Purification and Characterization of Two Plasma Membrane Domains from Ejaculated Bull Spermatozoa

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Abstract. Plasma membranes were detached from ejaculated bull spermatozoa by a brief sonication in a moderately hypotonic medium, and the released plasma membranes were partially purified by differential centrifugation. The resulting fraction was enriched 8- and 15-fold in alkaline phosphatase and 5′ nucleotidase activities, respectively, compared with the starting sonicated spermatozoa. This total plasma membrane fraction was separated into two distinct fractions by equilibrium density centrifugation on a continuous linear sucrose gradient. Two peaks of light scattering material were formed at densities of 1.117 and 1.148 g/ml. The denser peak contained most of the protein of the plasma membrane fraction, whereas nearly all the concanavalin A binding activity was found in the lighter peak. The two bands had distinctly different polypeptide compositions when analyzed by SDS PAGE. Polyclonal antibodies were raised in rabbits against a major integral membrane glycoprotein of each fraction (M_r of 92,000 in the light peak and 98,000 in the dense peak). The two antigens were detected on the surface of intact spermatozoa by indirect immunofluorescence microscopy. The 92-kD protein (present in the lighter band) was detected only on the plasma membrane of the acrosomal and anterior postacrosomal regions of the head. The 98-kD antigen, present in the heavier band, was localized to the surface of the postacrosomal region of the head, to the principal piece of the tail, and to the connecting piece between the head and tail. The exclusive localization of the 92-kD polypeptide to the surface of the anterior portion of the head was confirmed by immunoelectron microscopy. These data show that the two fractions isolated on the sucrose gradient originate from different regions of the sperm cell plasma membrane.

The mammalian spermatozoon is a highly polarized cell that has several distinct morphological regions. Within the head, two distinct portions are represented by the acrosomal and postacrosomal regions, whereas the tail is characterized by an anterior portion, which contains mitochondria (the midpiece), and a posterior region, which comprises the principal and end pieces (for a review, see Fawcett, 1975). A corresponding regional diversification of the sperm cell surface has been revealed by a variety of techniques, including freeze-fracture (Friend, 1982), lectin binding (Kinsey and Koehler, 1976; Nicolson et al., 1977; Koehler, 1978; Aguas and da Silva, 1983), lipid probe binding (Bearer and Friend, 1982), and immunolocalization (Myles et al., 1981; Gaunt et al., 1983; Primakoff and Myles, 1983; Naz et al., 1984).

Although the presence of different domains on the surface of the mammalian sperm cell is well established, the degree of compositional overlap between them is not known. To understand how these domains are generated and maintained, it is important to know the composition of the different domains.

Plasma membrane (PM)[1]–enriched fractions from mammalian spermatozoa have been obtained and characterized (Gillis et al., 1978; Peterson et al., 1980; Noland et al., 1983; Russell et al., 1983). However, the separation and compositional analysis of the different domains has not been reported so far. In this study, we report the separation from ejaculated bull spermatozoa of two membrane fractions that contain vesicles with distinct ultrastructure and polypeptide composition. Major polypeptides of each fraction are localized within different regions of the cell surface of intact sperm, which indicates that the fractions contain membranes that originate from different domains of the spermatozoon surface.

Materials and Methods

Materials

The following reagents were purchased from the following sources: Hanks' solution, Difco Laboratories Inc., Detroit, MI; iodoacetamide, DL-dithiothreitol, benzamidine, β-glycerophosphate, wheat germ agglutinin, concanavalin A (Con A), *N*-benzoyl-L-arginineethylester-HCl, *p*-nitrocatecholsulfate, poly-L-lysine, L-cysteine sulfinic acid, 2,6-dichlorophenol-indophenol, phenazine methosulfate,

1. *Abbreviations used in this paper:* Con A, concanavalin A; EM, electron microscopy; HTB, hypotonic Tris buffer; OAM, outer acrosomal membrane; P92, 92-kD polypeptide; P98, 98-kD polypeptide; PM, plasma membrane; TBS, Tris-buffered saline; TS, total sonicate.

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Triton X-100, AMP sodium salt, barium hydroxide, 1-0-methyl-α-D-glucopyranoside, and polyvinylpyrrolidone-360, Sigma Chemical Co., St. Louis, MO; Percoll, protein A, Sephadex G-50 medium, and Ficoll-400, Pharmacia Fine Chemicals, Uppsala, Sweden; sucrose (RNAse-free), protein assay kit, Coomassie Brilliant Blue R-250, low molecular weight standards for SDS PAGE, and Biogel-P2, Bio-Rad Laboratories, Richmond, CA; p-nitrophenylphosphate, BDH Chemicals Ltd., Poole, England; ^{3}H-AMP ammonium salt and Na^{125}I, Amersham International plc, Amersham, England; N-acetylglucosamine, P-L Biochemicals, Inc., Milwaukee, WI; nitrocellulose paper (type HAHY), Millipore Corp., Bedford, MA; bovine serum albumin (BSA, fraction V), Boehringer Mannheim GmbH, Mannheim, FRG; radioactive low molecular weight standards for SDS PAGE, New England Nuclear, Langen, FRG; goat anti-rabbit rhodaminated IgG, Cappel Laboratories, Cochranville, PA.

Cell Preparation

Fresh ejaculates from fertile bulls were kindly supplied by the "Centro per il potenziamento zootecnico" (Zorlesco, Milan). Sperm concentration, motility, and viability were checked by technicians of the Center, and ejaculates with <40% motile sperm cells were discarded. Ejaculates were kept in a thermos bottle until use. Experiments were always initiated within 3 h after ejaculation.

At the beginning of the experiment, the temperature of the ejaculates was slowly lowered to 4°C in the cold room, and all subsequent operations were done in the cold, unless otherwise specified. Ejaculates were diluted in Hanks' solution to obtain a sperm concentration of 5–8 × 10^6 cell/ml. A self-generating Percoll gradient was used to separate viable spermatozoa from cytoplasmic droplets and soluble seminal plasma components. The gradient was obtained by centrifugation, for 30 min at 21,000 g_{max} in a Beckman 50.2 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA), of a mixture that contained 4 ml of sperm suspension in Hanks' and 20 ml of a Percoll solution (9 parts Percoll + 1 part of 10× phosphate-buffered saline [PBS], pH 7.4). Two peaks were obtained: the less dense one contained cytoplasmic droplets, broken spermatozoa, and cellular debris, whereas the denser one was highly enriched (>99%) in viable spermatozoa, as checked by phase-contrast light microscopy. The latter fraction was used for cell fractionation experiments.

Cell Disruption and Fractionation

Spermatozoa obtained from the Percoll gradient were pelleted away from Percoll by dilution with Hanks' solution and centrifugation (at 350 g_{max}, 20 min), followed by two washes with Tris-buffered saline, pH 7.4 (TBS). The final pellets were resuspended in hypotonic Tris buffer (HTB; 34 mM NaCl, 0.1 mM KCl, 5 mM benzamidine, 1 mM EDTA, 2.5 mM Tris-HCl, pH 7.4) to a final protein concentration of 8–16 mg/ml. 2-ml aliquots of the cell suspension were sonicated for 3 s on ice with a Branson B-15 sonifier with a microtip (Branson Sonic Power Co., Danbury, CT) at a power of 50 W. The resulting total sonicate (TS) was centrifuged for 10 min at 1,000 g_{max}. The pellet (low speed pellet) was saved for biochemical and morphological analyses, and the supernate was the starting material for plasma membrane purification. 1.3-ml aliquots of the supernate were centrifuged for 10 min at 6,000 g_{max} in the Sorvall SA-600 rotor (DuPont Co., Wilmington, DE) to sediment sperm tail fragments. The pellets were resuspended in HTB and centrifuged for 15 min at 6,000 g_{max}. The pellets (6,000 g pellets) were saved, and the supernates, which resulted from the 2× 6,000 g centrifugation, were pooled and centrifuged for 40 min at 100,000 g_{max} in the Beckman 50 Ti rotor. The supernate was decanted, and the membrane pellet (PM) was resuspended by gentle homogenization with a Dounce homogenizer in 0.25 M sucrose, 1 mM EDTA, 5 mM benzamidine, 3 mM imidazole-HCl, pH 7.0, to a protein concentration of 1–2 mg/ml.

For further subfractionation, 1.5 ml of the PM suspension (containing ~3 mg protein) was layered over a 16.5-ml continuous linear sucrose density gradient (0.5–1.8 M sucrose) that contained 1 mM EDTA, 3 mM imidazole-HCl, pH 7.0, and was centrifuged overnight in the Beckman SW 27.1 rotor at 24,000 rpm. 1.3-ml fractions were collected with an Auto Densiflow probe (Buchler Instruments Inc., Fort Lee, NJ) connected to a peristaltic pump.

For protein and enzyme assays, gradient fractions were analyzed directly. For SDS PAGE, they were first diluted with 3 vol of water, sedimented into pellets (45,000 rpm, 1 h, Beckman 50 Ti rotor), and then resuspended in small volumes of water.

Carbonate Procedure

Peripheral membrane proteins were extracted from vesicles of the PM fraction by incubation in 100 mM Na_2CO_3, pH 11 (Fujiki et al., 1982). The membrane vesicles were separated from the released proteins by centrifugation (1 h at 230,000 g_{max}).

Biochemical Assays

Protein determinations were done according to Bradford (1976) using the Bio-Rad protein assay kit.

Enzyme activities were measured on freshly prepared fractions, or on fractions stored at −70°C (up to a month) and thawed once. There were no differences between the activities measured in fresh and stored, freeze-thawed samples.

Acid phosphatase was determined according to Appelmans and de Duve (1955). The reaction was stopped by addition of trichloroacetic acid, and the inorganic phosphate released was determined by the method of Ames (1966). The activity of alkaline phosphatase was determined by the release of p-nitrophenol from p-nitrophenylphosphate. Incubation mixtures of 0.33 ml total volume, which contained 70 mM KCl, 5 mM Mg acetate, 15 mM p-nitrophenylphosphate, 5 mM glycine-KOH, pH 9, and various amounts of fractions, were incubated for 30 min at 37°C. The reaction was stopped by addition of 0.5 ml of 0.16 N NaOH, and the generation of product was measured by the absorption at 400 nm. Arylsulfatase, 5′nucleotidase, and succinate dehydrogenase were measured as described by Yang and Srivastava (1974), Stanley et al. (1980), and Arrigoni and Singer (1962), respectively.

For acrosin activity determinations, samples in TBS were sonicated for 10 s three times (at a power of 50 W), then acidified to pH 2.7 with HCl and incubated for 1 h, and finally centrifuged for 15 min in a Microfuge B (Beckman Instruments, Inc.). The supernate, which contained released acrosin, was passed through a 3-ml Sephadex G-50 medium column equilibrated with 0.1 M glycine-HCl buffer, pH 2.7, to separate acrosin from its natural inhibitor present in the spermatozoon. 0.25-ml fractions were collected from the column, and 0.1-ml aliquots of the fractions were assayed for acrosin activity by following the hydrolysis of N-benzoyl-L-arginineethylester in 0.9 ml of 50 mM $CaCl_2$, 50 mM Tris-HCl, pH 8.5. The reaction was started by addition of 2.5 µl of a 100 mM solution of the substrate, and ultraviolet absorption at 253 nm was monitored continuously on a double beam spectrophotometer.

SDS PAGE

For SDS PAGE, samples were solubilized by addition of 2 vol of a solution that contained 95 mM DL-dithiothreitol, 6.7% SDS, 0.005% bromophenol blue, 0.52 M sucrose, 0.3 M Tris-HCl, pH 8.9, boiled for 2 min, and then alkylated with a 10-fold excess of iodoacetamide. SDS PAGE was done essentially as described by Maizel (1971) on 8–15% gradients or 8% polyacrylamide slab gels, 1.5-mm thick. After electrophoresis, gels were stained with silver (Switzer et al., 1979).

Preparation of Antibodies

Polypeptides of the PM fraction were purified by preparative SDS PAGE and used to raise antibodies in rabbits. 0.5–1.2 mg of protein from the PM fraction or subfractions thereof were loaded onto a 1.5-mm-thick polyacrylamide gel in one, 15-cm-wide, slot. After electrophoresis, two strips on either side of the gel were rapidly stained with Coomassie Brilliant Blue, and the polypeptides of interest were excised from the remaining portion of the gel. The excised bands were homogenized in PBS with a Potter-Elvejhem homogenizer, using a motor-driven pestle. Homogenized gel bands were used immediately or stored at −20°C. Rabbits were immunized by multiple intradermal injections of the gel homogenates at biweekly intervals. Each rabbit received a total amount of protein deriving from two gels.

Ig fractions were prepared from immune and preimmune sera by repeated precipitations with 33% $(NH_4)_2SO_4$. The final Ig solutions were dialyzed against PBS, 0.05% NaN_3 and stored at 4°C.

Radioimmunoblotting

Electrophoretic transfer of polypeptides to nitrocellulose and radioimmunostaining of the blots with rabbit Igs and ^{125}I-protein A were done as previously described (Borgese et al., 1982).

Glycoprotein Identification with ^{125}I-labeled Con A

SDS polyacrylamide gels, which contained the PM fraction or subfractions thereof, were fixed in 25% isopropanol, 7% acetic acid and washed extensively with water and then with 0.5 M Na^+ phosphate buffer, pH 6.5. Gels were then equilibrated in incubation buffer (0.5 M NaCl, 3% BSA, 0.05 M Na^+ phosphate buffer, pH 6.5) and incubated for 24 h in the same buffer that contained 2.5 × 10^6 cpm/ml of ^{125}I-Con A, iodinated by the method of Greenwood et al.

(1963). Gels were washed for a few days with several changes of incubation buffer. The last washes were without BSA. Gels were then dried and exposed to Kodak-Omat AR films. The specificity of Con A binding to sugar residues was determined by including 1-O-methyl-α-D-glucopyranoside in the Con A incubation mixture. For a quantitative analysis, bands from autoradiograms were cut and treated for a spectrophotometric quantitation, according to Suissa (1983).

Preparation of Protein-Colloidal Gold Conjugates

Colloidal gold particles of ~5-nm diam were prepared according to Faulk and Taylor (1971). Gold-protein A conjugation was done by the method of Slot and Geuze (1981), and Con A was conjugated by the method of Horisberger and Rosset (1977).

Immunolocalization of Sperm Surface Polypeptides

2-4 × 10^9 spermatozoa were pelleted after dilution of fresh ejaculates with cold Hanks' solution and centrifugation for 5 min at 350 g_{max}. Cells were washed twice with Hanks' and sedimented as above. The final pellet was suspended in freshly prepared 3% paraformaldehyde, 0.2% glutaraldehyde, 0.12 M phosphate buffer, pH 7.4 (for immunoelectronmicroscopy), or in 3% paraformaldehyde alone in the same buffer (for indirect immunofluorescence), and fixed for 1 h at 0°C. The fixed cells were rinsed twice with 0.12 M phosphate buffer.

For indirect immunofluorescence, the fixed and washed cells were resuspended in 0.12 M Tris-HCl, pH 7.4, and drops of the suspension were deposited on polylysine-covered glass slides for 30 min, to allow the spermatozoa to adhere to the substrate. Slides were then rinsed once with Tris-HCl buffer and twice with 0.12 M glycine-NaOH, pH 7.4. After incubation for 30 min in 0.5 M NaCl, 5% BSA, 0.02 M Na^+ phosphate buffer, pH 7.4, the slides were covered with the same buffer that contained different dilutions of Ig fractions prepared from immune and preimmune rabbit sera, and incubated for 2 h at room temperature. Then they were washed five times with 0.5 M NaCl, 0.02 M phosphate buffer, pH 7.4, and incubated for 90 min with goat anti-rabbit rhodaminated IgG diluted 1:60 in chicken egg albumin buffer. The slides were then washed thoroughly with 0.5 M NaCl, 0.02 M phosphate buffer, pH 7.4, and finally with 5 mM phosphate buffer, pH 7.4. Slides were mounted and observed with a Zeiss Photomicroscope III (Carl Zeiss, Oberkochen, FRG).

For immunolocalization at the electron microscope (EM) level, cells were first embedded in agarose blocks (De Camilli et al., 1983). After two washes with 0.12 M glycine-NaOH, pH 7.4, small agarose pieces were incubated overnight at 4°C in the presence of Ig in 0.5 M NaCl, 5% BSA, 0.02 M Na^+ phosphate buffer, pH 7.4. The agarose blocks were then washed in 0.5 M NaCl, 20 mM phosphate buffer, pH 7.4, for 3 h (five changes). After incubation for 2 h with 0.3 ml of gold-protein A properly diluted in PBS that contained 0.5% BSA, blocks were rinsed twice with PBS-0.5% BSA and three times with PBS. The samples were then processed for EM (see below).

Localization of Con A Binding Sites on the Sperm Surface

6 × 10^7 spermatozoa, prepared as described under Cell Preparation, were incubated with 1 ml of gold-Con A complex (0.8 optical density at 520 nm) in TBS for 1 h at 4°C. Control samples were incubated with gold-Con A premixed with 0.1 M 1-O-methyl-α-D-glucopyranoside. After the cells were washed three times in TBS, they were fixed and processed for EM (see below).

Electron Microscopy

Cells, subcellular fractions, or agarose blocks were fixed in suspension in ice-cold 2% glutaraldehyde, 0.12 M cacodylate buffer, pH 7.4 for 1-2 h. Samples were then sedimented into pellets, which were washed and postfixed with ice-cold 1.5% osmium tetroxide, 0.12 M cacodylate buffer, pH 7.4, for 1 h. Block staining was in 0.5% Mg-uranyl acetate in Veronal buffer, pH 6.0. Dehydration was followed by embedding in Epon 812. Oriented, thin sections were cut on a Reichert Ultracut (C. Reichert AG, Vienna, Austria), stained with uranyl acetate and lead citrate, and examined with a Philips 400 electron microscope (Philips Industries, Eindhoven, The Netherlands).

Results

Preparation and Characterization of a PM-enriched Fraction from Bull Spermatozoa

To optimize conditions of cell disruption that lead to the most efficient and selective detachment of the PM from bull spermatozoa, the distribution of traditional PM marker enzymes between a supernate and a low speed pellet (500 *g*, 10 min) was determined. The best disruption condition was 3 s of sonication (at a power of 50 W) of the cells suspended in a moderately hypotonic buffered solution (HTB). Under these conditions, ~25% and 40% of the PM marker enzymes alkaline phosphatase and 5′nucleotidase were recovered in the low speed supernate, respectively, whereas only ~5% of the total protein was released (Table I). Other enzyme activities were measured to test for the release of other cell components. Release of succinate dehydrogenase, a mitochondrial enzyme, was not detected (data not shown), which indicates that mitochondrial fragments had not been released to any great extent. 80% of the arylsulfatase, a marker of acrosomal content, was released to the supernate, which showed that the acrosomal membrane became leaky. Only ~5% of the acrosin activity (measured in one experiment, in which the inhibitor benzamidine was omitted from the buffers) was released (data not shown). This enzyme is known to be difficult to solubilize and is thought to be in part associated with the inner acrosomal membrane (Morton, 1976). The behavior of acid phosphatase, also believed to be a marker for acrosomal content, was intermediate to that of the other two acrosomal enzymes (~19% release).

The disruption procedure seemed to preferentially release the PM, also on the basis of morphological criteria. Fig. 1

*Table I. Distribution of Protein and Enzyme Activities Between Bull Spermatozoon Subcellular Fractions**

Fraction	Protein (17)‡	Alkaline phosphatase (10)	5′ Nucleotidase (5)	Arylsulfatase (3)	Acid phosphatase (2)
Retained with cells after sonication					
Low speed pellet	94.7 ± 3.0	74.6 ± 2.7	62.9 ± 2.5	19.0 ± 1.4	81.4 ± 10.5
Released by sonication					
6,000 *g* pellet	1.3 ± 0.1	2.8 ± 0.3	7.0 ± 1.4	0.1 ± 0.1	3.6 ± 1.4
PM	1.5 ± 0.1	11.0 ± 0.9	20.6 ± 1.5	0.2 ± 0.2	11.3 ± 4.5
100,000 *g* supernate	2.5 ± 0.3	11.3 ± 1.8	10.4 ± 2.3	80.7 ± 5.4	3.7 ± 3.6

* Values given are percentage of recovered constituent in subcellular fractions ± SE. Recoveries in the sum of the fractions of TS constituents were: protein, 94.3 ± 3.0%; alkaline phosphatase, 86.3 ± 4.8%; 5′ nucleotidase, 101.5 ± 4.8%; arylsulfatase, 86.7 ± 4.8%; acid phosphatase, 103.4 ± 1.1%. The protein content of TS was 7.6 ± 0.6 mg/10^9 spermatozoa in the unfractionated ejaculate. Enzyme activities in the TS (in nmol product formed/min per 10^9 spermatozoa in the unfractionated ejaculate) were: alkaline phosphatase, 47.7 ± 8.6; 5′ nucleotidase, 12.0 ± 3.5; arylsulfatase, 112,150 ± 9,140; acid phosphatase, 22.6 ± 12.1. 35.2 ± 4.9% of the spermatozoa of the fresh ejaculate were recovered in the TS (average of 10 experiments).

‡ Numbers in parentheses indicate the number of experiments.

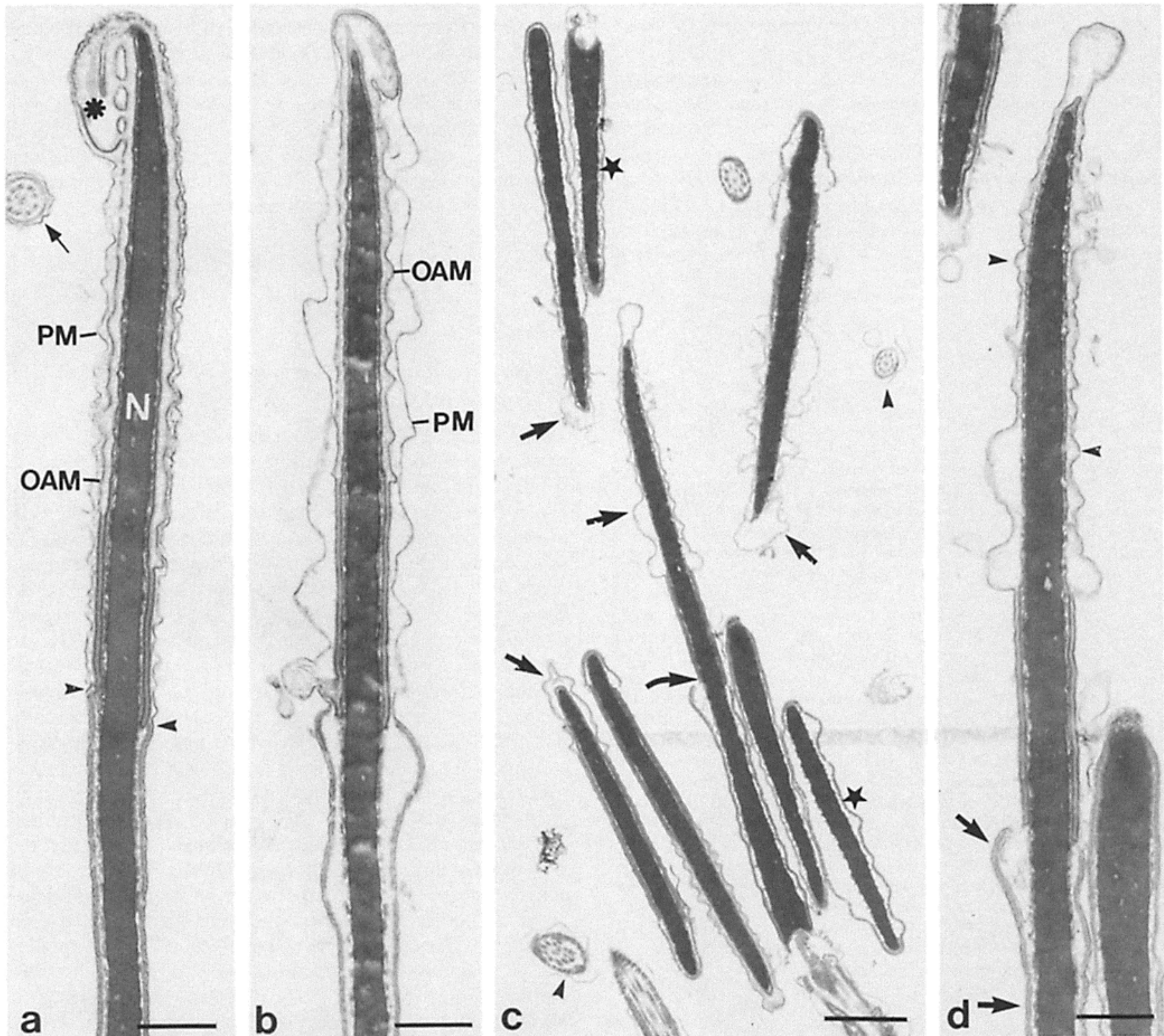

Figure 1. Electron micrographs of bull spermatozoa at various steps during cell disruption. (*a*) Longitudinal section of a washed bull spermatozoon fixed under isotonic conditions. The ultrastructure of a freshly ejaculated, untreated spermatozoon is shown. The anterior portion of the nucleus (*N*) is surrounded by the acrosome (asterisk). The *PM* and *OAM* are closely adjacent. The limits between the acrosomal region and the postacrosomal region are marked by arrowheads. The PM is also present on a cross-sectioned tail (arrow). (*b*) After Percoll gradient centrifugation, spermatozoa were resuspended in HTB. Swelling of the intracellular space between PM and OAM is evident. (*c*) After a 3-s sonication of the material shown in *b*, most of the cells are deprived of PM. Swelling of the acrosome has occurred in most of the spermatozoa (arrows). Sheets of PM are still present around some of the cross-sectioned tails (arrowheads). Some of the profiles represent only the postacrosomal region (stars). The curved arrow indicates the junctional point between the acrosomal and the postacrosomal region. (*d*) Higher magnification of one of the cells shown in *c*. The PM is no longer present on the acrosomal region, but has remained attached to the cell in the postacrosomal region (arrows). Note swelling of the acrosome, which is still limited by OAM (arrowheads). Bars: (*a*, *b*, and *d*) 0.5 μm, (*c*) 1 μm.

illustrates the ultrastructure of spermatozoa at various stages of the disruption procedure. Cells fixed in isotonic solution showed closely apposed PM and outer acrosomal membrane (OAM) (Fig. 1 *a*). Exposure of the cells to hypotonic medium resulted in a swelling of the space between PM and OAM, but most of the cells retained their PM (Fig. 1 *b*). A 3-s sonication resulted in detachment of PM from many of the cells, swelling and clearing of the acrosome, but retention of the acrosomal membrane (Fig. 1, *c* and *d*). 120 profiles for each sperm region were counted. The PM was absent from the acrosomal region of 74% of the profiles, from the postacrosomal region of 54% of the profiles, from 64% of the observed profiles of the principal piece of the tail, and from 25% of the tail midpiece profiles. The inner acrosomal membrane remained attached to the cell in 100% of the profiles after sonication, and 95% of the observed head profiles also had the OAM. Unfortunately, the low release of OAM could not be confirmed biochemically, because of the lack of well-

established marker enzymes for the acrosomal membranes.

A fraction enriched in PM was obtained by pelleting membranes from the low speed supernate obtained from the sonicated cells. Table I shows how various marker enzymes were distributed during the fractionation procedure. The relative specific activities of marker enzymes in the PM fraction can be derived from the data of the table, by dividing the enzyme recovery values by the protein recovery value (1.5%). Thus, the PM fraction contained ~10 and 20% of the total alkaline phosphatase and 5'nucleotidase, respectively, with relative specific activities of ~7 and 14. The higher recovery of 5' nucleotidase than of alkaline phosphatase in the PM fraction can be explained by the localization of alkaline phosphatase to other membranes besides the PM (Gordon, 1973). Alternatively, the two enzymes could be localized on different regions of the PM, which were released with different efficiencies by the sonication procedure. Arylsulfatase, a marker of acrosomal contents, was recovered largely in the high speed supernatant. Unexpectedly, 11% of acid phosphatase was found in the PM fraction and had a relative specific activity of 7.5.

Examination of the fractions by EM showed that the low

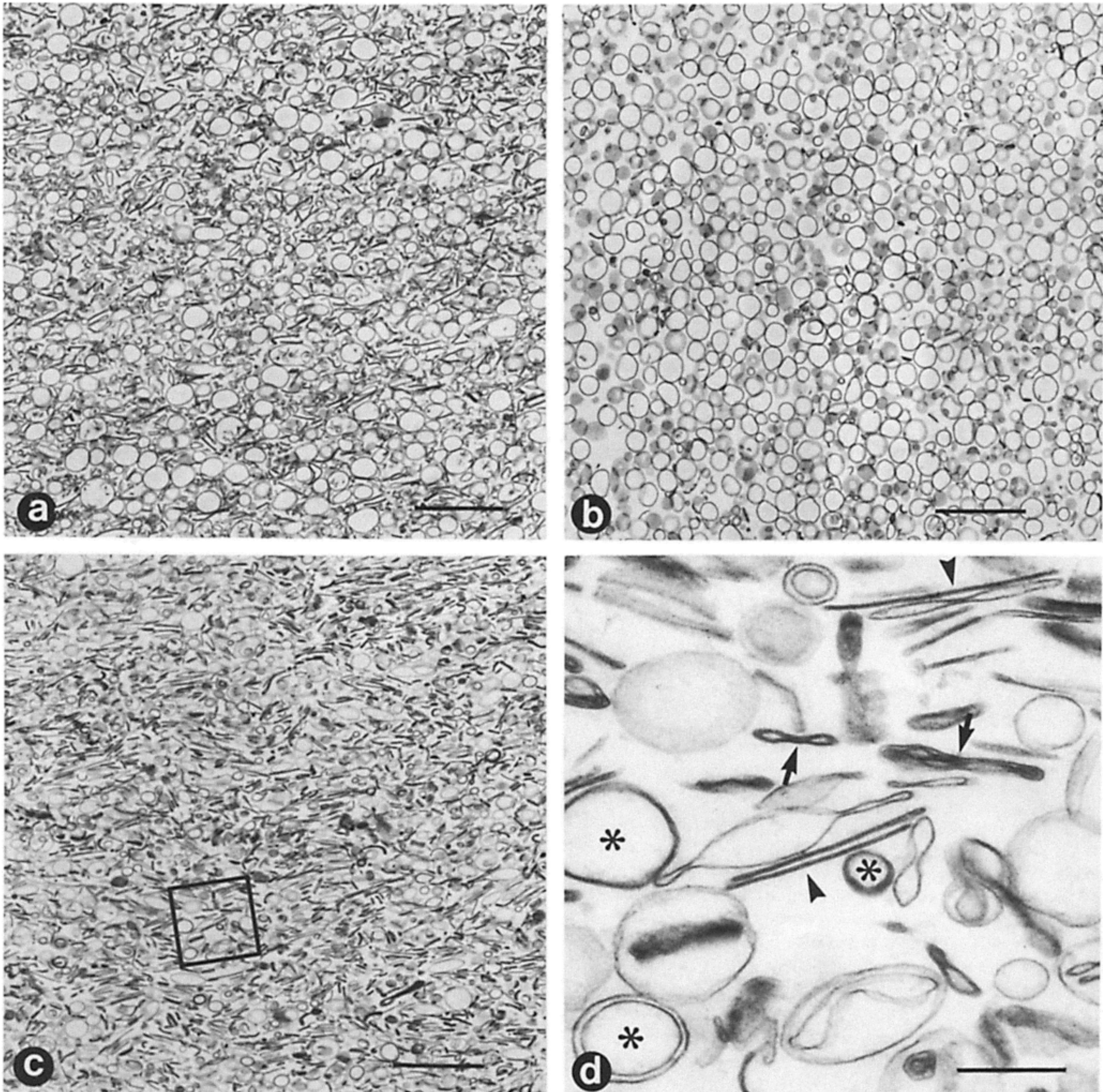

Figure 2. Electron micrographs of the PM fraction and subfractions thereof. (*a*) PM fraction. A mixed population of round-shaped and flattened vesicles is present. After equilibration on a continuous sucrose gradient, the PM fraction gives rise to two distinct peaks (*b–d*). (*b*) Peak II, which equilibrates at $\rho = 1.148$, is composed primarily of round-shaped vesicles. (*c*) Peak I, which equilibrates at $\rho = 1.117$, is enriched in flattened structures. (*d*) Enlargement of the area framed in *c*: flattened single-walled (arrowheads) or double-walled (arrows) vesicles are seen. Double-walled spherical vesicles (asterisks) are also characteristic of this membrane fraction. Bars: (*a*, *b*, and *c*) 2 μm; (*d*) 0.4 μm.

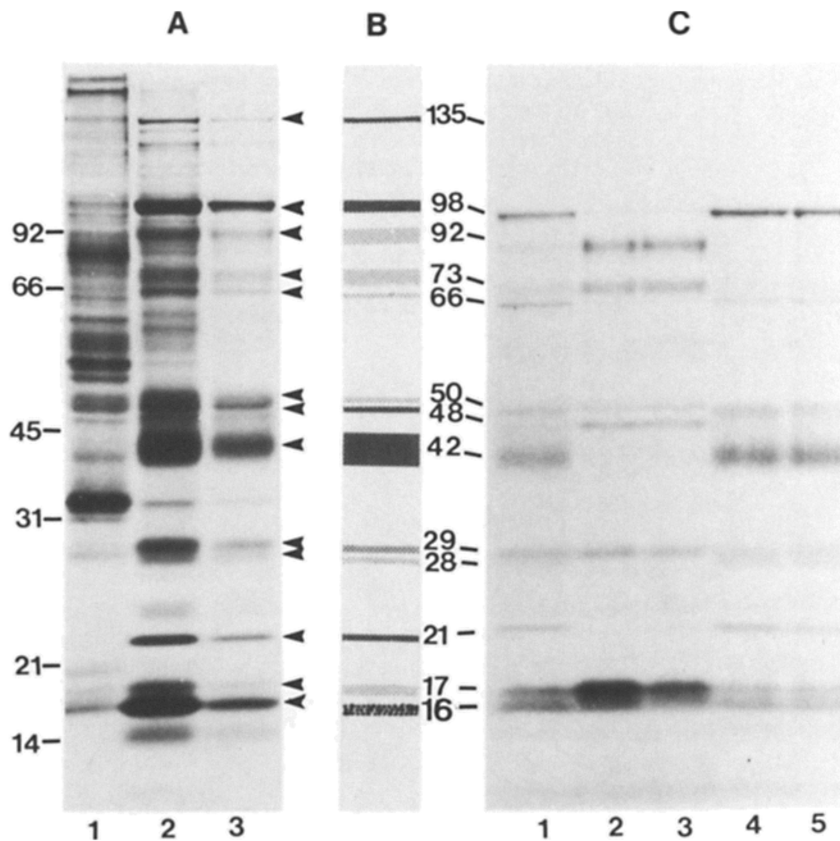

Figure 3. Silver-stained SDS polyacrylamide gradient gels (8–15%) of TS, PM, and PM subfractions. (*A*) Lane *1*, TS (12 μg protein); lanes *2* and *3*, PM fraction (12 and 6 μg of protein, respectively). Arrowheads point to the principal polypeptides, which are also schematically represented in *B*. Their apparent molecular weights (× 10^{-3}) are indicated on the right side. (*C*) Lane *1*, PM fraction; lanes *2* and *3*, fractions 5 and 6 from the sucrose gradient, respectively (peak I); lanes *4* and *5*, fractions 9 and 10 from the sucrose gradient (see Fig. 6), respectively (peak II). All lanes in this panel contained 6 μg of protein. Numbers on the left of *A* indicate molecular weights ×10^{-3} of standards (Bio-Rad, low molecular weight).

speed pellet contained the head and the midpiece of the sperm cells, the 6,000 *g* pellet was enriched in fragments from the principal piece of the tail, whereas the 100,000 *g* supernate contained small membrane fragments (micrographs not shown). These membrane fragments could explain the presence of PM marker enzymes in the 100,000 *g* supernate (Table I). The PM fraction (Fig. 2*a*) was composed of a heterogeneous population of round-shaped vesicles and flattened membranous structures.

The PM fraction was enriched in polypeptides that were barely visible or not detectable at all in the TS (Fig. 3*A*). To determine which of these polypeptides might be integral membrane proteins, the PM fraction was extracted with 0.1 M Na_2CO_3, pH 11, and the polypeptide compositions of the membrane pellet and the alkaline extract were analyzed by SDS PAGE (Fig. 4). Only low molecular weight polypeptides (<21 kD) and some material not entering the gel were extracted by the alkaline treatment. The major polypeptides, with M_r >21,000 in the PM fraction, are therefore candidates for integral membrane proteins. These polypeptides also partitioned into the detergent phase of Triton X-114 after phase separation (not shown), which also indicates that they are integral membrane proteins (Bordier, 1981).

Subfractionation of PM Vesicles

After equilibrium density centrifugation of the PM fraction on a linear sucrose gradient, two bands of light-scattering material could be seen (Fig. 5). Stars and asterisks show the positions of the two peaks in Fig. 6. A minor peak (I) equilibrated at 1.117 g/ml and a major peak (II) equilibrated at a density of 1.148 g/ml. Most of the protein of the gradient fractions (Fig. 6*a*) was contained in the major peak (II).

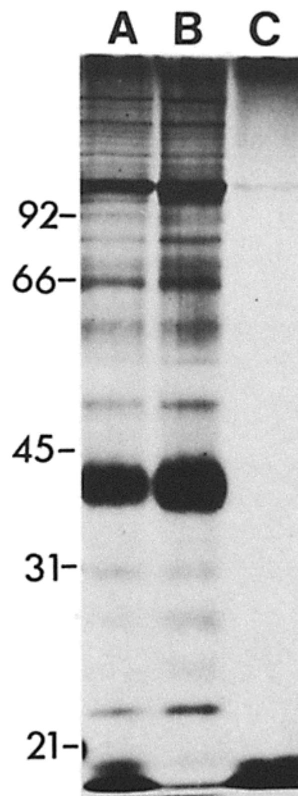

Figure 4. Silver-stained SDS polyacrylamide gel (8%) of the PM fraction before and after alkali treatment. Lane *A*, untreated PM fraction (7 μg of protein); lane *B*, pellet deriving from carbonate treatment of 10 μg protein of the PM fraction; lane *C*, supernate deriving from carbonate treatment of 10 μg protein of the PM fraction. Numbers on the left indicate the molecular weights (× 10^{-3}) of Bio-Rad low molecular weight standards.

Alkaline and acid phosphatase activities (Fig. 6, *b* and *c*) were enriched in the major peak 1.5-fold with respect to the starting PM fraction. The distribution of 5′ nucleotidase could not be determined, because the activity was partially lost after the

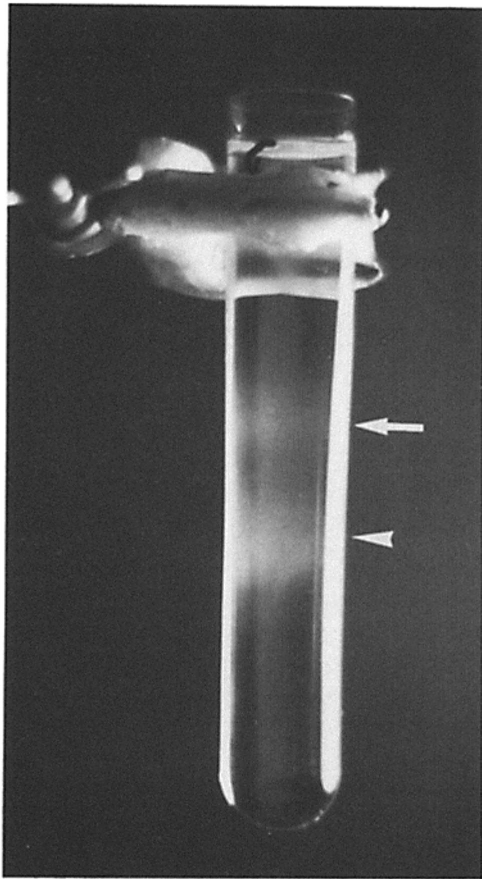

Figure 5. Presence of two peaks of light-scattering material after sucrose gradient density equilibrium centrifugation of the PM fraction. Conditions of centrifugation are described under Materials and Methods. Arrow and arrowhead point to peaks I and II, respectively.

sucrose gradient centrifugation.

The morphologically heterogeneous structures in the total PM fraction were separated into two distinct sets of vesicles on the sucrose gradient (Fig. 2). The higher density peak (II) contained mostly spherical vesicles (Fig. 2*b*), and the lower density peak (I) was enriched in flattened membranous structures (Fig. 2*c*).

Peaks I and II displayed very different polypeptide compositions (Fig. 3*c*). Most of the major polypeptides of the PM fraction were present in either one or the other of the two peaks of the gradient. In particular, the 92-, 73-, and 48-kD bands as well as the carbonate extractable 17-kD polypeptide were exclusive to peak I, whereas peak II contained the 98- , 66-, 42-, and 21-kD polypeptides.

Immunocytochemical Localization of Polypeptides of the Two Membrane Subfractions

To determine the origin of the two membrane fractions, polyclonal antibodies against major polypeptides exclusive to each of the fractions were raised in rabbits and used for immunolocalization studies. Antibodies were raised either against the 92-kD polypeptide or the 98-kD polypeptide (P92 and P98), characteristic of peaks I and II, respectively (see Fig. 3). The specificities of the antibodies for the immunogen were demonstrated by immunoblotting. When tested against the total M fraction, each antiserum reacted only with the polypeptide that had been used as immunogen (Fig. 7). Additional antigens were not revealed when the antisera were tested against TS (data not shown). The antibodies were then used to immunolocalize the two membrane antigens on intact ejaculated bull spermatozoa. Fig. 8 shows that the 92-kD antigen was localized on the anterior portion of the head region, which includes the acrosomal and part of the postacrosomal region. Immunolocalization at the EM level con-

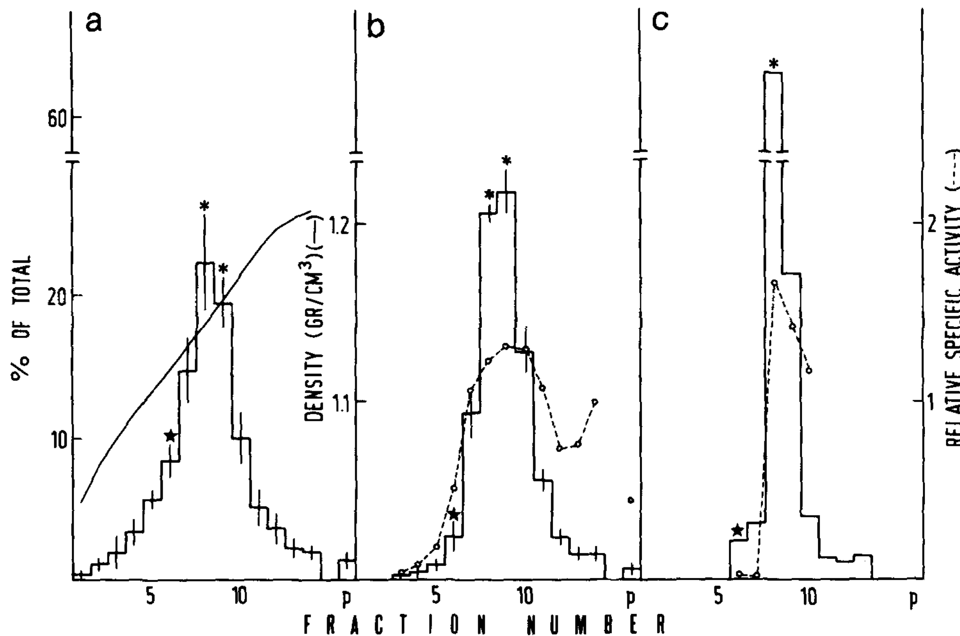

Figure 6. Distribution of constituents of the PM fraction after sucrose density gradient centrifugation. The results are presented as percentage of protein (*a*) or percentage of enzyme activities (*b*, alkaline phosphatase, and *c*, acid phosphatase) ± SE (vertical bars) of the total recovered constituent. The percentage of protein and alkaline phosphatase activity recoveries (averages ± SE of six and five experiments for protein and alkaline phosphatase, respectively) were 109 ± 10 and 136 ± 7, respectively. The recovery of acid phosphatase, determined in a single experiment, was 70% (protein recovery was 67% in this experiment). The continuous line in *a* shows the sucrose density distribution along the gradient (values are the means of five experiments). The dashed lines in *b* and *c* represent the distribution of specific enzyme activity, relative to the starting PM fraction, along the gradients (i.e., percent recovery of enzyme activity/percent recovery of protein in each fraction for each experiment, with sum of constituents in each fraction = 100%). Stars and asterisks indicate the positions of peaks I and II, respectively; *P* indicates pellet.

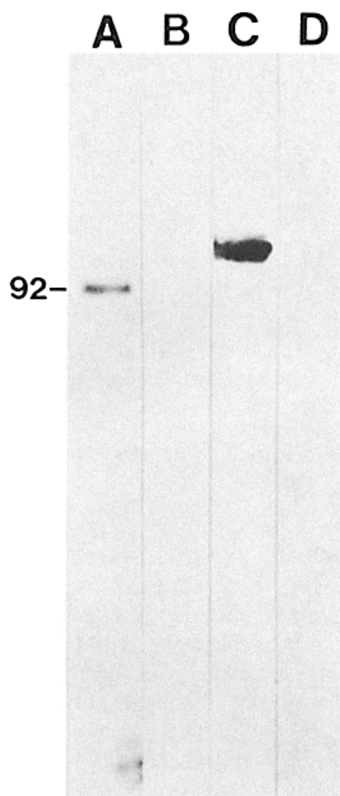

Figure 7. Radioimmunostaining of electroblotted PM proteins with anti-P92 and anti-P98 antibodies. The total PM fraction (50 μg of protein/lane) was run on a 8% gel and then transferred to a nitrocellulose filter. Strips were incubated with the following Ig preparations: lane *A*, anti-P92 (0.02 mg/ml); lane *B*, preimmune Ig from the same rabbit as in A (0.02 mg/ml); lane *C*, anti-P98 (0.05 mg/ml); lane *D*, preimmune Ig from same rabbit as in *C* (0.05 mg/ml). Bound antibodies were revealed with ^{125}I-protein A. The molecular weight ($\times 10^{-3}$) of a standard is indicated on the left.

firmed the immunofluorescence results (Fig. 8, *e–i*). P92 was found on the PM, delimiting the acrosomal region and the anterior part of the postacrosomal region, whereas labeling was low or completely absent on the rest of the cell surface. During the preparation for immunolocalization, cells occasionally lost their PM, exposing a ruptured OAM. However, no labeling of the OAM was ever observed (micrographs not shown). Thus, P92 was a component of the PM. Its localization to the acrosomal region indicated that the membranes in peak I came from the PM of the acrosomal region.

The distribution of P98, enriched in peak II, was more complex (Fig. 9). Surface immunofluorescence localized this polypeptide to the surface of the postacrosomal region and on the principal piece of the tail (Fig. 9). A bright fluorescent spot was also present on the connecting piece, between the neck region of the head and the midpiece of the tail. This region was also weakly positive with preimmune Igs (Fig. 9 *d*). Postacrosomal region of the head, connecting piece, and principal piece of the tail also gave a positive signal with an antiserum raised against the 42-kD polypeptide of peak II (results not shown). Thus, these three noncontiguous regions may have these two polypeptides in common.

Distribution of Con A Receptors between the Two Membrane Subfractions

Con A receptors are known to be concentrated mainly in the head region of mammalian spermatozoon PM (Koehler, 1978; Virtanen et al., 1984). As shown in Fig. 10, we confirmed this finding also for the bull spermatozoon. Con A–colloidal gold conjugate binding was restricted to the surface of the acrosomal (Fig. 10 *a*) and, with a lower density, to the postacrosomal region (Fig. 10 *b*) of the spermatozoon head, whereas gold particles were absent from the tail (Fig. 10, *c* and *d*). Cells incubated with Con A–gold in the presence of 1-*O*-methyl-α-D-glucopyranoside showed no significant labeling (not shown).

A number of polypeptides of the PM fraction, with M_r of 48,000 and above, bound ^{125}I-labeled Con A (Fig. 11). The major Con A binding species (92, 73, and 48 kD) were recovered with the light fraction of the sucrose gradient (peak I: Fig. 11, lane *C*), while only two weakly positive polypeptides (135 and 98 kD) were enriched in the heavy fraction (peak II: Fig. 11, lane *B*). The major polypeptide of peak II (42 kD) showed hardly any labeling. The presence of ~10% of the other major Con A binding species of peak I in peak II (quantitated by spectrophotometry of silver grains: Suissa, 1983) was probably due to a small degree of cross-contamination between the two membrane subfractions.

Discussion

The mammalian spermatozoon is one of the most striking examples of a polarized cell. In this study, we have separated and characterized two PM subfractions from bull spermatozoa, and presented evidence that they derive from different domains of the cell surface.

A very light sonication under moderately hypotonic conditions was found to be the most efficient and selective means to release the PM from the sperm. 38% of the activity of the traditional PM marker enzyme, 5′ nucleotidase, was released into the low speed supernate. 8-fold and 15-fold enrichments with respect to the TS were obtained, respectively, for alkaline phosphatase and 5′ nucleotidase activities in the final PM fraction, with a 10–20% recovery of their respective total activities. About 95% of the cells retained their OAM after this treatment. Thus, the membranes of the PM fraction were probably derived from the cell surface, with little contamination by OAM and mitochondria.

Nearly all the major polypeptides of the PM fraction were resistant to alkali extraction and therefore are likely to be integral membrane proteins. Only a group of low molecular weight components (M_r < 21,000) were extracted by the pH 11 treatment. Russell et al. (1983) have described a group of polypeptides in that molecular weight range in the boar, which they believe to be peripheral components of the membrane contributed by the seminal plasma. Other more loosely bound peripheral components may have been lost during our fractionation procedure.

When the PM fraction was subjected to density equilibrium centrifugation on a continuous linear sucrose gradient, it separated into two peaks: the lower density peak contained a small fraction of the total protein, but nearly all the Con A binding activity of the starting PM fraction, and the higher density peak contained most of the protein and phosphatase

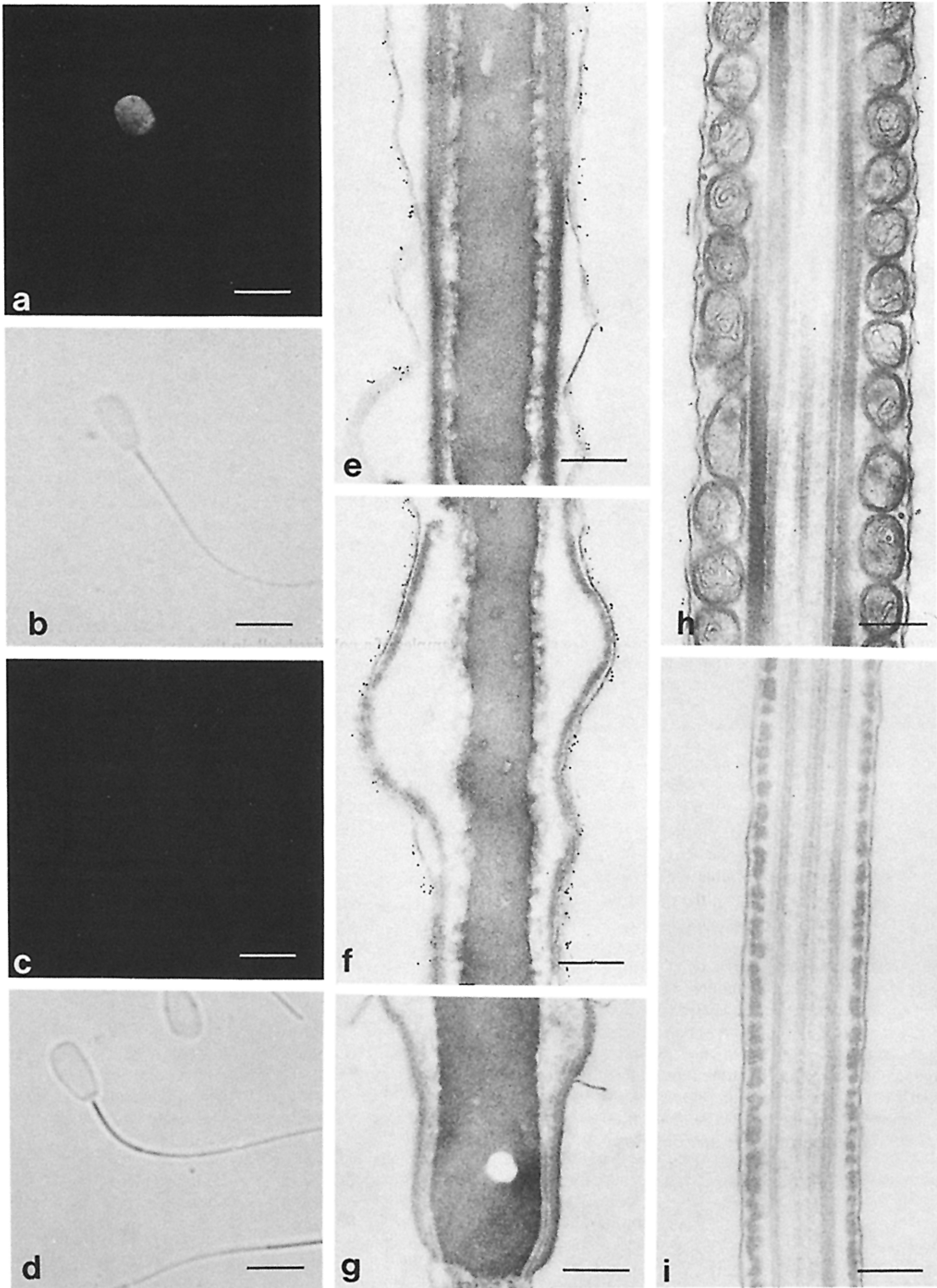

Figure 8. Immunolocalization of the 92-kD polypeptide of peak I. (*a* and *c*) Immunofluorescence images obtained with anti-P92 and preimmune Ig (both at 0.2 mg/ml), respectively. (*b* and *d*) Phase contrast images of the same fields shown in *a* and *c*, respectively. (*e–i*) Protein A–gold ultrastructural immunolocalization of P92. Cells were incubated with anti-P92 at a concentration of 1 mg/ml. Immunoreactivity is restricted to the PM of the acrosomal (*e*) and of the anterior part of the postacrosomal region (*f*). Immunoreactivity is almost undetectable on the plasma membrane of the posterior part of the postacrosomal region (*g*) and on the midpiece (*h*) and principal piece (*i*) of the tail. Bars: (*a–d*) 25 μm; (*e–i*) 0.2 μm.

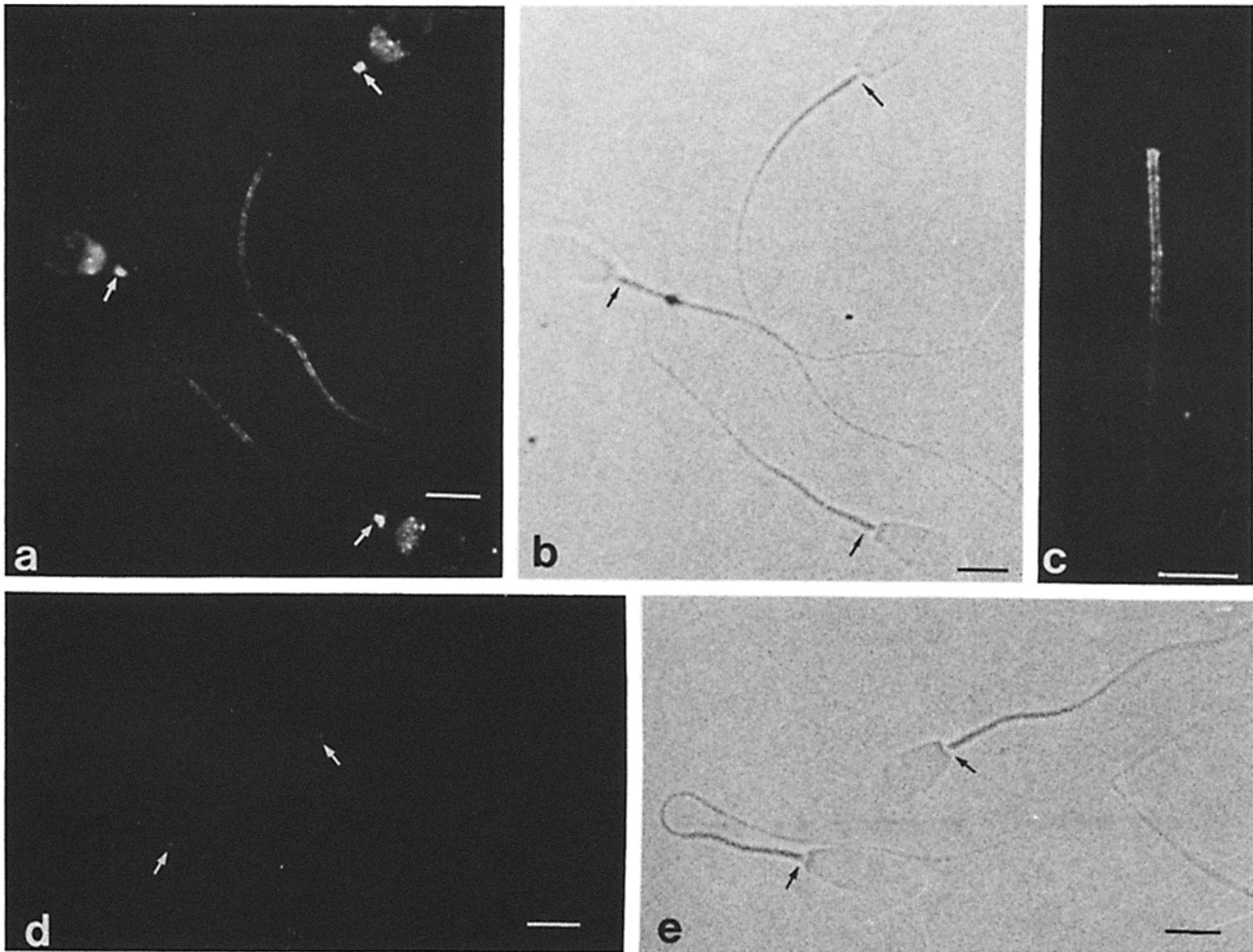

Figure 9. Immunolocalization of the 98-kD polypeptide of peak II. (*a* and *c*) Immunofluorescence images obtained with anti-p98 at a concentration of 0.7 mg/ml. Immunofluorescence is restricted to the posterior part of the head, and to the connecting (arrows) and principal pieces of the tail. It is absent from the midpiece. At higher magnification (*c*), it can be seen that immunofluorescence on the principal piece of the tail is more intense on the borders, as expected for a PM antigen. Differences in the intensity of immunofluorescence on the positive segment are due to out-of-focus effects. (*d*) Immunofluorescence image obtained with preimmune Ig (0.7 mg/ml). (*b* and *e*) Phase contrast images of the same fields shown in *a* and *d*, respectively. Arrows indicate the connecting piece. Bars, 20 μm.

activities of the PM fraction. Ultrastructural analysis revealed that the two peaks were composed of different kinds of vesicles. Flattened and/or double-walled vesicles were enriched in peak I, whereas spherical vesicles bounded by single bilayers were concentrated in peak II. Striking differences between the polypeptide compositions of the two peaks were also observed. Each band contained specific polypeptides that were either absent or very poorly represented in the other one.

Having obtained the separation of two different membrane populations, the problem was to determine whether both of them were derived from the PM, and if so, from which regions. Monospecific, polyclonal antibodies raised to specific polypeptides of each fraction demonstrated that peak I contains membranes that derive from the PM of the anterior portion of the head of the spermatozoon. This conclusion was confirmed by the observation that Con A receptors, which were shown by EM localization studies to be concentrated at the surface of the anterior portion of the head, were highly enriched in peak I. In the case of peak II, antibodies against two of its major polypeptides stained the surface of the sperm cell, which demonstrates that this fraction also was derived from the cell surface. However, because of the triple localization of the two antigens, to the posterior portion of the head, the principal piece of the tail, and the connecting piece, we cannot decide whether the vesicles in peak II derive from all these three regions, or only from one or two of them. Because these three regions appear to share two antigens, one wonders whether they have identical polypeptide composition and derive from the same PM during biogenesis. However, in other species, these regions show differences in antigen composition (Myles et al., 1981). The relationship between these three surface regions in the bull remains to be defined by further studies.

Previous studies have been published on mammalian sperm cell PM isolation and characterization by other authors (Gillis et al., 1978; Peterson et al., 1980; Noland et al., 1983; Russell et al., 1983). However, until the present, the separation of vesicle populations that derive from different domains of the cell surface had not been reported. Noland et al. (1983) reported the separation of two membrane peaks from bovine epidydimal sperm disrupted by nitrogen cavitation, but con-

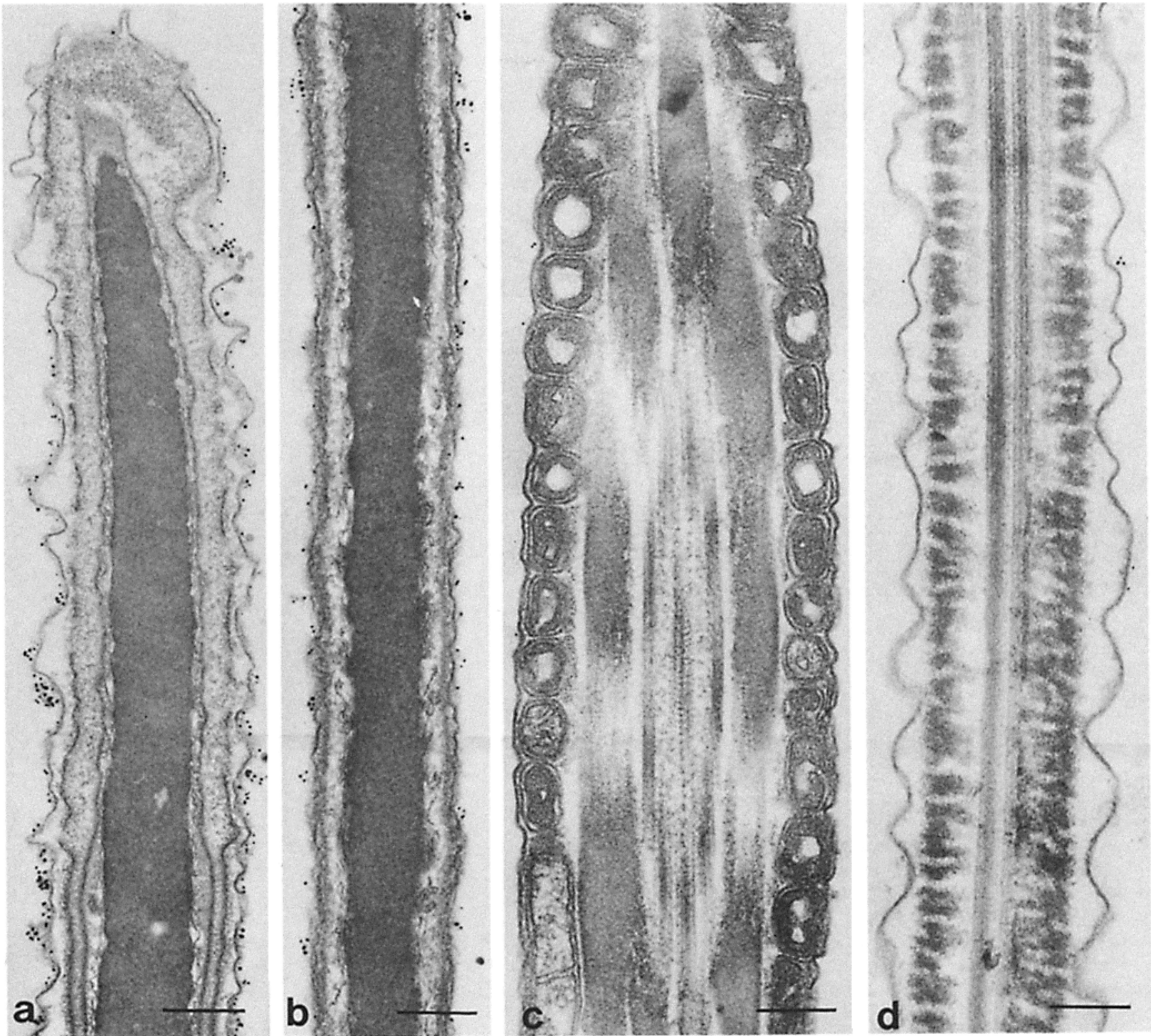

Figure 10. Thin section electron micrographs of ejaculated bull spermatozoa incubated with Con A–gold. Labeling of the PM was very intense over the acrosomal region (*a*), less intense on postacrosomal region (*b*), and almost undetectable on the midpiece (*c*) and principal piece (*d*) of the tail. Bar, 0.2 μm.

cluded that one of the peaks derived from cytoplasmic droplets. The ejaculated spermatozoa we used, purified on a Percoll gradient, were devoid of cytoplasmic droplets, as assessed by phase-contrast microscopy. The most extensive biochemical characterization of mammalian sperm cell PM has been done on the boar (Russell et al., 1983). 20 major polypeptides were detected on one-dimensional gels with Coomassie Blue staining, in good agreement with our SDS PAGE pattern of the PM fraction. Two-dimensional gels, however, revealed at least 250 spots. We do not know whether our PM fraction would also reveal such a complex pattern with a more refined analysis.

The most striking finding of this study is the distinctly different polypeptide composition of two membrane fractions derived from the surface of the same cell. This different composition could be so clearly demonstrated because of the excellent separation between the two fractions, thanks to the homogeneous physical properties of each vesicle population. How domains with such different molecular components are generated and maintained remains a mystery. The problem of the generation of membrane domains in polarized cells has been investigated most intensely in epithelial cells, where it has been found that newly synthesized surface molecules characteristic of the basolateral or apical regions are delivered directly to their respective domains (Simons and Fuller, 1985). In epithelial cells, tight junctions have also been implicated in the maintenance of distinct membrane domains (Gumbiner and Louvard, 1985). Sperm cells, of course, do not have any intercellular junctions, and the definition of the different domains may thus be more complex. For example, it has generally been thought that acrosomal and postacrosomal regions represent two domains, and that some barrier to

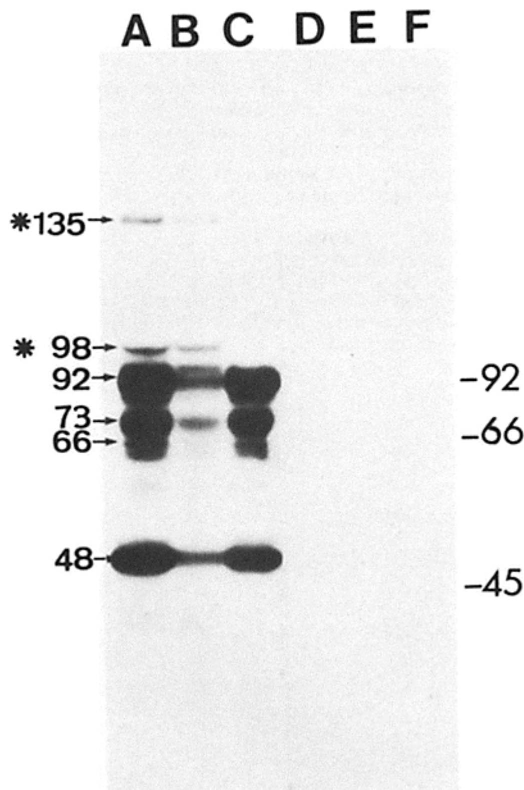

Figure 11. Binding of ^{125}I–Con A to glycoproteins of the PM fraction and of PM subfractions, separated by SDS PAGE (7–10% polyacrylamide gradient). Lanes contained: *A* and *D*, total PM fraction (79 μg of protein); *B* and *E*, band II (45 μg of protein); *C* and *F*, band I (16 μg of protein). Lanes *D–F* were incubated with ^{125}I–Con A in the presence of 0.5 M 1.0-methyl-α-D-glucopyranoside. Numbers on the left indicate M_r (× 10^{-3}) of the major Con A binding species of the PM fraction (see Fig. 3 for reference). Asterisks indicate glycoproteins characteristic of band II, while all other glycoproteins were concentrated in band I. Molecular weights (× 10^{-3}) of standards (Bio-Rad, low molecular weight) are indicated on the right.

protein diffusion might exist between these two regions. However, in the present study it was found that P92 extended over the entire acrosomal region and to part of the postacrosomal region of the head, which suggests that an additional barrier to the diffusion of some protein species exists within the postacrosomal region. The exact degree of spatial overlap between P92 and antigens characteristic of the postacrosomal region remains to be defined by double-labeling at the EM level.

A recent study (Virtanen et al., 1984) on the cytoskeletal structure of human sperm cells has shown the presence of distinct cytoskeletal domains, which seem to correspond well to the surface regions observed in this and other studies (Koehler, 1978; Myles et al., 1981; Bearer and Friend, 1982; Eddy and Koehler, 1982; Gaunt et al., 1983; Wolf and Voglmayr, 1984). This suggests a role for the cytoskeleton in the organization of different membrane domains. The possibility of purifying and characterizing different PM regions should represent a starting point for the study of the biogenesis of these domains as well as of the functions that their molecular constituents play in the biology of sperm maturation and sperm–egg interaction.

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