Biochimica et Biophysica Acta, 778 (1984) 25–37
Elsevier

BBA 72279

ISOLATION AND CHARACTERIZATION OF A PLASMA MEMBRANE FRACTION FROM SEA URCHIN SPERM EXHIBITING SPECIES SPECIFIC RECOGNITION OF THE EGG SURFACE

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(Received June 6th, 1984)

Key words: Plasma membrane isolation; Sperm-egg recognition; Fertilization; (Sea urchin)

A method is described for isolating preparative quantities of plasma membranes from sea urchin sperm. The final membrane fraction is homogeneous by sucrose density sedimentation and is enriched in adenylate cyclase as well as in the four glycoproteins accessible to radioiodination of intact sperm. The electrophoretic profiles of sperm membranes from three sea urchin species are very similar. The membrane preparation consists primarily of sealed vesicles which release carboxyfluorescein when exposed to detergents or distilled water. Ninety-two percent of the ^{125}I-labeled vesicle material binds to wheat germ lectin columns, suggesting a right-side-out orientation. The isolated sperm membrane vesicles exhibit species specific adhesion to the surfaces of sea urchin eggs; this adhesion is blocked by pretreatment of the vesicles with trypsin or egg jelly. This method will be useful for isolating biologically active sperm membrane components involved in sperm-egg recognition during fertilization.

Introduction

The plasma membrane of the sea urchin sperm enables this highly specialized cell to sense from its environment exactly when to initiate the intracellular changes required for fertilization. These changes, which precede sperm-egg fusion, include increased motility and respiration, the exocytosis of the acrosome granule, and the formation of the acrosome process. At the molecular level, these events require calcium uptake [1,2], intracellular alkalinization [3–5], and a depolarization of the cell membrane potential [6]. All of these events are triggered when the sperm contacts the jelly coat surrounding the egg.

The specific mechanisms by which environmental cues are transduced across the sperm plasma membrane remain largely unknown. One approach to an understanding of these mechanisms is to isolate the sperm plasma membrane, study its components, and determine their response to egg jelly. The plasma membranes of intact sea urchin sperm have been externally labeled, the cells extracted with detergents, and the labeled proteins identified after electrophoretic separation [7–9]. Although useful for the identification of externally disposed membrane proteins, this approach cannot be used for studying ion fluxes or other phenomena requiring compartmentalization. A combination of syringe shearing and hypotonic lysis, followed by sucrose gradient sedimentation, has been used to isolate membranes from sea urchin sperm [10,11]. This method has produced a useful initial characterization of the membrane, but is unsuitable for the large scale preparations required for biochemical purification of individual membrane components. Other techniques have been used successfully to isolate membranes from both

invertebrate and mammalian sperm, for example homogenization [12,13], sonication [14,15], hypotonic lysis [16], and nitrogen cavitation [17]. While appropriate for some experiments, these methods all share the disadvantage of disrupting sperm so thoroughly that sucrose gradients are required to separate the membranes from the cellular debris.

Here we present a rapid and simple method for isolating a plasma membrane fraction from sea urchin sperm which does not require sucrose gradient centrifugation. The technique is therefore useful for making very large quantities of membranes that would be difficult to prepare on sucrose gradients. The final preparation compares favorably with others in terms of purity. A large percentage of the preparation appears to be in the form of sealed, right-side-out vesicles that exhibit species specificity in binding to sea urchin eggs.

Materials and Methods

Gametes. Sperm and eggs were spawned into beakers of millipore-filtered seawater (MFSW) by injecting *Strongylocentrotus purpuratus*, *Strongylocentrotus franciscanus*, *Lytechinus pictus* or *Arbacia punctulata* with 0.5 M KCl. Sperm concentration was determined spectrophotometrically [18]. Experiments were performed with *S. purpuratus* except where noted otherwise.

Membrane vesicle preparation. Sperm were washed twice in MFSW (4°C) by centrifugation for 15 min at 2000 × *g*. The cells were resuspended at a dilution of 1:20 in buffer A, consisting of millipore-filtered sea water containing 20 mM Tris base, 2 mM benzamidine-HCl, 0.01% (w/v) streptomycin sulfate, and 0.01% (w/v) penicillin G. Buffer A was adjusted to pH 9.0 with 1 M HCl before addition to sperm. The cell suspension was incubated without agitation for 5 to 15 h at 15°C. Following incubation, cellular debris was pelleted by centrifugation for 30 min at 7000 × *g* (4°C). The supernatant was collected and re-centrifuged under the same conditions. The second 7000 × *g* supernatant was then centrifuged for 1 h at 40000 × *g* (4°C) to pellet the sperm membrane vesicles. In some cases, the final centrifugation was for 1 h at 100000 × *g*. All chemicals were from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Electron microscopy fixation and staining. Samples were fixed overnight as pellets in 3% glutaraldehyde in millipore-filtered sea water (MFSW) (5°C), washed in MFSW without glutaraldehyde, then post-fixed for 30 min in 1% OsO_4 in MFSW. After a wash in deionized water, the material was dehydrated with an ethanol-propylene oxide series, then embedded in Epon. Staining was with 2% aqueous uranyl acetate for 1 h, followed by lead citrate for 10 min.

Enzyme and compositional assays. Protein was determined using bovine serum albumin as a standard, [19]. Lipid content was analyzed gravimetrically after three extractions with 2:1 chloroform/methanol. DNA was measured by the diphenylamine method, as modified by Giles and Myers [20], using deoxyadenosine as a standard [21]. Hexose content was determined by phenol-sulfuric acid assay, with galactose as a standard [22]. Cytochrome oxidase activity was measured in the presence of 0.1% Tergitol NP-10 [23]. Adenylate cyclase activity was assayed with [^{3}H]ATP as a substrate using a 10 min incubation period [24].

Electrophoresis. Proteins were analyzed on 7% polyacrylamide slab gels [25], and stained either with 0.1% Coomassie brilliant blue R, silver nitrate [26], or periodic acid Schiff's reagent [27]. Two-dimensional gels [28] were silver stained as above. Molecular weight standard proteins were myosin (205000), β-galactosidase (130000), phosphorylase *b* (97000), bovine serum albumin (66000), ovalbumin (43000), and carbonic anhydrase (29000).

Solubility of sperm membrane vesicle components. For tests of detergent solubility, the 40000 × *g* sperm membrane vesicle pellet was resuspended in either millipore-filtered sea water, 0.5 M NaCl with 100 mM EDTA (pH 8.0), or 5 mM Tris (pH 8.0) with 5 mM benzamidine, to a final protein concentration of 0.5 mg/ml. One hour (room temperature) after addition of the desired detergent, the sample was centrifuged for 1–4 h at 170000 × *g* in a Beckman airfuge.

Sucrose gradient centrifugation. For sucrose gradient centrifugation, a 20–60% sucrose step gradient, in 5% increments of 1.0 ml each, was layered in a Beckman 16 × 102 mm Ultraclear centrifuge tube and allowed to diffuse 40 h at 5°C before use [29]. The membrane pellet was resus-

pended to a protein concentration of 1.25 mg/ml in 0.5 ml of either buffer A, adjusted to pH 8.0, or 10 mM Tris (pH 8.0) with 5 mM benzamidine, then layered over a gradient prepared with the same buffer. The tubes were centrifuged for 6 h at 27000 rpm in a Beckman SW-27 rotor.

^{125}I labeling of intact sperm and sperm membrane vesicles. Sperm were suspended in millipore-filtered sea water (MFSW) containing 50 mM Tris (pH 7.5) at a concentration of $4 \cdot 10^7$ cells/ml. 54 ml of this sperm suspension were reacted with 2 mCi of $Na^{125}I$ (New England Nuclear, Boston, MA) in a 250 ml beaker coated with 500 μg of Iodogen (Pierce Chemical Co., Rockford, IL) [9,30]. After 10 min, the sperm were transferred to a clean test tube and washed twice in MFSW [9] before isolation of sperm membrane vesicles or precipitation with trichloroacetic acid. Sperm membrane vesicles used in lectin affinity chromatography were labeled with one iodobead (Pierce Chemical Co.) and 500 μCi of $Na^{125}I$ per 1.0 mg of protein in 1.0 ml volume. The reaction was terminated after 10 min at room temperature by removing the sperm membrane vesicles to a clean tube without the bead.

Protease digestions. Sperm, at a concentration of $6 \cdot 10^8$ cells/ml, were incubated in millipore-filtered sea water with trypsin or chymotrypsin at 0.1 mg/ml for 30–60 min at 15 °C. The digestion was stopped by the addition of 0.15 mg/ml soybean trypsin inhibitor, and sperm membrane vesicles were isolated as described above. In other experiments the sperm membrane vesicles were isolated first, then treated with 0.1 mg/ml trypsin, chymotrypsin, or pronase. In this case, the reaction was stopped by adding an equal volume of 2-times concentrated Laemmli electrophoresis sample buffer and heating to 100 °C for 10 min.

Wheat germ agglutinin (WGA) affinity chromatography. Wheat germ agglutinin was purified from raw wheat germ (purchased from a local health food store) by affinity chromatography on an *N*-acetyl-D-glucosamine (GlcNAc)-conjugated Sepharose 4-B column [31]. The isolated WGA was coupled to Affigel-10 (Bio-Rad Labs, Richmond, CA) according to the manufacturer's instructions, then poured into a series of pasteur pipette columns, each receiving 1.0 ml of resin. The columns were equilibrated at room temperature with millipore-filtered sea water containing 5 mM benzamidine and 10 mM sodium azide (Buffer B), and GlcNAc at concentrations ranging from 0.0125 to 400 mM.

The sperm membrane vesicle sample used in the wheat germ agglutinin binding assay was first iodinated, then applied to a 1×30 cm column of Sephadex G-25 (Pharmacia, Piscataway, NJ) equilibrated in buffer B to remove free ^{125}I. The combined flow-through (V_o) fractions from this column were concentrated by centrifugation for 1 h at $40000 \times g$ (4 °C), then the pellet was banded on a sucrose gradient as previously described. After collection, the band of sperm membrane vesicles was diluted with buffer B to 26.7 μg protein/ml (550000 cpm/ml) and loaded in 60 μl aliquots on the WGA-Affigel columns. The total amount of sperm membrane vesicles loaded represents less than 10% of the capacity of the columns.

The sperm membrane vesicles were eluted from the WGA columns with 2.0 ml of the original GlcNAc equilibration buffer, applied in ten 100 μl, and four 250 μl portions over a 2 h period. The entire 2.0 ml eluate was counted in a Beckman Biogamma II counter. To test whether the eluted counts included any free ^{125}I, a 500 μl sample of each was re-applied to Sephadex G-25, and the percentage of cpm in the V_o and V_i fractions determined.

Carboxyfluorescein labeling of sperm membrane vesicles (SMV). In experiments designed to determine whether the sperm membrane vesicles were tightly sealed, sperm were incubated in buffer A containing 50 mM sodium carboxyfluorescein (Eastman Chemicals, Rochester, NY) [32]. The final $40000 \times g$ sperm membrane vesicle pellet was resuspended in 1.0 ml buffer B, then sieved on a 1×30 cm column of Sephadex G-25 (in the same medium) to separate free carboxyfluorescein from SMV-associated carboxyfluorescein. Flow-through fractions, representing the latter, were combined. Sperm membrane vesicles samples were subsequently re-run on the same column; an increase in the A_{492nm} [33] was interpreted as carboxyfluorescein leakage from the sperm membrane vesicles.

To study the binding of carboxyfluorescein-labeled sperm membrane vesicles to eggs, sperm membrane vesicles were isolated in the presence of

1 mM sodium carboxyfluorescein, then run over a Sephadex G-25 column in millipore-filtered sea water to remove free dye. Some of the carboxyfluorescein-loaded sperm membrane vesicles were incubated as a 0.1 mg protein/ml suspension with either 0.1 mg/ml native trypsin or heat denatured trypsin (boiled 20 min) for 1 h at room temperature. Before the sperm membrane vesicles were added to eggs, the trypsin was inactivated by adding 0.2 mg/ml soybean trypsin inhibitor to each tube for 10 min. Other sperm membrane vesicles were mixed with equal volumes of either soluble egg jelly (0.035 mg/ml hexose) or fucoidin (final concentration 0.5 mg/ml) for 10 min before observation of egg binding.

Eggs were washed, their jelly coats solubilized, and the 20000 × *g* supernatant of jelly prepared as previously described [34]. Such washed, dejellied eggs still retain tightly associated jelly components that are not released from the vitelline layer until its elevation at fertilization [34]. These eggs were then rewashed by settling in millipore-filtered sea water containing 0.1 mg/ml soybean trypsin inhibitor. The soybean trypsin inhibitor was added to inhibit the activity of the cortical granule protease which alters the egg surface making it unreceptive to sperm [35]. In some experiments, the eggs were treated with 0.01 M dithiothreitol at pH 9.1 for 10 min to destroy the vitelline layer [36].

To observe sperm membrane vesicle binding to eggs, 100 μl of carboxyfluorescein-labeled sperm membrane vesicles (50 μg protein) were mixed for 30 min at 23°C with 100 μl of a 2% (v/v) suspension of eggs. After the incubation, the eggs were washed three times in 5 ml portions of buffer B, then sedimented by gentle hand centrifugation. The supernatant was removed by aspiration, and the washed eggs viewed with a fluorescence microscope.

Results

Composition and yield

The distribution of protein, lipid, and DNA during the isolation procedure is shown in Table I. The final 40000 × *g* pellet (SMV) consists of 68% lipid, 31% protein, and 1% hexose by weight, with no detectable DNA. Liberation of membrane material from the cells reaches maximal yield by 5 h. Additional incubation times up to 16 h do not alter yield or composition of the final pellet, but if sperm are incubated over the same period at pH 8 (the normal pH of seawater) or at 0°C, separation of the membrane from the cells fails to occur. A gradual decrease in yield is observed if the osmotic strength of buffer A is lowered by more than 10% by dilution with distilled water.

TABLE I

FRACTIONATION OF PROTEIN, LIPID AND DNA DURING SPERM MEMBRANE VESICLES ISOLATION

Figures are average percentages from three experiments. n.d., undetectable.

Fraction	% Distribution [a]		DNA [b]
	Protein	Lipid	
Whole sperm	100	100	29.0
First 7000 × *g* pellet	94.3	90.6	34.5
Second 7000 × *g* supernatant	5.6	9.4	0.3
40000 × *g* supernatant	4.8	1.4	1.2
40000 × *g* pellet	0.8	5.6	n.d.

[a] Percentages are normalized to 100% recovery, assuming loss during assay to be equal for each fraction. Actual % recovery of protein and lipid varies from 77 to 96%.

[b] DNA units are nmoles deoxyadenosine/100 mg protein. Limit of detection is 0.3 nmoles deoxyadenosine/100 mg.

Microscopy

As seen with both the light and the electron microscope, when whole sperm are suspended for 5 h in buffer A, plasma membranes appear to vesiculate from the surface of the cell, leaving the nucleus, mitochondrion, and axoneme intact. Electron micrographs of the 40000 × *g* pellet show that the preparation consists of vesicles, ranging in size from 0.05 to 0.7 μm in diameter. The vesicles appear free of contaminating mitochondria, nuclei, acrosome granules and axonemes (Fig. 1A, 1B).

Marker enzyme analysis

Cytochrome oxidase activity, used as an indicator of mitochondrial contamination, is reduced 23-fold in the sperm membrane vesicles, as compared to whole sperm. The first order rate constant for cytochrome oxidase activity, determined according to Smith [37], is 1.79 $min^{-1} \cdot (mg\ protein)^{-1}$ for the sperm membrane vesicles, and 40.9 $min^{-1} \cdot (mg\ protein)^{-1}$ for whole sperm. On the

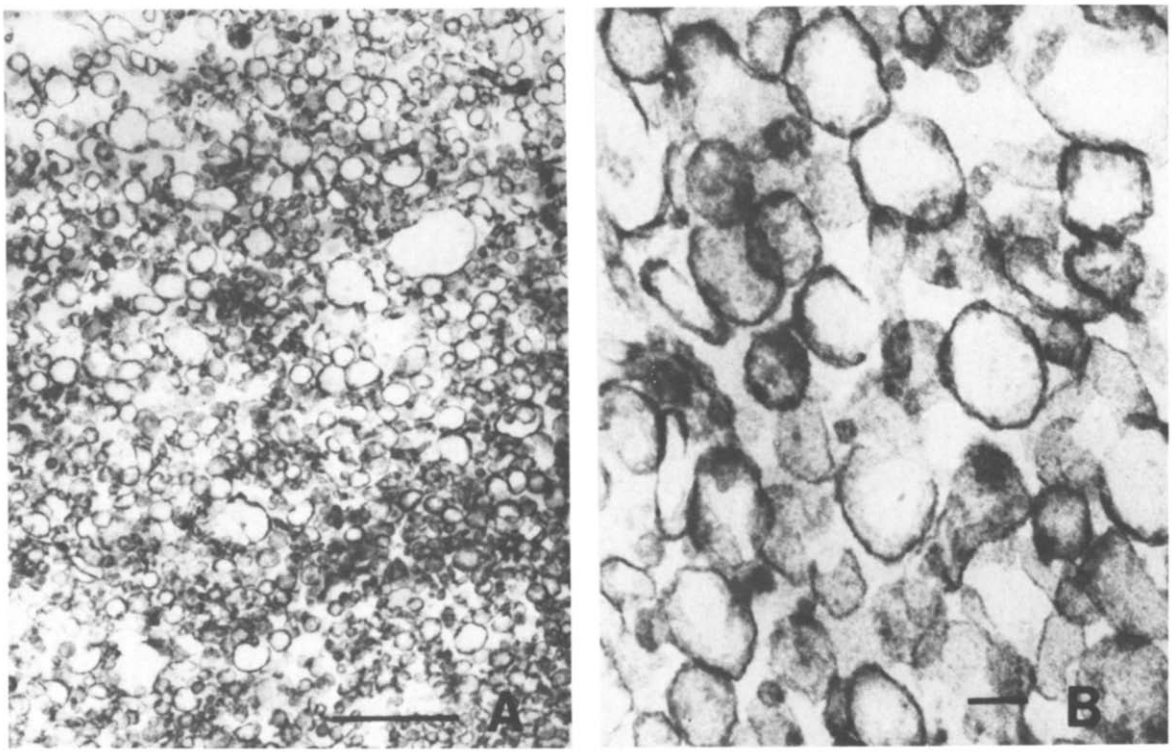

Fig. 1. Electron micrographs showing 40000 × *g* pellet of *S. purpuratus* sperm membrane vesicle isolation. Bar indicates 1.0 μm in A, and 0.1 μm in B.

other hand, specific activity of the plasma membrane marker adenylate cyclase is increased 6-fold in the sperm membrane vesicles over whole sperm treated with buffer A, when enzyme activity is measured in the presence of 0.1% Triton X-100 (Table II). This concentration of detergent increases the activity of buffer A-treated sperm by only 1.2-fold, but multiplies measurable adenylate cyclase activity in sperm membrane vesicles by more than 17.5-fold. The enormous increase in enzyme activity seen with addition of detergent to the sperm membrane vesicles suggests that adenylate cyclase is relatively inaccessible in the untreated vesicles. This result would be expected in the sperm membrane vesicles were sealed in a right-side out orientation.

TABLE II

ADENYLATE CYCLASE ACTIVITY IN *L. PICTUS* SPERM AND SPERM MEMBRANE VESICLES

Specific activity units are nmol/min per mg protein. Values represent the average of three separate determinations with different batches of sperm and their sperm membrane vesicles.

	Specific activity		Fold activation by detergent
	no detergent	+0.1% Triton X-100	
Sperm (treated with buffer A 5 h)	3.53 ± 0.61	4.27 ± 0.90	1.2
Sperm membrane vesicles	1.44 ± 0.20	25.26 ± 0.89	17.6

Electrophoretic characterization

Electrophoretic separation of the polypeptide components of the sperm membrane vesicles yields a consistent pattern that is remarkably similar among three species of sea urchins (Fig. 2). Bands co-migrating with sperm specific histone H-1 (M_r 33000) and the other histones (H) are only minor contaminants in the membrane preparation. As Table III indicates, in *S. purpuratus* more than one-third of the total Coomassie-staining protein is distributed among three major bands, of approximate M_r 150000, 140000 and 80000. These bands, in addition to two minor bands (M_r 210000 and 60000), stain for carbohydrate with periodic acid Schiff's reagent. The actual number of

TABLE III

MAJOR POLYPEPTIDES OF *S. PURPURATUS* SPERM MEMBRANE VESICLES SEPARATED BY ELECTROPHORESIS

M_r ($\times 10^{-3}$) [a]	% Coomassie staining protein [b]	Periodic acid-Schiff's staining	^{125}I labeling in intact sperm
350	2.73	–	–
210	1.22	+	+
190	1.39	+	–
170	0.92	–	–
150	11.54	–	–
140	13.70	+	+
120	1.99	–	–
94	2.81	–	–
80	11.83	+	+
60	6.16	+	+
52	4.84	–	–
49	4.82	–	–
38	8.91	–	–

[a] Molecular weight determinations were the average of five experiments.

[b] Percent Coomassie staining protein determined from densitometric scan of a typical 7% polyacrylamide gel at 550 nm.

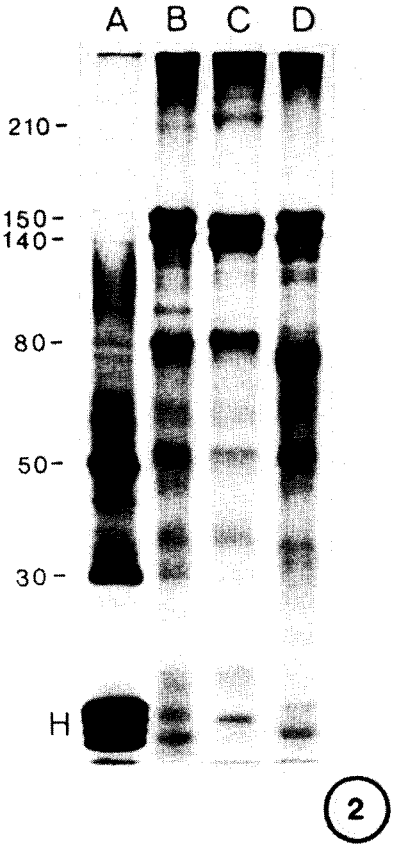

Fig. 2. Electrophoretic profile of sperm membrane vesicles from 3 species of sea urchin, stained with Coomassie blue (75 μg protein per lane). A, 10% trichloroacetic acid precipitate of *S. purpuratus* sperm; B, *S. purpuratus* sperm membrane vesicles; C, *S. franciscanus* sperm membrane vesicles; D, *L. pictus* sperm membrane vesicles.

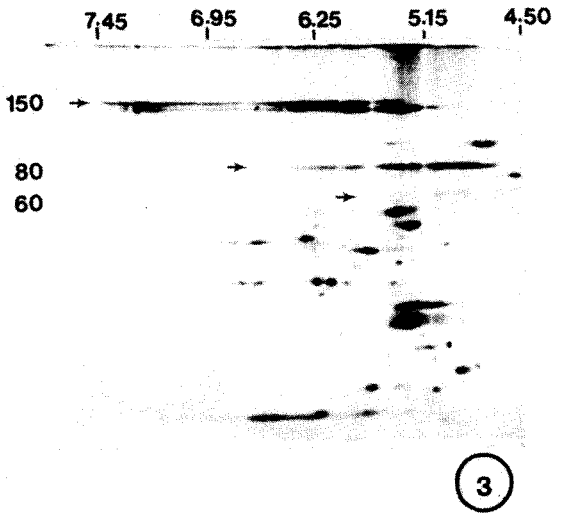

Fig. 3. Two-dimension gel of *S. purpuratus* sperm membrane vesicles (30 μg protein) stained with silver. Isoelectric focusing reference points (horizontal axis) determined by direct measurement of gel with flat-surface pH electrode. Vertical axis is 7% Laemmli electrophoresis. Arrows indicate the M_r 150000, 80000 and 60000 components.

polypeptides present in the membrane preparation is several times that visible in one-dimensional, Coomassie blue-stained gels, as revealed in Fig. 3 by two-dimensional electrophoresis and silver staining. Those bands identified as glycoproteins by periodic acid Schiff's staining tend to run as streaks in the isofocus dimension, suggesting charge heterogeneity in their carbohydrate residues.

The major proteins found in the sperm membrane vesicles cannot be solubilized without the use of detergent or chaotropic agents harsh enough to totally dissolve the membrane structure, such as 2 M urea or 6 M guanidinium chloride. Clear differences in solubility can be observed between the proteins if they are extracted from sperm membrane vesicles with various ionic and non-ionic detergents, then centrifuged at 170000 × *g* for 1 h at room temperature. Fig. 4 shows the results obtained by treating freshly isolated sperm membrane vesicles with MFSW containing 3% Triton X-100, sodium cholate, or SDS. After centrifugation, the M_r 80000 and 60000 proteins remain in the supernatant, the M_r 190000 and 150000 components sediment, and most of the other bands are found in both supernatant and pellet. This pattern of distribution remains unaltered by ultracentrifugation for up to 4 h, the replacement of calcium and magnesium with 100 mM EDTA, or lowering of the ionic strength to 10 mM (5 mM Tris plus 5 mM benzamidine HCl, pH 8.0).

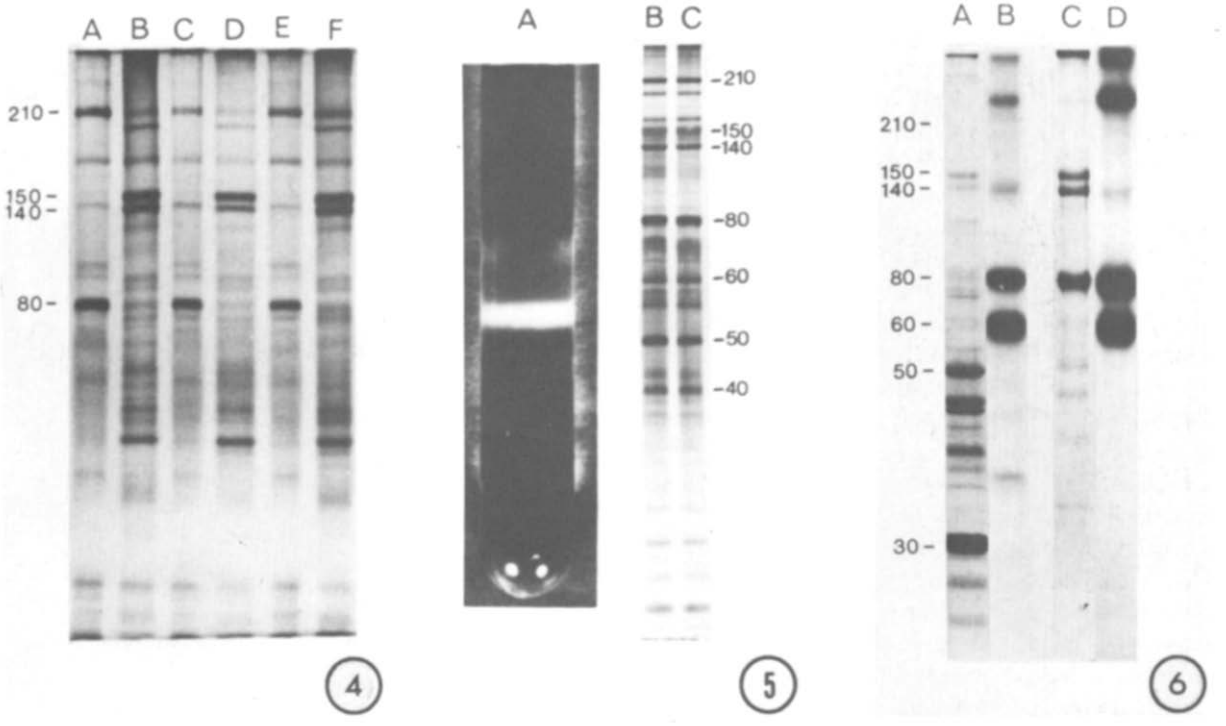

Fig. 4. Differential solubility of *S. purpuratus* sperm membrane vesicle proteins in detergent solutions. Lanes A, C, and E are supernatants and lanes B, D, and F are pellets from 170000 × *g* centrifugation. Detergents used were: A, B, 3% Triton X-100; C, D, 3% cholate; E, F, 3% SDS. 4 μg protein per lane, stained with silver.

Fig. 5. Sucrose equilibrium centrifugation of isolated *S. purpuratus* sperm membrane vesicles. A, band formed in sucrose with 10 mM Tris buffer; B and C, silver stained, 7% gels run in the presence of 5 M urea (5 μg protein per lane). B, sperm membrane vesicles before equilibrium centrifugation; C, sperm membrane vesicles after equilibrium centrifugation.

Fig. 6. Electrophoresis of trichloroacetic acid precipitates of ^{125}I-labeled whole *L. pictus* sperm (A, B) and sperm membrane vesicles made from those sperm (C, D). Lanes A and C are Coomassie stained, 7% gels (50 μg protein per lane). Lanes B and D are autoradiograms.

Sucrose gradient centrifugation

The 40 000 × *g* pellet of sperm membrane vesicles migrates as a single band when resuspended and centrifuged to equilibrium on a sucrose density gradient (Fig. 5). If the sedimentation is performed in millipore-filtered sea water, the membrane preparation reaches equilibrium at the 20–25% sucrose interface. When centrifuged in the less dense 10 mM Tris buffer [29], the band equilibrates at the 30–35% sucrose interface, corresponding to a density of 1.17 g/cm^3. Bands of membrane analyzed after sucrose gradient sedimentation show an electrophoretic profile identical to that of the original 40 000 × *g* pellet (Fig. 5), indicating that sucrose density centrifugation does not yield a purer fraction of sperm membranes.

External accessibility of sperm membrane proteins

External accessibility of sperm membrane proteins in both whole sperm and sperm membrane vesicles was studied by surface labeling with ^{125}I and by digestion with proteolytic enzymes. When whole sperm are gently labeled with ^{125}I, preserving their motility and ability to fertilize eggs, four protein bands are labeled, of approximate M_r 210 000, 140 000, 80 000 and 60 000 (Figs. 6A and 6B). Occasionally, an additional labeled band is seen at M_r 40 000, but this band is not consistently present. Of the labeled proteins, only the M_r 150 000 and 140 000 proteins are abundant enough to be clearly visible by protein staining in polyacrylamide gels of whole sperm. All four consistently labeled proteins are major components of the sperm membrane vesicles (Figs. 6C and 6D). The ^{125}I labeling pattern, like the electrophoretic gel pattern, is quite similar in the three species *S. purpuratus*, *S. franciscanus* and *L. pictus*. Sperm membrane vesicle preparations derived from labeled whole sperm have a specific activity 4–8-times greater than a 1% Triton X-100 extract of the same cells. When sperm membrane vesicles are labeled with ^{125}I after isolation, the labeling pattern is similar to that seen in intact cells, except that the M_r 150 000 protein is also labeled.

Trypsin and chymotrypsin are useful enzymes to digest the surfaces of whole sperm because neither they nor the agent used to inactivate them (soybean trypsin inhibitor) affects the ability of sperm to fertilize eggs at the concentrations used here. When sperm membrane vesicles are isolated from whole sperm that have been treated with trypsin or chymotrypsin, the most obvious changes are a reduction in the amount of the M_r 140 000 protein and the appearance of a new band at M_r 94 000 (Fig. 7). Both of the enzymes also cleave the M_r 60 000 protein that labels with ^{125}I (data not shown). When isolated sperm membrane vesicles are treated with trypsin or chymotrypsin, the previously insensitive M_r 350 000, 210 000, and 80 000 bands are lost. The rest of the bands are not proteolyzed by trypsin or chymotrypsin unless a detergent is added to solubilize the preparation. The M_r 150 000 protein appears insensitive to trypsin, chymotrypsin, and pronase in both whole sperm and in sperm membrane vesicles (in the absence of detergent).

Are the sperm membrane vesicles sealed?

To answer this question, sperm membrane vesicles prepared in 50 mM sodium carboxyfluorescein and sieved on Sephadex G-25 were

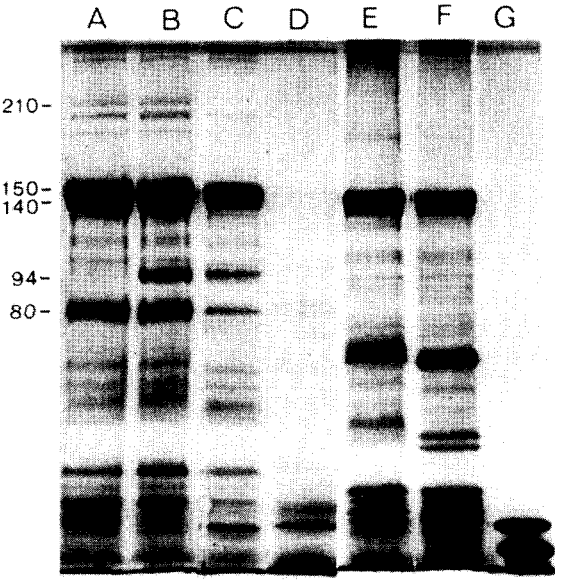

Fig. 7. Protease sensitivity of *S. purpuratus* sperm membrane proteins. Gels are 7%, stained with Coomassie blue. Lanes A–F, 45 μg protein per lane, Lane G, 10 μg protein. Lane A, sperm membrane vesicles from control sperm; B, sperm membrane vesicles from trypsin-treated sperm; C, sperm membrane vesicles treated with trypsin; D, sperm membrane vesicles treated with trypsin and 1% Triton X-100; E, sperm membrane vesicles treated with chymotrypsin; F, sperm membrane vesicles treated with pronase; G, trypsin and soybean trypsin inhibitor.

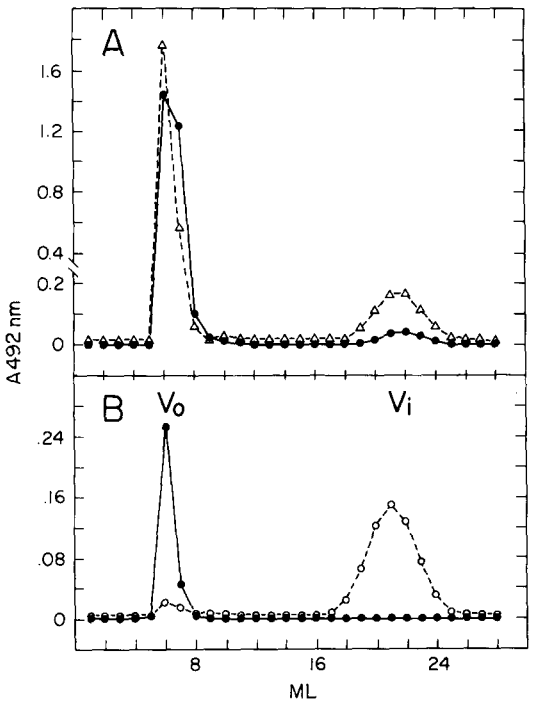

Fig. 8. Chromatography of carboxyfluorescein labeled *S. purpuratus* sperm membrane vesicles on Sephadex G-25. (A) Re-chromatography after 15 min (●) and after 22 h (△). (B) Re-chromatography of control sperm membrane vesicles (●) and sperm membrane vesicle sin the presence of 1% Triton X-100 (○).

re-run on the same column after 15 min (●) and 22 h (△). The data (Fig. 8A) show that very little carboxyfluorescein leaks from sperm membrane vesicles during 22 h at 24°C. When other carboxyfluorescein-sperm membrane vesicle fractions are made 1% in Triton X-100 before reapplication to the G-25 column, the carboxyfluorescein appears in the V_i fractions (Fig. 8B), demonstrating its release from sperm membrane vesicles dissolved by the detergent.

Carboxyfluorescein self-quenches its fluorescence emission while concentrated inside vesicles. Dissolving such vesicles, with the concomitant dilution of carboxyfluorescein, causes a large increase in fluorescence emission [32]. This type of fluorescence rise is seen when sperm membrane vesicles containing carboxyfluorescein are diluted into millipore-filtered sea water containing 1% Triton X-100, which causes a 7.8-fold increase in fluorescence emission. Dilution of sperm membrane vesicles containing carboxyfluorescein into distilled water (1 : 10) causes a 6.8-fold fluorescence increase. The latter experiment shows the sperm membrane vesicles to be osmotically sensitive, which is additional evidence of their sealed nature.

Sidedness of sperm membrane vesicles

The lectin wheat germ agglutinin binds to the entire surface of *S. purpuratus* sperm (Podell, S.B. and Vacquier, V.D., in the press). Since the sugar residues of glycoproteins are normally exposed only on the exterior surface of the plasma membrane, the lectin wheat germ agglutinin should bind only to those sealed vesicles whose glycoproteins are oriented right side out [37,38]. When ^{125}I-labeled sperm membrane vesicles are applied to WGA affinity columns, 12% of the cpm associated with the vesicles adhere non-specifically to the columns, even in the presence of 200 mM GlcNAc (Fig. 9). Since wheat germ agglutinin binding to intact sperm is completely inhibited by GlcNAc at much lower concentrations than this [40], the non-specific binding of sperm membrane vesicles may be due to aggregation of the vesicles. Of the remaining ^{125}I associated with sperm membrane vesicles, 8% fails to bind the WGA columns in the absence of GlcNAc, indicating either an inside-out orientation or an association with

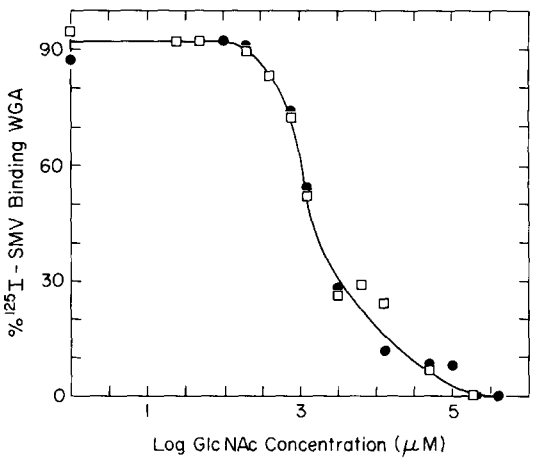

Fig. 9. Binding of *S. purpuratus* sperm membrane vesicles to wheat germ agglutinin affinity columns. ●, □ represent duplicate experiments performed with two different sperm membrane vesicle preparations.

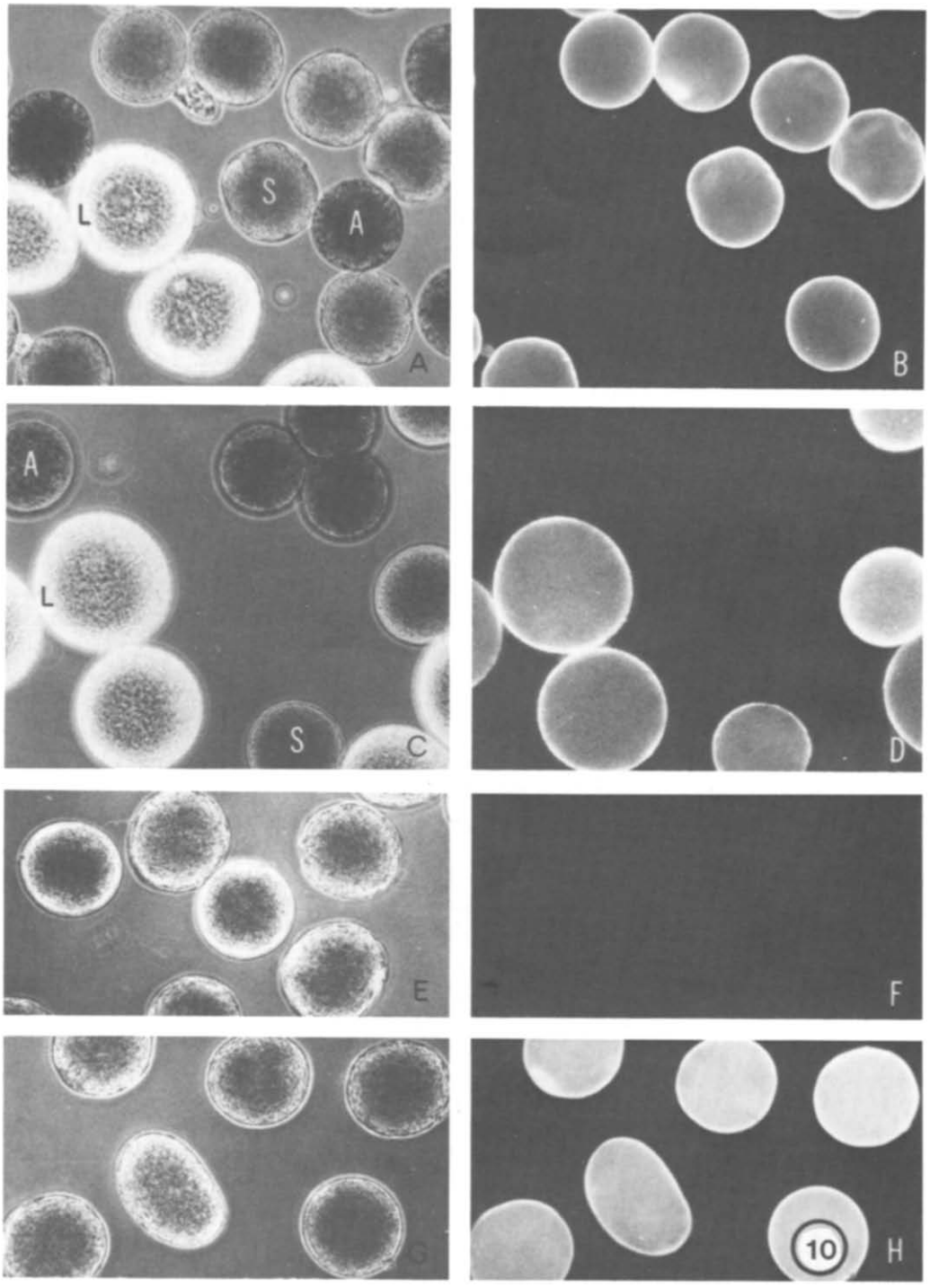

Fig. 10. Interaction of carboxyfluorescein-loaded sperm membrane vesicles with dejellied eggs. Panels on the left are phase contrast, and those on the right are the corresponding fluorescence micrographs. Species of eggs are marked S, *S. purpuratus*; L, *L. pictus*; A, *A. punctulata*. Sperm membrane vesicles in A and B are *S. purpuratus*; C and D. are *L. Pictus*. E, F, G, and H are *S. purpuratus* eggs. E, F, *S. purpuratus* sperm membrane vesicles treated with trypsin; G, H, *S. purpuratus* sperm membrane vesicles treated with heat inactivated trypsin. Magnification approx. 220×.

material that does not bind wheat germ agglutinin. The rest of the ^{125}I associated with the sperm membrane vesicles (92% of the available ^{125}I) binds to the WGA-Affigel and can be competitively removed with GlcNAc. Binding is half maximal at 2 mM GlcNAc, and is completely abolished by 200 mM GlcNAc (Fig. 9). This specific affinity of the vast majority of the sperm membrane vesicle preparation for wheat germ agglutinin suggests that most of the individual sealed vesicles are oriented right-side-out [38].

Interaction of sperm membrane vesicles with eggs

When sperm membrane vesicles containing carboxyfluorescein are mixed with the pH 5.0 treated and washed eggs, the sperm membrane vesicles bind to the egg surfaces in a species specific manner. Fig. 10 (A,B) shows that *S. purpuratus* sperm membrane vesicles bind to *S. purpuratus* eggs, but not to *L. pictus* or *A. punctulata* eggs. *L. pictus* sperm membrane vesicles bind to both *L. pictus* and *S. purpuratus* eggs (Fig. 10 C,D). This is interesting since *L. pictus* sperm can fertilize *S. purpuratus* eggs (Vacquier, V.D., unpublished data). Trypsinization of sperm membrane vesicles, followed, by inactivation of the enzyme with soybean trypsin inhibitor, completely destroys the ability of *S. purpuratus* sperm membrane vesicles to bind eggs (Figs. 10E–10H). Addition of egg jelly [34] to sperm membrane vesicles before addition to eggs also completely inhibits sperm membrane vesicles binding to egg surfaces. Sperm membrane vesicles bind only to unfertilized eggs. When living eggs with sperm membrane vesicles bound to their surfaces are fertilized with sperm, all the visible fluorescence detaches from the elevating fertilization envelope and disperses as a cloud in the surrounding seawater. Sperm membrane vesicle binding to eggs is markedly reduced after dithiothreitol treatment of the eggs to disrupt the vitelline layer.

Discussion

The method described here for isolating sea urchin sperm plasma membrane is rapid, simple, and suitable for large-scale preparations. Several lines of evidence indicate that the sperm membrane vesicle preparation is derived primarily from plasma membrane. All four marker proteins that label with ^{125}I on the external surface of whole sperm are enriched when analyzed on polyacrylamide gels (Fig. 6). Total ^{125}I surface label is also concentrated 4–8-times in the sperm membrane vesicles. Although the activities of several enzymes commonly used to identify plasma membranes, such as alkaline phosphatase, and 5′-nucleotidase are difficult to detect in sea urchin sperm [11], adenylate cyclase activity is enriched 6-fold.

Experiments designed to identify possible contaminants in the membrane preparation have shown non-plasma membrane components to be minimal. Sperm organelles, such as nuclei, mitochondria, acrosome granules, and axonemes are not found in electron micrographs of the sperm membrane vesicle pellet. No DNA is detectable. Because mature sea urchin sperm do not possess cytoplasmic organelles such as Golgi, endoplasmic reticulum, or lysosomes, contamination from these sources cannot occur. The sperm membrane vesicle preparation is homogeneous, since it migrates on equilibrium sedimentation gradients as a single, sharp band corresponding to a density of 1.17 g/cm^3. The sperm membrane vesicles do contain measurable cytochrome oxidase activity, but the levels of this mitochondrial marker enzyme are reduced 23-fold when compared to whole sperm.

Characterization of the membrane preparation described here shows it to be comparable to that obtained by Cross [11] using a different isolation method. Although he presents no data on lipid or DNA quantitation, equilibrium sedimentation, two-dimensional electrophoresis, sidedness, sealing, or egg binding properties, his one-dimensional electrophoretic profiles are similar to ours, as are his results using ^{125}I labeling and periodic acid Schiff's staining. Cytochrome oxidase levels in his preparation are also similar to ours. Subsequent work describing adenylate cyclase activity in sperm plasma membranes isolated by the method of Cross [41] claims an 8-fold enrichment of this enzyme over whole sperm homogenates in the purest ('top') membrane fraction. However, the maximum levels of adenylate cyclase activity measured in their preparation (2.1 nmol cAMP/mg protein per min) are 12-fold lower than in the sperm membrane vesicle preparation described here (25.26 nmol

cAMP/mg protein per min). Perhaps these differences can be attributed to the dissimilar conditions under which adenylate cyclase activity was assayed [42].

It is interesting to note that electrophoretic characterization of the proteins present in the sperm membrane vesicles shows a consistent pattern among three different sea urchin species. The four proteins that label with ^{125}I and stain with periodic acid Schiff's reagent (arguing for their external disposition in intact sperm) cannot be extracted from sperm membrane vesicles without totally dissolving the membrane in detergents or chaotropic agents. This suggests that these four proteins are integral, rather than peripheral constituents of the membrane.

At least some of the sperm membrane vesicles formed in the presence of carboxyfluorescein appear sealed, since this water soluble dye is not washed off and does not leak out in millipore-filtered sea water, but can be released by detergents or hypoosmotic conditions. A large percentage of the sperm membrane vesicles contain membrane glycoproteins that are accessible to wheat germ agglutinin binding, indicating a right-side-out orientation [38]. This conclusion is supported by the fact that 94% of the adenylate cyclase activity in the sperm membrane vesicles is masked without the use of detergents, compared to only 25% in hypotonic lysates from whole sperm incubated under the same conditions.

The patterns of ^{125}I labeling and protease sensitivity show slight differences in the sperm membrane vesicles when compared to whole sperm, suggesting that the conformation of some membrane proteins may have been altered, or that the vesicles contain a mixture of right-side-out and inside-out segments. Some conformational alterations could be caused by exposure to a medium one pH unit higher than normal sea water (pH 8.0 vs. pH 9.0). Nevertheless, at least some of the sperm membrane proteins have retained their native structure through the isolation procedure, as shown by the species-specific binding of sperm membrane vesicles to sea urchin eggs (Fig. 10).

The most significant aspect of the membrane isolation described here is that the sperm membrane vesicles bind to sea urchin eggs in a species-specific manner that exactly parallels the in vivo specificity of sperm-egg binding among *S. purpuratus*, *L. pictus*, and *A. punctulata*. For numerous reasons, it is unlikely that the adhesion is caused by bindin, the polypeptide responsible for attachment of the sperm to the egg after the acrosome reaction has occurred [43]. No band co-migrating with bindin is seen in polyacrylamide gels of sperm membrane vesicles. Sperm membrane vesicle binding to dithiothreitol-treated eggs is greatly decreased, whereas the adhesion of bindin to similar eggs is not (Vacquier, V.D., unpublished data). When eggs with bound carboxyfluorescein-containing sperm membrane vesicles are fertilized, the fluorescence detaches completely as the fertilization envelope elevates. No such detachment occurs with bindin-phospholipid particles under the same conditions. Sperm membrane vesicle binding to homologous eggs is not affected by fucoidin at 100-times the concentration that inhibits bindin agglutination of eggs by 50% (0.5 mg/ml) [44].

Soluble egg jelly is a potent inhibitor of sperm membrane vesicle binding to eggs. The sperm membrane vesicles will not bind to fertilized eggs, which have lost all their tightly associated jelly [34]. It is possible that the adhesion of sperm membrane vesicles to eggs is mediated by affinity of one of the sperm membrane proteins for the intact egg-jelly matrix. The sperm membrane vesicles we have characterized should be valuable in future experiments as a starting material for the isolation of biologically active sperm membrane proteins. The vesicles isolated by this method may also prove useful as a model system for studying the biochemical mechanisms of sperm-egg interaction.

Acknowledgement

We thank Drs. Nicholas L. Cross, Meredith Gould and Gregory S. Kopf for their criticisms of this work, Dr. Cindy A. Lewis for taking the electron micrographs, and Dr. Daniel S. Friend for his help and interest. This work was supported by an NSF predoctoral fellowship to S.B.P. and NIH grant HD-12896 to V.D.V.

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