4 M NaCl RR	0.6M NaCl RR	0.9M NaCl RR	1.3 M NaCl RR	2.6 M NaCl RR	pH 3 RR	1.3 M sucrose RR	Nakane Fix RR	Tannic Acid RR
Plasma Membrane	E	++	++	+	+/-	-	-	-	++	++	+++
	P	+	+	+/-	-	-	-	-	+	+	++
Acrosomal Membrane	P	+++	+++	+++	++	+	+	+/-	+++	+++	+++
	E	Contents of the acrosome lay adjacent to this membrane surface									

\- little or no increase in electron density above unstained preparations

\+ slight electron dense deposits

++ moderate electron dense deposits

+++ heavy electron dense deposits

Table 3

Summary of the effects of alterations in the conditions of ferritin exposure

Membrane and Surface		Conditions of exposure										
		Native Ferritin	CF	Pre-fix. CF	0.2 M NaCl CF	Pre-fix. 0.2 M NaCl	0.4 M NaCl CF	Pre-fix. 0.4 M NaCl	0.6-0.9 M NaCl CF	pH 5 CF	pH 4 CF	pH 3 CF
Plasma Membrane	E	-	++	++	+/-	+	-	-/+	-	+/++	+	-
	P	-	+	++	-	-/+	-	-	-	+	-/+	-
Acrosome Membrane	P	+	+++	+++	+++	+++	+/++	++	-/+	+++	++	-/+
	E	-	-/+	++*	-/+	-/+	-	-	-	-/+	-/+	-

\- little or no binding

\+ low density binding

++ high density binding

+++ very high density binding

* may include binding to acrosomal contents

DISCUSSION

Background

Cationic probes, such as RR, CF, and colloidal iron hydroxide have been used to detect anionic sites on the external surface of various cell membranes (Luft, 1971b; Danon et al., 1972; Skutelsky and Danon, 1976; King and Preston, 1977; Simionescu et al., 1981a) and on extracellular basement membranes (Kanwar and Farquhar, 1979a; Vaccaro and Brody, 1979; 1981). Anionic sites have been attributed to sialic acid of glucoconjugates on cell surfaces (Nicolson, 1973; Luft, 1976; Simionescu et al., 1981b) and glycosaminoglycans in extracellular basement membranes (Kanwar and Farquhar, 1979; Vaccaro and Brody, 1981; Charonis et al., 1983). Insight into the biochemical nature of the anionic sites was obtained through the use of selective lytic enzymes, with the cationic probes detecting the effectiveness with which the enzymes removed the anionic sites (Kanwar and Farquhar, 1979b; Vaccaro and Brody, 1981; Simionescu et al., 1981b). Reports of the detection of anionic sites on the cytoplasmic surface of organelles has been limited in comparison. Hackenbrock and Miller (1975) reported a random CF-binding distribution on the cytoplasmic surface of outer mitochondrial membranes within a mitochondrial fraction isolated from rat livers. Within a Golgi fraction from rat livers, Abe et al. (1976) found preferential CF labeling of small vesicles and associated tubular network, rather than central regions of cisternae or secretory granules.

The anionic sites of the cytoplasmic surfaces of membranes are probably not due to either carbohydrates or glycosaminoglycans, because of the manner in which membrane biosynthesis occurs. It is currently

acknowledged that much of membrane asymmetry is established during membrane biogenesis. The glycosyltransferases involved in glycolipid, glycoprotein, and glycosaminoglycans synthesis are membrane-bound within the luminal surface of the endoplasmic reticulum, Golgi apparatus, or on the external surface of the plasma membrane (Dawson, 1978; Struck and Lennarz, 1980; Wagh and Bahl, 1981). In biological systems which have been extensively studied such as the erythrocyte membrane, all carbohydrate of both glycoproteins and glycolipids, is located on the external surface (Steck, 1974; Marchesi et al., 1976; Rothman and Lenard, 1977). The surface anionic charge of erythrocytes is largely due to sialic acid (Nicolson, 1973; Pinto da Silva et al., 1973) and this property has been used to separate "rightside-out" from "inside-out" vesicles of erythrocyte membrane vesicles (Heidrich and Leutner, 1974). In contrast, the anionic sites on the cytoplasmic surface of membranes are probably not due to carbohydrate, but rather lipid or protein.

Cationic Ferritin (CF) and Ruthenium (RR) Results

The results of the two cationic probes agreed with each other with respect to the degree to which they labeled the P- and E-surfaces of the plasma and outer acrosomal membranes. Both probes revealed a dense concentration of anionic sites on the P-surface of the outer acrosomal membrane. The salt concentrations required to compete with either RR or CF binding to the outer acrosomal membrane were similar to those obtained in previous work in other systems (Kanwar and Farquhar, 1979a; Charonis and Wissig, 1983). A salt concentration of 1.3 M NaCl was required to displace RR from binding sites within continuous capillary basement membrane (Charonis and Wissig, 1983). These sites have been tentatively

identified immunocytochemically as heparin sulfate (Charonis et al., 1983). In addition, 0.4 - 0.5 M KCl or pH 3 displaced CF binding to heparin sulfate within the glomerular basement membrane (Kanwar and Farquhar, 1979a,b). Since similar conditions were needed to displace the cationic probes from the P-surface of the outer acrosomal membrane, anionic sites of similar charge density are likely present.

Fixation Effects

Although there was strong CF binding to the unfixed as well as to prefixed P-surface of the outer acrosomal membrane, binding to the P- and E-surfaces of the plasma membrane, and E-surface of the acrosomal membrane was enhanced by prefixation. This increase in CF binding after prefixation is consistent with findings from other workers (Grinnell et al., 1976; Wessells et al., 1976; Burry and Wood, 1979). That this enhanced CF binding could be inhibited by increased salt concentrations suggests that the CF was not being covalently bound to unreacted aldehyde groups. Rather, aldehyde fixation more likely increased the density of available anionic sites by eliminating positively charged amino groups of proteins and/or lipids through the formation of Schiff's bases or by causing conformational changes which expose additional anionic sites (Burry and Wood, 1979). Prefixation enhancement of CF binding to phospholipid vesicles (specifically phosphatidyl ethanolamine) and protein (BSA) has been previously reported (Burry and Wood, 1979).

Separation of the Plasma Membrane from the Acrosomal Membrane

Separation of the plasma membrane from the outer acrosomal membrane of caudal epididymal or ejaculated sperm has long been observed in a

variety of species and fixatives (Bedford, 1965; Fawcett and Phillips, 1969; Phillips, 1975; Peterson et al., 1978; Russell et al., 1979). We doubt that the separation of the plasma membrane from the outer acrosomal membrane represents a physiological change, but rather an artifact induced by the fixation procedure, and we offer the following hypothesis. There is apparently little material present between the two membranes which allows glutaraldehyde to cross-link the membranes together, because subsequent treatments that do not disrupt covalent bonds can result in increased separation of membranes. Because high salt, low pH, and to a lesser extent, incubation overnight in the secondary fixative, all seem to induce separation of the plasma membrane from the acrosomal membrane of aldehyde fixed sperm, this suggests to us that ionic interactions may help hold the fixed membranes together. Our results show that the P-surface of the acrosomal membrane has a high density of anionic sites, whereas the P-surface of the plasma membrane contains a lower negative charge density. Fixation increased the CF binding to the P-surface of the plasma membrane, suggesting an increase in available anionic sites. Thus, this increase in the number of negatively charged sites on the plasma membrane may reduce an ionic interaction that holds these two membranes together (perhaps directly or through intermediate molecules). For example, within biological systems studied to date, amino lipids (phosphatidyl ethanolamine, generally neutral, and phosphatidyl serine, generally negatively charged) tends to be concentrated on the cytoplasmic surface of membranes (Op den Kamp, 1979; Alsteil and Branton, 1983). Glutaraldehyde has been shown to react with amino lipids (Roozemand, 1969; Wood, 1973; Nir and Hall, 1974), eliminating the positive charge of

the amino group (Burry and Wood, 1979). Further experimentation will be necessary to test our hypothesis.

It is apparent that stabilization of the plasma membrane by either RR (through its binding to the external surface), or tannic acid and RR may override interactions (ionic) which would otherwise allow separation. Increased separation of the two membranes only occurs when there is no extensive stabilization of the plasma membrane.

The inability of added NaCl to inhibit RR staining of tannic acid fixed sperm is not surprising. Tannic acid is known to act as a mordant between fixed biological structures and heavy metals (Simionescu and Simionescu, 1976a,b). It appears that tannic acid may also act as a mordant for RR, thereby stabilizing the membrane.

Nature of the Anionic Sites on the P-Surface of the Outer Acrosomal Membrane

As mentioned, anionic sites on the P-surface of the acrosomal membrane are likely due to lipids and/or proteins. Such anionic sites may be due to carboxyl, phosphate (Kerbs and Beavo, 1979), or sulfate (Huttner, 1982) groups.

Although peripheral membrane proteins might be present, and calmodulin has been proposed to be present between the plasma and acrosomal membranes (Jones et al., 1980), our results suggest that the anionic sites are not due to calmodulin. Calmodulin is acidic (pI 3.9), binds four Ca^{2+}, and has been localized in the acrosomal region of guinea-pig sperm using immunofluorescence (Dedman et al., 1977; Jones et al., 1980). However, the results of our salt-desalt experiment suggests that if a cytoplasmic protein is responsible for the anionic sites, it is

intimately associated with the membrane and is not removed by exposure to either high salt (0.6 M) or acidic pH (pH 3, citrate/phosphate buffer). In addition, none of the proteolytic enzymes under the conditions used inhibited CF binding to the P-surface of the outer acrosomal membrane, suggesting that either the anionic sites are not protein, or that if they are, they are inaccessible to proteolytic enzymes.

The extraction of fixed sperm with lipid solvents inhibited much of the RR staining of the P-surface of the acrosomal membrane, suggesting lipids are responsible for at least some of the anionic sites. Acetone does not solubilize charged phospholipids as readily as chloroform:methanol:water (Christie, 1982). Thus, our result that acetone was less effective at inhibiting subsequent RR staining is not surprising. The anionic sites might, of course, reside in proteins which are not cross-linked by glutaraldehyde and thus, can be subsequently extracted with lipid solvents or during the rehydration. Future work will be necessary to eliminate this possibility.

If anionic lipids are responsible for at least a portion of the P-surface anionic sites, it raises some interesting biological questions. What anionic lipids might be involved? For example, the anionic lipid phosphatidyl serine is also an amino lipid which may be cross-linked by glutaraldehyde, suggesting that it might not be extracted following glutaraldehyde fixation. However, there are other anionic lipids such as phosphatidic acid, cholesterol sulfate, desmosterol sulfate, and polyphosphatidylinositol which are not cross-linked by glutaraldehyde. Preliminary work by Friend (1982b) indicates that polyphosphatidylinositol may be present on the P-surface of the outer acrosomal membrane. What is responsible for maintaining the lipid

asymmetry across the acrosomal membrane? Within other biological systems such as the red blood cell and the blood platelet, cytoskeletal components have been postulated to serve in maintaining the asymmetric distribution of anionic lipids across the bilayer (Haest et al., 1978; Bearer, 1982). Because asymmetric CF labeling of coiled segments of the outer acrosomal membrane remains despite a 30 min pre-exposure to proteinases at 37°C, we doubt that cytoskeletal components are needed to actively maintain anionic site asymmetry. Rather, we suggest that the integral membrane proteins (visible as intramembrane particles) are responsible for maintaining anionic asymmetry either by maintaining "shells" of anionic lipids around themselves, or by providing the anionic sites which are inaccessible to protease digestion.

Biological Implications

The presence of a dense concentration of anionic sites on the P-surface of the acrosomal membrane has functional implications. In addition to serving to help hold the plasma membrane to the outer acrosomal membrane, the fixed outer acrosomal membrane binds Ca^{2+}, as suggested by Roomans (1975) and Friend (1977). Using potassium-pyroantimonate to localize high cation concentrations, Friend reported pyroantimonate deposits on the P-surface of the acrosomal membrane, with dense concentrations at the tip of the acrosome and near the tip of the nucleus. Our localization of anionic sites was uniform throughout the P-surface of the outer acrosomal membrane. When Roomans added 60 mM Ca^{2+} to his fixative, the electron density and thickness of the outer acrosomal membrane of human sperm increased, while the electron density of the plasma membrane remained essentially unchanged. This increase in

thickness was aysmmetric, with more of the increase occurring on the P-surface than on the E-surface. Mg^{2+} did not produce a similar increase in thickness, while strontium (Sr^{2+}) mimicked the Ca^{2+} effect. Interestingly, Yanagimachi and Usui (1974) have found that both Ca^{2+} and Sr^{2+} can stimulate the guinea-pig acrosome reaction, while Mg^{2+} can not. Roomans suggested that Ca^{2+} may bind to lipids of the outer acrosomal membrane. Our work demonstrates the presence of a dense concentration of anionic sites on the P-surface of the acrosomal membrane. These sites may be the targets for Ca^{2+} in the induction of membrane fusion.

The Acrosome Reaction of A_2C-Capacitated Sperm

The alternating electron dense and lucent ridges that appeared in many of the acrosome reacted sperm on the E-surface of the acrosomal membrane were not observed in nonacrosome reacted sperm. While it is possible that these ridges are A_2C induced artifacts, we suggest that they are not due to the presence of A_2C. Somewhat similar structures have been reported in boar sperm after A23187 and Ca^{2+} induced acrosome reactions (Peterson et al., 1978; Russell et al., 1979), but were described as "microfilaments," and were present on the plasma membrane. While there may be species differences, we interpret these structures (within the guinea-pig sperm) as occurring on the acrosomal membrane, since they have a periodicity similar to the intramembrane particles present in freeze-fracture of the acrosomal membrane. Further work will be required to determine their nature.

The marked decrease in RR staining after fusion of the plasma membrane with the outer acrosomal membrane is intriguing. There are

several possible explanations: 1) Upon fusing, an intermixing of some of the components of the plasma and acrosomal membranes may occur, resulting in a rearrangement (dilution) of anionic sites; 2) upon fusion, the permeability of the membranes to RR may decrease, thus limiting the access of RR to anionic sites; 3) Ca^{2+} may activate an enzyme responsible for the cleavage of anionic sites on the P-surface of the acrosomal membrane. This enzymatic cleavage may be responsible for both changing the anionic and fusigenic properties of the acrosomal membrane (Llanos et al., 1982); 4) finally, the influx of Ca^{2+} across the plasma membrane may bind to anionic sites on the acrosomal membrane, thus inhibiting subsequent RR binding, although the presence or absence of 2 mM Ca^{2+} in the primary and secondary fixatives had no effect on the RR deposition within non-acrosome reacted sperm (data not illustrated).

FUTURE DIRECTIONS

With the dense concentration of anionic sites on the P-surface of the outer acrosomal membrane convincingly established, further efforts should be placed on clarifying the biochemical nature of the anionic sites and understanding their role in membrane fusion.

Our work using lipid solvents suggests that anionic lipids are responsible for at least a portion of the anionic sites; however, further work is needed to support this contention. A first step should include the digestion of Polytron-disrupted sperm with phospholipases prior to CF labeling. Phospholipases vary in their phospholipid specificity and their ability to cleave phospholipids within bilayers without disrupting the integrity of the bilayer (Chap et al., 1979).

Biochemical analysis of the anionic sites would be greatly aided by the refinement of procedures for the isolation of a purified acrosomal membrane fraction. Preliminary reports of a procedure to isolate acrosomal membranes have been published (Winer and Olson, 1982), but attempts to follow the outlined procedure were only moderately successful.

The isolation of an acrosomal membrane fraction would allow both lipid and protein analysis of the acrosomal membrane components, without contamination from other sperm components. This isolation of an acrosomal fraction would also allow harsher and longer exposure to lytic enzymes since long exposures to lytic enzymes often released the acrosomal membrane from the nuclear envelope, thus resulting in loss of the means by which the acrosomal membrane is identified when acrosomal contents are extracted.

Future efforts should also include experimentation designed to further our understanding of the role of the anionic sites of the acrosomal membrane. Since within reacted sperm the acrosomal membrane loses the ability to bind RR, the effect of adding Ca^{2+} to either washed or unwashed Polytron-disrupted sperm may help distinguish if Ca^{2+} is simply binding to anionic sites and preventing subsequent cationic probe binding, or whether endogenous lytic enzymes activated by Ca^{2+} cleave the anionic sites. The disruption of sperm by Polytron treatment allows access to the P-surface of both the plasma and acrosomal membranes. The addition of gold-labeled liposomes of varying lipid composition (Hong et al., 1983) to Polytron-disrupted sperm should allow tests of the fusigenicity of each membrane upon the addition of Ca^{2+}.

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