ISOLATION AND CHARACTERIZATION OF PLASMA MEMBRANE-ASSOCIATED CORTICAL GRANULES FROM SEA URCHIN EGGS

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ABSTRACT

Cortical granules, which are specialized secretory organelles found in ova of many organisms, have been isolated from the eggs of the sea urchins *Arbacia punctulata* and *Strongylocentrotus purpuratus* by a simple, rapid procedure. Electron microscope examination of cortical granules prepared by this procedure reveals that they are tightly attached to large segments of the plasma membrane and its associated vitelline layer. Further evidence that the cortical granules were associated with these cell surface layers was obtained by ^{125}I-labeling techniques. The cortical granule preparations were found to be rich in a proteoesterase, which was purified 32-fold over that detected in a crude homogenate. Similarly, the specific radioactivity of a ^{125}I-labeled, surface glycoprotein was increased 40-fold. These facts, coupled with electron microscope observations, indicate that the isolation procedure yields a preparation in which both the cortical granules and the plasma membrane-vitelline layer are purified to the same extent.

Gel electrophoresis of the membrane-associated cortical granule preparation reveals the presence of at least eight polypeptides. The major polypeptide, which is a glycoprotein of apparent mol wt of 100,000, contains most of the radioactivity introduced by ^{125}I labeling of the intact egg. Lysis of the cortical granules is observed under hypotonic conditions, or under isotonic conditions if Ca^{2+} ion is present. When lysis under isotonic conditions is induced by addition of Ca^{2+} ion, the electron-dense contents of the granules remain insoluble. In contrast, hypotonic lysis results in release of the contents of the granule in a soluble form. However, in both cases the ^{125}I-labeled glycoprotein remains insoluble, presumably because it is a component of either the plasma membrane or the vitelline layer. All of these findings indicate that, using this purified preparation, it should be possible to carry out in vitro studies to better define some of the initial, surface-related events observed in vivo upon fertilization.

KEY WORDS egg surface isolation · egg surface iodination · cortical granule enzymes · vitelline layer · plasma membrane · cortical granules

The eggs of many species of invertebrates and vertebrates contain specialized organelles, the cortical granules (perhaps more appropriately termed

cortical vesicles) located in the peripheral cytoplasm (19), apposed or attached (3, 11, 28, 29) to the cytoplasmic face of the plasma membrane. Upon fertilization of the echinoid egg, these organelles fuse with the plasma membrane of the egg and release their contents into the extracellular space (12, 18, 34, 46). This process of exocytosis, formation of the hyaline layer, elevation of the fertilization envelope, and other less well-defined events at the surface of the egg constitute the "cortical reaction," a prerequisite for normal development of the activated egg. Sperm are not necessary for initiation of this reaction as it can be induced by parthenogenic agents. Moreover, because the cortical granules are tightly associated with the plasma membrane, it seems possible that some of the events in the "cortical reaction," such as fusion of the cortical granule membrane with the plasma membrane, and release of components normally involved in formation of new external layers of the zygote, can be studied in vitro with isolated preparations of plasma membranes containing associated cortical granules.

Over 20 years ago, the isolation of "cortical strips" from eggs attached to a glass slide was briefly reported by Allen (2). Several years later, Sakai (35) described the preparation of egg "cortices." More recently, Vacquier (47) reported a method for isolating cortical material from *Strongylocentrotus purpuratus*. By this method, unfertilized eggs attached to protamine-coated culture dishes are ruptured, and the egg cytoplasm is washed away. This technique produces a layer of cortical granules free of most other subcellular organelles, but still adhering to the plasma membrane attached to the culture dish. Although this preparation allows examination of some of the morphological and osmotic characteristics of the cortical granules, the physiologically relevant fusion of the cortical granule membrane with the plasma membrane is not observed, possibly because the plasma membrane is firmly attached to the culture dish. Schatten and Mazia (36) have very recently utilized a variation of Vacquier's procedure to attach the surface complex of fertilized eggs to dishes. This technique enabled them to visualize passage of the sperm through to the inner side of the egg plasma membrane.

Our objective has been to devise a procedure that will yield preparations of purified cortical granules associated with the plasma membrane and the vitelline layer in suspension. Such a procedure has been devised using eggs of *Arbacia punctulata*; it also appears to be applicable to the eggs of *S. purpuratus*. In this study we report the major ultrastructural characteristics, osmotic behavior, and some of the chemical properties of this preparation.

MATERIALS AND METHODS

Materials

Carrier-free $Na^{125}I$ was obtained from New England Nuclear, Boston, Mass. Chloramine-T was purchased from Fisher Scientific Company, Pittsburgh, Pa. The ^3H-methyl ester of α-tosyl-L-arginine hydrochloride ([^3H]TAME) was purchased from Biochemical and Nuclear Corporation, Burbank, Calif. Artifical seawater (Instant Ocean) was purchased from Aquarium Systems, Inc., Eastlake, Ohio. All other reagents were of the highest purity available.

A. punctulata were purchased from either Connecticut Valley Biological Supply Company, Southampton, Massachusetts, or Florida Marine Biological Specimen Company, Panama City, Florida, and maintained in aquaria at 18°C. *S. purpuratus* were purchased from Pacific Bio-Marine Laboratories Inc., Venice, California, and maintained at 10°C.

Unless otherwise noted, eggs of *A. punctulata* and *S. purpuratus* were dejellied by acid treatment as previously described (39). Experiments were performed in artificial seawater (seawater A); aquarium water to which penicillin G (35 μg/ml) and streptomycin sulfate (50 μg/ml) were added after the water was passed through a 0.22 μm cutoff Millipore filter (seawater B) (Millipore Corp., Bedford, Mass.); or Ca^{2+}- and Mg^{2+}-free, artificial seawater, pH 8.0, containing (g/liter): NaCl, 29.34; KCl, 0.745; $NaHCO_3$, 0.2100; ethylene glycol bis (β-aminoethyl ether) *N,N,N′,N*-tetraacetate (EGTA), 9.51 (seawater C). In seawater C the eggs spontaneously dejelly.

Procedure for Isolation of Membrane-Associated Cortical Granules

Before homogenization, 1-2 ml (packed volume) of eggs were dejellied by gentle resuspension with a Pasteur pipette in 10 ml of ice-cold seawater C in a 12-ml conical glass centrifuge tube and immediately centrifuged in an IEC clinical centrifuge (model CL, Damon/IEC Div., Damon Corp., Needham Heights, Mass.) for 7 s to achieve a final speed of 2,000 rpm. The resulting pellet of dejellied eggs was diluted $^1/_{10}$ (vol/vol) in ice-cold seawater C and uniformly resuspended with a Pasteur pipette. 3 ml of the egg suspension were transferred to a homogenizing vessel (Vitro "200", VWR Scientific, Baltimore, Md.) and homogenized by hand with five strokes of a Teflon pestle. The homogenate was quickly added to a graduated cylinder containing 9 vol of

seawater C and mixed to uniformity by inversion. 10-ml aliquots of diluted homogenate were poured into conical centrifuge tubes and centrifuged for 20 s at 3,000 rpm. The supernate was removed and the surface of the granule-enriched pellet (P_1) was washed with 1 ml of seawater C to remove the top, fluffy layer. The washed P_1 pellet was resuspended in 1 ml of seawater C, diluted to 10 ml, mixed by inversion, and centrifuged for 1 min. The resulting supernate was removed and the pellets (P_2) from all of the centrifuge tubes were combined and resuspended in a volume equal to that of the original homogenate. This suspension was centrifuged for 1 min as before, and the supernate was removed. The pellet of purified membrane-associated cortical granules (P_3) was resuspended in 0.5–1.0 ml of seawater C and stored on ice. Occasionally, a dense pellet of highly pigmented cell debris was present in P_1 or P_2. In these instances the less dense, faintly colored, membrane-associated cortical granules were carefully separated from this material with a Pasteur pipette during resuspension of the sediments. All fractions were saved for protein determinations and for electron microscopy. Because the cortical granule preparation was found to be fragile to vigorous pipetting, the pellets were passed through a Pasteur pipette not more than four times during each resuspension step.

Preparation and Characterization of ^{125}I-Labeled Ghosts

Dejellied eggs were iodinated by adding 200 μl of a fresh solution of chloramine-T (3 mg/ml) in seawater A or B to a 100-μl suspension of eggs (10% vol/vol, 4 mg egg protein/ml) containing 100 μCi of carrier-free $Na^{125}I$. After 30 s, the reaction was stopped by the addition of 10 ml of seawater A or B. This resulted in a 33-fold isotopic dilution of the radioactive iodide in solution. The cells were immediately washed 4–5 times with 10 ml of fresh seawater A or B. Radioactivity was determined either in a Packard autogamma counter or a Packard scintillation counter (Packard Instrument Co., Downers Grove, Ill.), using Gammafluor scintillation fluid (Yorktown Research Inc., S. Hackensack, N. J.).

Ghosts that have been previously shown to contain a receptor for sperm (37) were prepared from ^{125}I-labeled eggs as follows: ^{125}I-labeled eggs (0.8 ml, 8 mg protein) were lysed by suspension in 10 ml of distilled water followed by centrifugation for 10 min at $\times$ 1,000 g. The pellet was washed with 10 ml of distilled water and centrifuged again at $\times$ 1,000 g for 10 min. The pooled supernate fractions containing the cytosol and small particulate material were combined. The pellet, containing the ghosts, was suspended in a small volume of seawater A. Lipids were extracted from ^{125}I-labeled ghosts (2 mg protein) with 20 vol of $CHCl_3$-CH_3OH (2:1) for 30 min at 37°C as previously described (39). To establish that the majority of the ^{125}I was incorporated into protein, the residual, $CHCl_3$-CH_3OH insoluble fraction was incubated at 37°C for 18 h with 500 μg of Pronase (Sigma Chemical Co., St. Louis, Mo.) with 0.4 ml of 50 mM Tris-HCl, pH 7.0, containing 10 mM $CaCl_2$. After Pronase digestion, the distribution of radioactivity in trichloroacetic acid (TCA) soluble and insoluble fraction was determined. Ghosts obtained from ^{125}I-labeled eggs (900 μg protein) were also treated with a mixture of 100 μg of DNAse (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio) and 100 μg of RNAse (Worthington Biochemical Corp., Freehold, N.J.) in 1.5 ml of 5 mM Tris-HCl, pH 7.2. After 1 h at room temperature, the entire reaction mixture was centrifuged at $\times$ 39,000 g for 10 min, and radioactivity in the supernate and pellet was determined.

Preparation of ^{125}I-Labeled Plasma Membrane-Associated Cortical Granules

^{125}I-labeled material was prepared by two methods: (*a*) to 10 ml of a 10% suspension of acid dejellied eggs containing 0.5 mCi carrier-free $Na^{125}I$, a solution of 30 ml of chloramine-T (3 mg/ml) in seawater A was added. After 30 s, the reaction was stopped by diluting the reaction mixture with 500 ml of seawater A. The eggs were allowed to settle, and the supernate seawater was removed by aspiration. The eggs were then washed two times in 20 ml of seawater A followed by two washes in 20 ml of seawater C. The eggs were resuspended in 10 ml of seawater C, and the membrane-associated cortical granules were prepared as described above; or (*b*) the same volumes were used as described above, but eggs were dejellied and labeled in seawater C. The reaction was stopped by addition of unlabeled KI to a final concentration of 5 mM in seawater C.

Sucrose Gradient Centrifugation

2-ml portions of the homogenate, or P_3, prepared as described above, were applied to 39-ml centrifuge tubes containing 33 ml of a continuous gradient of 27–78% (wt/vol) sucrose in seawater C. The samples were centrifuged in a Beckman Spinco SW 27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 3 h at 4°C at 26,000 rpm. The gradients were collected in 20 drop fractions for examination by electron microscopy, and for protein and ^{125}I determinations.

Electron Microscopy

Micropellets (6) of membrane-associated cortical granules (P_3) were readily formed from 300-μl aliquots of resuspended P_2 by centrifugation for 15 s in a Beckman microfuge (model 152, Beckman Instruments, Inc.) attached to a Powerstat variable transformer (type 116B) operated at 60 V. On some occasions, P_3 (see Results) was directly processed for electron microscopy in conical centrifuge tubes. Primary fixation for 2 h at 4°C was accomplished with either 3.0% glutaraldehyde

in seawater C or in a glutaraldehyde, paraformaldehyde fixative (3, 24) containing 0.05 M $NaPO_4$, as indicated in the figure legends. The vehicle (20) of all fixatives used was routinely adjusted to 1.0 OsM by addition of NaCl or distilled water as monitored with an Advanced Instruments Osmometer (model 3W, Advanced Instruments, Inc., Needham Heights, Mass.). The pH was routinely adjusted to 8.0 with NaOH after addition of aldehyde.

After fixation, samples were incubated for 1 h or overnight at 4°C in the medium used for fixation, but with aldehyde omitted. Preparations were then postfixed in 2.0% osmium tetroxide in seawater A. Dehydration, embedding, and poststaining were performed as reported earlier (6), with the exception of the rapid embedding technique used for the preparation displayed in Fig. 2. Details of this modified procedure will be reported at a later date (Decker and Lennarz, unpublished results).

Compositional and Enzymatic Analyses

Proteins were determined by the method of Lowry et al. (27). Hexose was measured by the phenol-sulfuric acid method of Dubois et al. (10). Fucose content was determined by the method of Dische and Shettles (8); uronic acid was measured by the carbazole method of Dische (7). The thiobarbituric acid method of Warren (49) was used to determine sialic acid content. Hexosamine was measured by the method of Elson and Morgan as modified by Davidson (5). Total and lipid phosphate were determined by the method of Bartlett according to Dittmer and Wells (9).

β-1,3-glucanohydrolase activity was measured by the method of Epel et al. (12) with the following modifications: the preparation was suspended in seawater C, and the release of glucose was determined by the Glucostat reagent obtained from Worthington Biochemical Corp. TAME hydrolase activity was monitored at pH 9 by the method of Roffman et al. (33). Incubations were carried out in a scintillation vial. The reaction mixture contained 25 μl 1 M Tris-HCl, pH 9.0; 1 μl 1 mM TAME-^{3}H (210 mCi/mmol), 5 μl 0.05 M TAME, and 50 μl enzyme (25–100 μg). To each vial, 10 ml of toluene-based solvent system containing 2,5-diphenyloxazole, (4 g/liter), and 1,4-bis [2-(5-phenyloxalolyl)]-benzene (0.5 g/liter) was added. The vials were shaken and radioactivity of the soluble [^{3}H]-CH_3OH formed during the reaction was determined with a scintillation counter.

SDS Polyacrylamide Gel Electrophoresis

Samples containing 25–100 μg protein were precipitated with ice-cold 10% TCA and washed with 10% TCA. The TCA insoluble material was solubilized by boiling in 100 μl 10 M urea, 1% SDS, 1% β-mercaptoethanol. Samples were electrophoresed in 4.5% acrylamide gels, and processed according to Kent et al. (25).

RESULTS

Preparation of Membrane-Associated Cortical Granules

Based on earlier studies (3), as well as on our examination of thin sections of *A. punctulata* eggs, it was clear that the cortical granules are very closely associated with the plasma membrane of the egg. As shown in Fig. 1, the major ultrastructural components of the periphery of intact *A. punctulata* eggs are the vitelline layer, the plasma membrane, and the cortical granules. It is evident that the limiting membrane of the cortical granule is tightly apposed to the plasma membrane, with a suggestion of membrane confluence in certain regions (inset, Fig. 1, arrow).

On the basis of the above observations, as well as the finding of Vacquier (47) that the cortical granules of *S. purpuratus* eggs remained associated with the plasma membrane attached to a synthetic surface, it seemed possible that egg plasma membranes prepared by a "suspension" method might retain attached cortical granules. If this were so, it might be expected that such membranes, with the cortical granules attached, could be readily isolated by either velocity or isopycnic centrifugation. Therefore, eggs were subjected to homogenization in Ca^{2+}- and Mg^{2+}-free seawater containing 25 mM EGTA (seawater C) to prevent Ca^{2+}-induced lysis (47) of the cortical granules. It was found that under these conditions, homogenization produced a component that was readily sedimented by low speed centrifugation. On the basis of phase contrast microscopy examination, the pellet obtained by such centrifugation appeared to be rich in large sheets with attached refractile bodies with the dimensions of cortical granules. Simple multiple washings of this pellet resulted in removal of any remaining cell debris.

It is apparent by electron microscope examination that this simple procedure yields a preparation containing highly purified cortical granules adherent to the plasma membrane, as well as the vitelline layer associated with the plasma membrane (Figs. 2 and 3). Thus, it is clear that in both *A. punctulata* (Fig. 2 and 3) and *S. purpuratus* (Fig. 4) the association between most of the cortical granules and the plasma membrane is sufficiently strong to survive the forces encountered during the isolation procedure. Only occasionally are free, detached granules observed. On

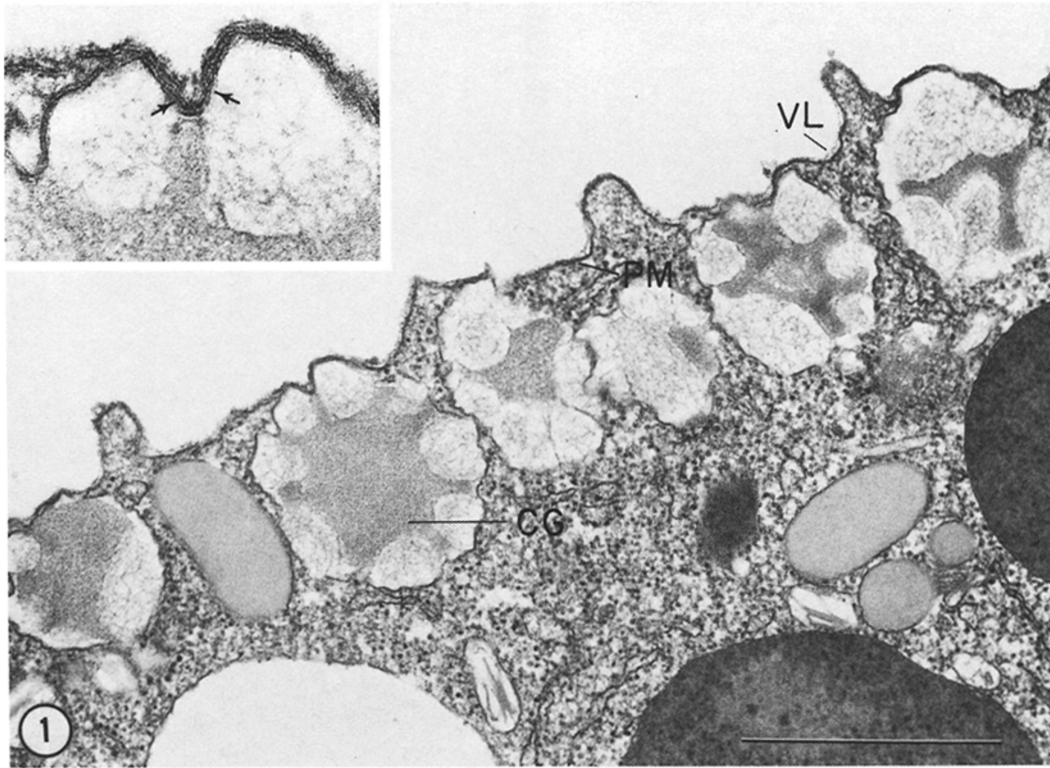


FIGURE 1 Electron micrographs of the cortical region of an *A. punctulata* egg. Thin section of a preparation fixed in glutaraldehyde and paraformaldehyde, and then postfixed in osmium tetroxide as described in Materials and Methods. The cortical granule (*CG*), plasma membrane (*PM*) and vitelline layer (*VL*) are readily visualized. × 35,000. Bar, 1 μm. At higher magnification (inset) there is a suggestion of confluence between the plasma membrane and the cortical granule membrane (arrows). × 105,000.

close inspection of the *A. punctulata* cortical fraction (Fig. 3), it is clear that the cortical granules are tightly apposed to the plasma membrane, and the vitelline layer is adherent to the exterior surface of the plasma membrane. This association is also evident in cortical granule preparations from *S. purpuratus* (Fig. 4). These cortical granules exhibit the lamellar core structures previously observed in the intact egg (34).

It is apparent from these ultrastructural studies that this simple procedure, which yields ~ 1 mg protein from 100 mg egg protein, and can be accomplished in < 1 h, yields highly purified preparations of membrane-associated cortical granules. Another indication of the purity of this cortical granule preparation was obtained by sucrose density centrifugation. As shown in Fig. 5, a single band of protein, with a buoyant density (1.29 gm/cc) only slightly greater than that detected in the crude homogenate, was observed.

Analytical Studies on Membrane-Associated Cortical Granules

From ultrastructural analysis of the cortical granule preparations, it is clear that multiple structural components are present, i.e. the contents of the granule, the membrane of the granule, the plasma membrane, and the vitelline layer. Therefore, it was not surprising that analysis of cortical granule preparations by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis revealed the presence of at least six protein bands (Fig. 6). It is of interest that the major protein band, with an apparent mol wt of 100,000, is a glycoprotein, as it stains with periodate-Schiff

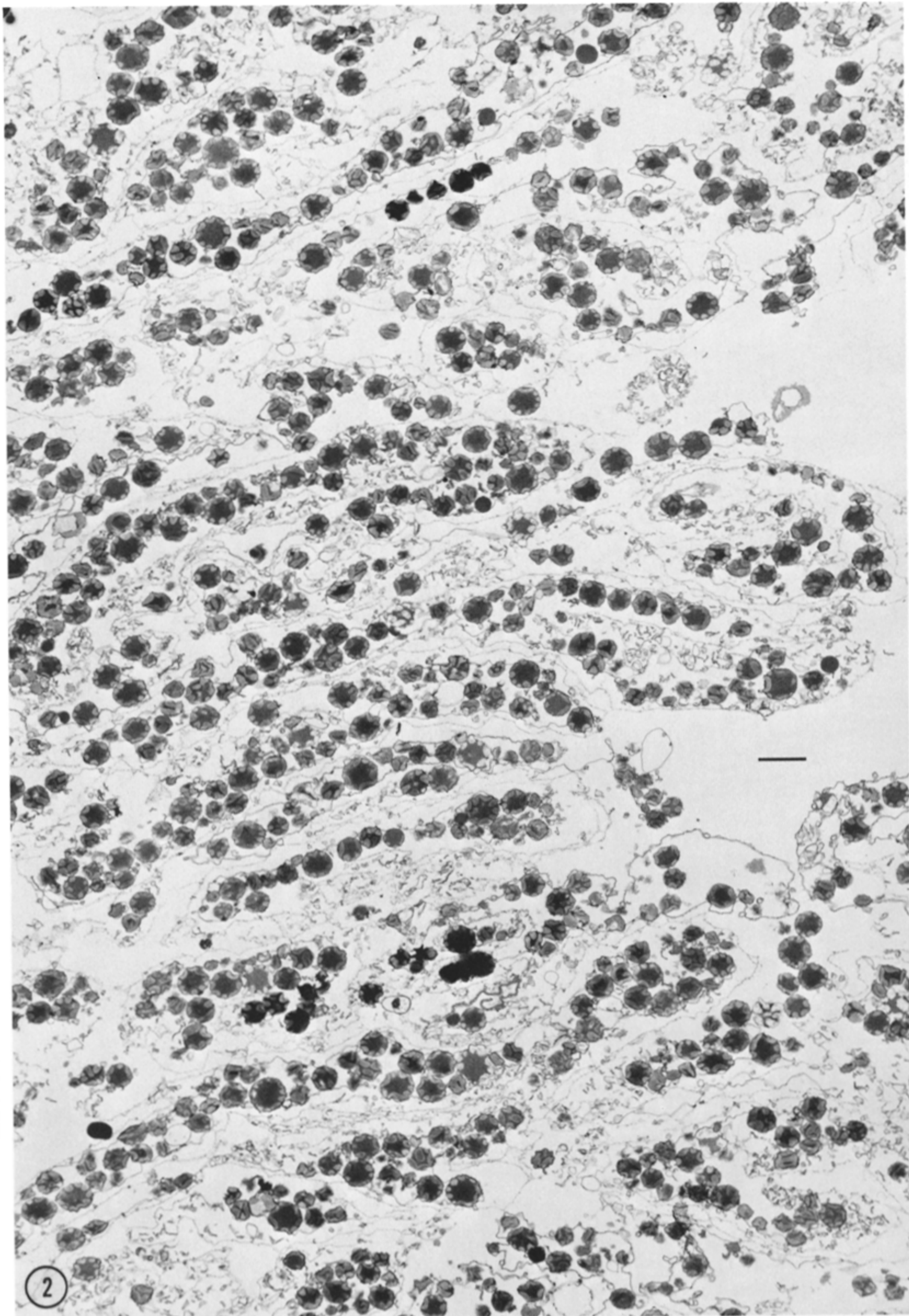
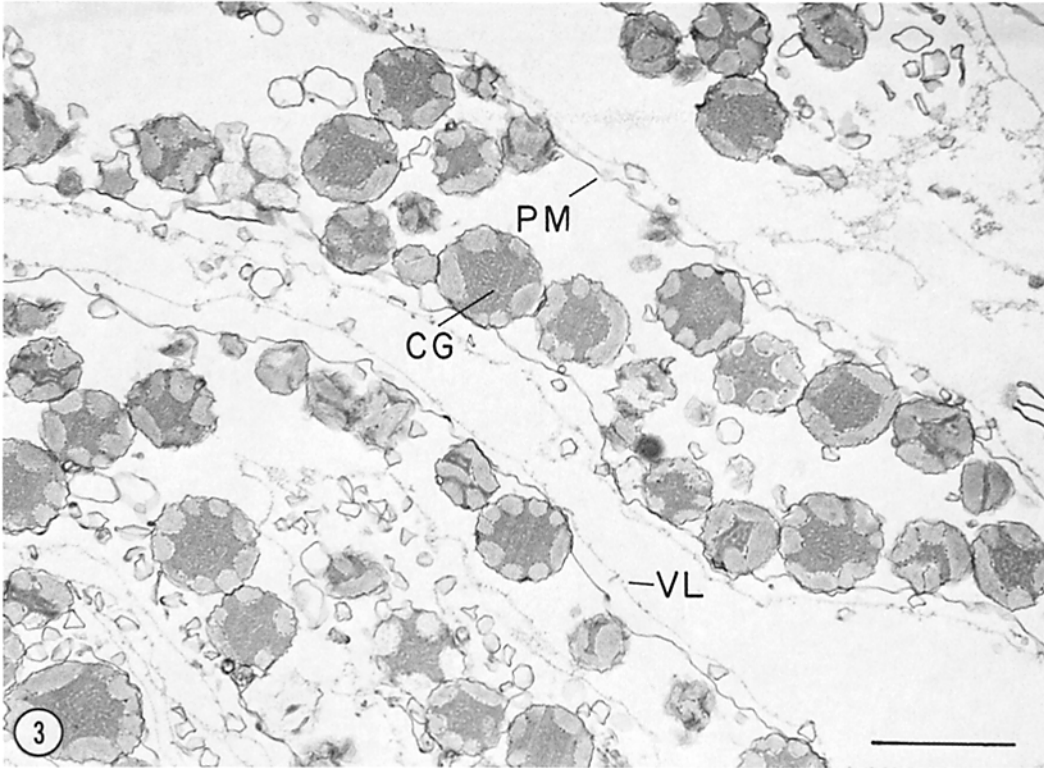


FIGURE 2 Thin sections of isolated membrane-associated cortical granules of *A. punctulata* prepared as described in Materials and Methods, and fixed in glutaraldehyde followed by osmium tetroxide. × 4,100. Bar, 2 μm.



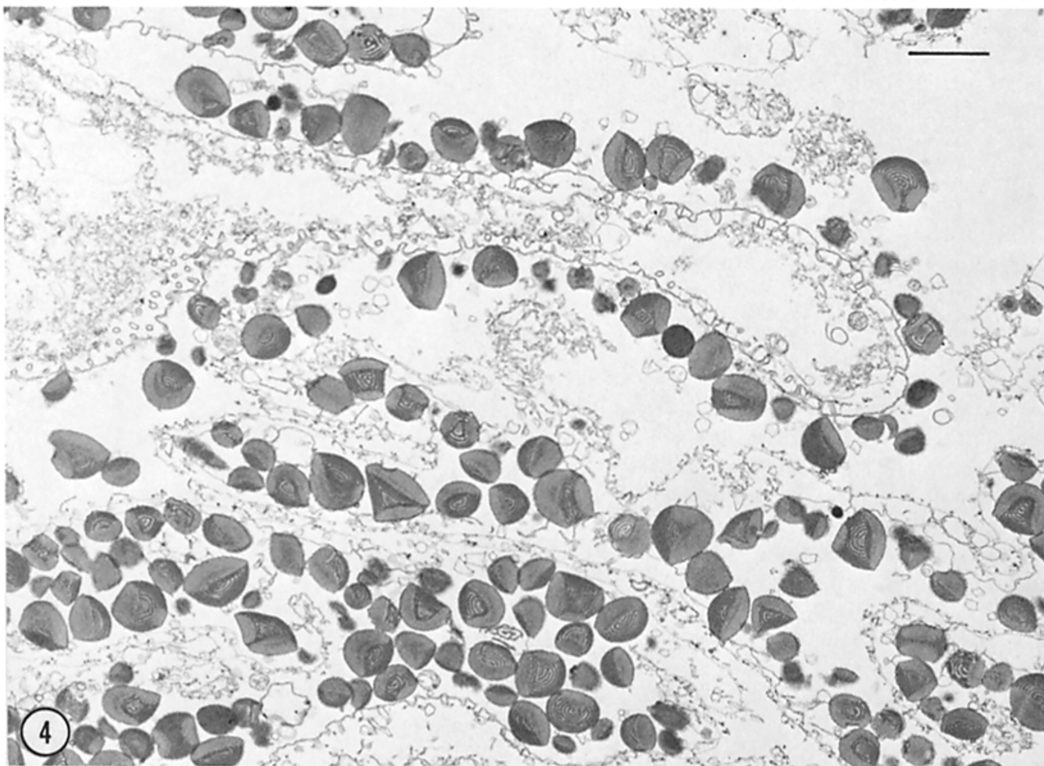


FIGURE 3 At higher magnification the close association of the cortical granules with the plasma membrane is readily evident. At many points the vitelline layer has separated from the plasma membrane. × 19,400. Bar, 1 μm.

FIGURE 4 The membrane-associated cortical granule preparations of *S. purpuratus* eggs demonstrate a structural arrangement similar to that of *A. punctulata*. Note the highly organized lamellae of the dense staining cortical granule matrix. × 5,500. Bar, 2 μm.

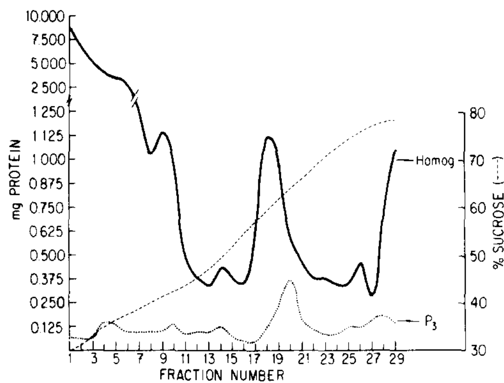


FIGURE 5 Profile of sucrose density gradient centrifugation of egg homogenate and purified membrane-associated cortical granules (P_3). Samples were analyzed as described in Materials and Methods.

reagent. Compositional analysis of the cortical granule preparation, shown in Table I, indicates that the preparation is relatively rich in neutral hexoses, hexosamine, and sialic acid. Of interest is the finding that uronic acids are absent. Finally, it should be noted that almost 25% of the total phosphate is present in phospholipids, which is not unexpected in view of the presence of both the cortical granule membrane and the plasma membrane in the preparation.

^{125}I *Labeling of Cell Surface Proteins*

To provide further evidence that the membranes associated with the cortical granules are derived from the cell surface, means to label the cell surface of intact eggs were investigated. A procedure for labeling the surface of *S. purpuratus* eggs using lactoperoxidase has been reported (42). However, despite repeated attempts, lactoperoxidase-catalyzed iodination of *A. punctulata* eggs did not yield significant labeling of cell surface components; virtually all the radioactivity incorporated was the result of ^{125}I-labeled lactoperoxidase binding to cells. Consequently, chemical iodination using chloramine-T was employed (38). Under the conditions used, eggs labeled by treatment with this reagent were fully fertilizable and capable of normal development for at least four cell divisions.

As shown in Table II, treatment of cells with $Na^{125}I$ plus chloramine-T results in a high level of incorporation of radioactivity into trichloroacetic acid-insoluble components associated with the egg. DNase and RNase do not degrade the labeled

FIGURE 6 SDS-polyacrylamide gel electrophoresis of membrane-associated cortical granules (P_3). Electrophoresis was performed on 100 μg of protein as described in Materials and Methods. The two lines near the bottom of the gel represent the boundaries of the tracking dye. The major protein (arrow) has an apparent mol wt of 100,000.

TABLE I
Compositional Analysis of Membrane-Associated Cortical Granules

Component	μmol/mg protein
	μmol
Neutral hexose	0.90
Fucose	0.08
Hexosamine	0.25
Sialic acid	0.12
Uronic acid	Not detectable
Total phosphate	0.30
Lipid phosphate	0.08

Purified granules (P_3) were analyzed as described in Materials and Methods. Results are expressed relative to the protein content.

Table II

Chloramine-T Catalyzed Iodination of Eggs

Deletion	TCA insoluble ^{125}I
	cpm
None	316,159
Eggs	570
Chloramine-T	880
Eggs and chloramine-T	477

Eggs were iodinated as described in Materials and Methods. The washed cells were precipitated and washed three times with 10% TCA, and radioactivity in the final pellet was determined.

material. About 20% of the radioactivity in the TCA insoluble material is soluble in $CHCl_3$, and probably represents labeled lipids and/or hydrophobic proteins. When the residue obtained after $CHCl_3$-CH_3OH extraction of the TCA-insoluble material is subjected to digestion with Pronase, all of the radioactivity is released in TCA soluble form. Thus, at least 80% of the ^{125}I incorporated into eggs in a TCA-insoluble form is linked to protein.

Most, if not all, of the ^{125}I-labeled protein is localized on the egg cell surface, because treatment of intact, labeled eggs with trypsin for 1 h results in the release of over 50% of the ^{125}I-labeled protein in TCA-soluble form (data not shown). Analysis of the ^{125}I-labeled proteins by SDS-polyacrylamide gel electrophoresis, before and after treatment with trypsin, revealed that all of the major labeled polypeptide peaks were found to be diminished in intensity. Thus, it appears that the majority of the labeled proteins on the surface of the egg are accessible to trypsin. After such trypsin treatment the cells are intact and viable, as over 95% of the cells can be parthenogenetically activated using the Ca^{2+} ionophore A23187 (44, 45).

Two additional lines of evidence indicate that the ^{125}I-labeled proteins are associated with cell surface components. In one experiment, cells were iodinated as described in Materials and Methods and divided into two equal aliquots. One aliquot was immediately precipitated with TCA to determine the extent of labeling in whole cells. A ghost pellet and cytoplasmic supernate were prepared from the second aliquot, and the distribution of radioactivity in TCA insoluble components was determined for both subcellular fractions. As shown in Table III, > 95% of the TCA-insoluble radioactivity that is incorporated into cells is recovered in the ghost pellet; virtually no radioactivity is associated with the cytoplasmic proteins. Analysis of the total protein content in both the cytoplasmic supernate and ghost pellet reveals that the ghost pellet contains only one-fifth of the total cell protein. Because all of the surface labeled ^{125}I-proteins are recovered in the ghost, this procedure results in a fivefold purification of the surface membranes.

In a second experiment designed to investigate the association of ^{125}I-labeled proteins with the cell surface of the egg, ghosts prepared from labeled cells were sedimented on a continuous sucrose gradient. A single peak of total protein, ^{125}I-labeled protein, lipid phosphate, and sperm receptor activity (37) was obtained at a buoyant density characteristic of membranes (data not shown).

Preparation of ^{125}I-Labeled, Membrane-Associated Cortical Granules from Labeled Eggs

Using the technique described above to label the cell surface of eggs, ^{125}I-labeled, membrane-associated cortical granules were prepared by the standard procedure. The results of two representative experiments shown in Table IV indicate that: (*a*) the recovery of protein in the purified membrane-associated cortical granule complex is 0.4–0.8% of the total protein in the homogenate, and (*b*) the specific radioactivity of ^{125}I-labeled protein is enriched 41-fold in the purified cortical granule complex. Although the recovery of ^{125}I-labeled proteins in the cortical granule complex was only 17–27% of that present in the crude

Table III

Surface Localization of ^{125}I-Labeled Proteins

	Intact cells		Cellular subfractions	
	Supernate	Pellet	Cytoplasmic supernate	Ghost pellet
^{125}I-protein, *cpm*	680	66,000	668	64,000
Total protein, %	0	100	80	20

As described in the text, eggs were labeled and divided in two equal aliquots. One aliquot was directly precipitated with TCA. The other was treated to prepare a cytoplasmic fraction and a ghost fraction, and then both were precipitated with TCA.

Table IV

Distribution of Proteins and Radioactivity in Membrane-Associated Cortical Granules Isolated from ^{125}I-Labeled Eggs

	Protein	Protein yield	Total radioactivity	Recovery of radioactivity	Specific activity	Purification
	mg	*%*	*cpm*	*%*	*cpm/mg protein*	*fold*
Experiment I						
Homogenate	100	(100.0)	1.5×10^6	(100)	1.5×10^4	(1)
P_3	0.75	0.75	4.1×10^5	27	5.5×10^5	37
Experiment II						
Homogenate	60	(100.0)	7.1×10^6	(100)	1.17×10^5	(1)
P_3	0.24	0.4	1.2×10^6	17	4.99×10^6	43

Eggs were labeled and membrane-associated cortical granules (P_3) were isolated from them as described in Materials and Methods. In Experiment I labeling was performed in seawater A, and in Experiment II it was carried out in seawater C.

homogenate, the majority (72%) of the label from the original supernate and washes of the cortical granule preparation could be recovered by high speed centrifugation. Thus, these labeled proteins probably represent small fragments of components of the cortical granule-plasma membrane complex that were generated during homogenization, but did not sediment under the low speed centrifugation used to pellet the complex.

Further evidence that the ^{125}I-labeled protein(s) highly enriched in the membrane-associated cortical granule complex are indeed an integral part of it was obtained by sucrose density centrifugation of the labeled preparation. Virtually all of the iodinated protein was found to sediment at the buoyant density (1.29 g/cm^3) of the cortical granule complex (see Fig. 5). Finally, to determine the distribution of ^{125}I label in the various protein components of the cortical granule complex, the labeled preparation was subjected to SDS-polyacrylamide gel electrophoresis. The results shown in Fig. 7 revealed that most of the radioactivity is found in the major glycoprotein present in the complex.

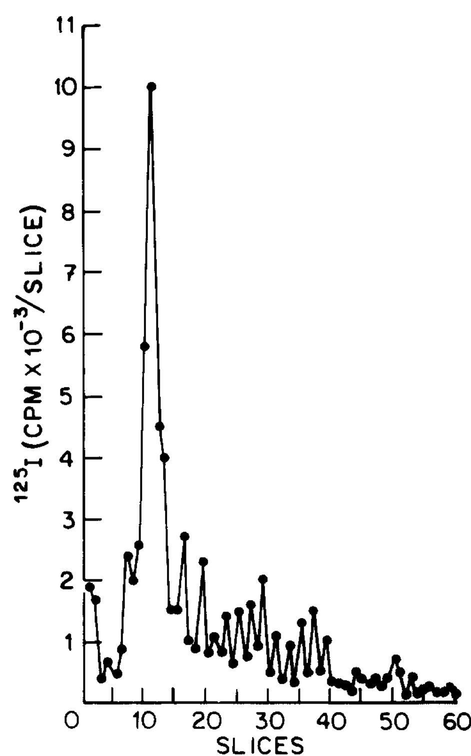


Figure 7 SDS-polyacrylamide gel electrophoresis of ^{125}I labeled, membrane associated cortical granules prepared from labeled eggs. Electrophoresis of 40 μg of protein was performed as described in Materials and Methods. The ^{125}I label, as well as the major Coomassie Blue and periodate-Schiff staining glycoprotein were localized in slices 10–14.

Enzymatic Components of Membrane-Associated Cortical Granules

Both an α-proteoesterase and a β-glucanase are released from eggs of *A. punctulata* after the cortical reaction, and it has been suggested that they are components of the cortical granule (see Discussion). To directly test this idea, membrane-associated cortical granules were analyzed for proteoesterase and β-glucanase. It was found that 25 μg of the cortical granule preparation contains proteoesterase activity equivalent to 50 ng of pure trypsin. Like trypsin, the proteoesterase is inhibited by soybean trypsin inhibitor; over 90% of the proteoesterase activity in 100 μg of the prep-

aration is inhibited by 1 μg of soybean trypsin inhibitor. A comparison of proteoesterase activity in the purified granule complex and in the crude homogenate revealed a 32-fold increase in its specific activity (Table V). In contrast, the β-glucanase was not enriched in the cortical granule complex.

Effect of Hypotonic Conditions or Calcium on Membrane-Associated Cortical Granules

Incubation of membrane-associated cortical granules in water leads to complete lysis of the granules and release of the internal contents in a soluble form. As seen in Fig. 8 *a*, most of the granule matrix has been dissipated, and the remaining membranes are largely vesiculated or sheet-like. Presumptive fragments of the vitelline layer are loosely associated with the large membrane sheets. Although treatment with Ca^{2+} under isotonic conditions also causes lysis of the granules, the electron dense matrix material is not solubilized (Fig. 8 *b*). Many membrane vesicles and sheets are also observed. In addition, the vitelline layer appears increased in thickness. Although an intact cortical granule can be seen in Fig. 8 *b*, in many experiments virtually all of the granules lysed.

To extend these electron microscope observations, the effects of hypotonic lysis and of isotonic lysis with Ca^{2+} on the release of total protein, ^{125}I-labeled protein, and proteoesterase activity were studied. The results of this study, summarized in Table VI, indicate that Ca^{2+}-induced lysis releases a relatively small proportion of both the total protein and the ^{125}I-labeled protein in a soluble form. However, most of the proteoesterase is solubilized. In the case of hypotonic lysis, a majority of the total protein and the proteoesterase is solubilized, but the ^{125}I-labeled protein remains insoluble. This finding, taken in conjunction with the electron microscope observations previously discussed, strongly indicates that, as expected, the ^{125}I-labeled glycoprotein is not a component of the electron-dense contents of the cortical granules.

DISCUSSION

Much of our current understanding of the structure, composition, and function of the cortical granules of ova of many species is based on early studies with sea urchin eggs (see 17, 43 for recent reviews). Deductions on the properties and function of the granules has been based primarily on two approaches, namely, electron microscope visualization of the granules before and after their fusion with the plasma membrane, and examination of the extracellular components released after fusion and exocytosis.

From electron microscope studies, it is clear that the limiting membrane of the cortical granules fuses with the plasma membrane at fertilization (1, 3, 11, 16). It seems possible that the resulting mosaic membrane may participate in some of the characteristic permeability changes observed after fertilization (13, 14). After fusion, the electron-dense core of the cortical granule is exocytosed and appears to interact with the vitelline layer and become part of the fertilization envelope (34, 43). The fate of the less electron-dense regions in the cortical granule surrounding this core is not known. Another structural component of the embryo, the hyaline layer, is believed to originate

TABLE V

Distribution of Proteoesterase and Glucanase in Membrane-Associated Cortical Granules

	Specific activity	Protein yield	Recovery of activity	Purification
		%	%	*Fold*
Proteoesterase*				
Homogenate	1.0×10^4	100	(100)	(1)
P_3	3.2×10^5	1	32	32
Glucanase‡				
Homogenate	45	100	(100)	(1)
P_3	40	1.1	0.97	Nil

The activity of both enzymes were determined in the crude egg homogenate and in purified cortical granules (P_3) as described in Materials and Methods.

* Expressed as cpm of [^{3}H]-CH_3OH released from ^{3}H-TAME/h/mg protein.

‡ Expressed as of glucose released/h/mg protein.

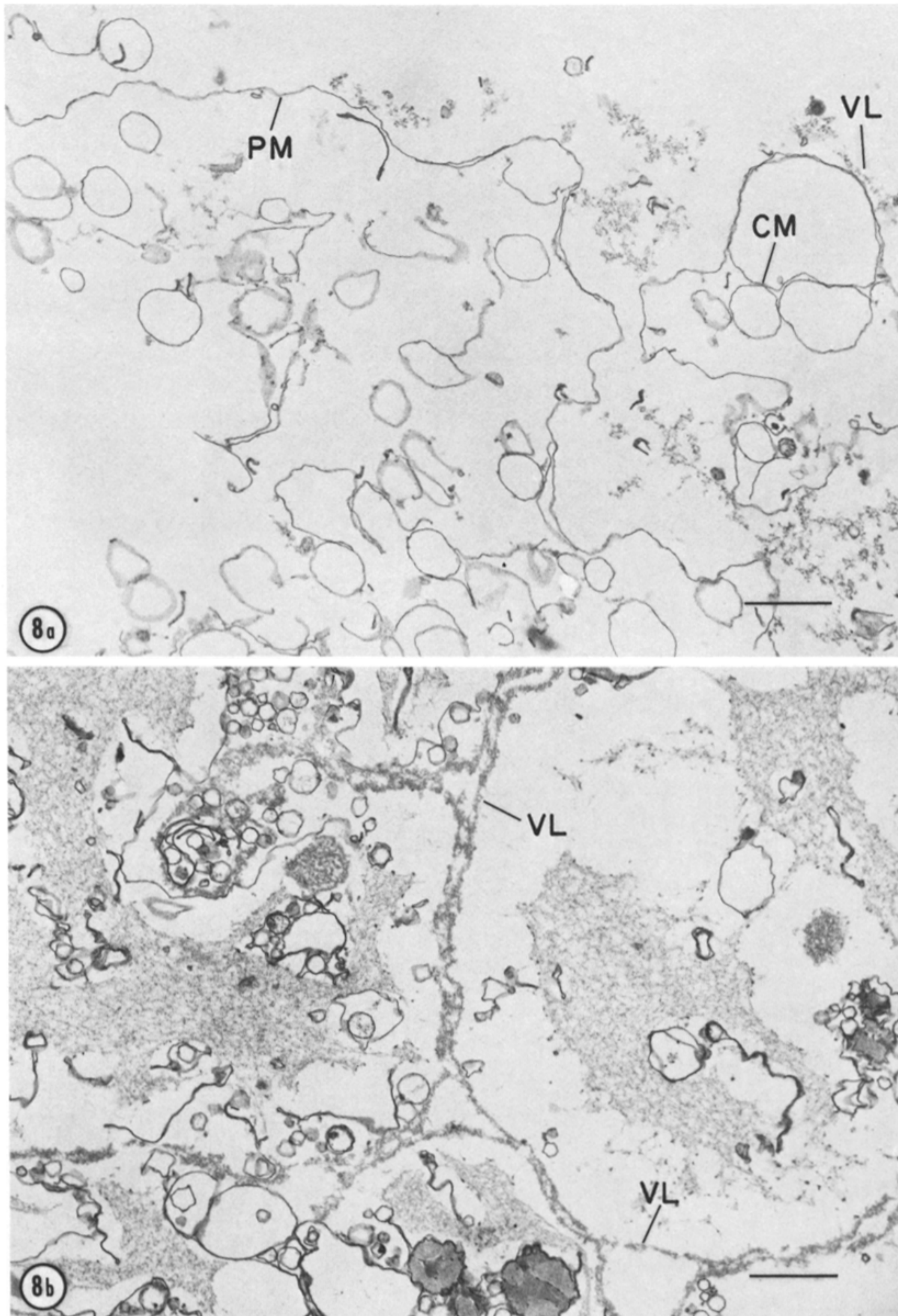


FIGURE 8 Electron micrographs of lysed cortical granule preparations. (*a*) membrane-associated cortical granules were lysed in water; a pellet was obtained by centrifugation and fixed as described in Materials and Methods. The plasma membrane (*PM*), as well as possible remnants of the cortical granule membrane (*CM*) and the vitelline layer (*VL*) are indicated. × 14,000. Bar, 1 μm. (*b*) membrane-associated cortical granules were treated with seawater A containing 10 mM Ca^{2+} for 10 min at room temperature; the pellet was fixed as described in Materials and Methods. Note that many of the membranes have extensively vesiculated, the vitelline layer appears thickened, and electron dense material typical of the granule matrix is still present. × 14,000. Bar, 1 μm.

from some component of the cortical granule (50).

The second approach, examination of the products released extracellularly upon fertilization, has revealed the presence of at least two enzymatic activities, a proteoesterase (15, 18, 46), and a β-glucanase (12). The proteoesterase activity consists of two components (4), one of which is believed to be involved in the prevention of polyspermy, probably by destruction of a receptor for sperm. The function of the β-glucanase is unknown (31).

An obvious limitation of this approach is that it does not allow one to determine unambiguously that the products released are solely of cortical granule origin. In an attempt to obviate this limitation, several investigators have devised methods for the isolation of intact cortical granules to study their chemical components (40, 41). Unfortunately, cortical granules isolated by these techniques are acknowledged to be both fragile and contaminated by yolk platelets. Clearly, to gain a detailed understanding of the chemical components of the granules and their relationship to the structural components formed upon fusion of the cortical granules with the plasma membrane of the egg, a pure, stable preparation is necessary. As discussed in the introduction, one approach to obtaining such a preparation has recently been reported by Vacquier (47, 48).

Our objective was to obtain purified preparations of intact cortical granules attached to plasma membranes in suspension. With this objective in mind, a procedure was devised for gently shearing eggs in Ca^{2+}- and Mg^{2+}-free seawater. The presence of EGTA in the homogenization medium was essential to minimize cortical granule breakdown (14, 47) and contamination with yolk platelets (40). This procedure yields large segments of the plasma membrane. Arrays of cortical granules, bounded by a typical bilayer membrane, are tightly apposed to one side of the plasma membrane. On the other side of the plasma membrane, a second layer, similar in appearance and dimensions to the vitelline layer as seen in thin section preparations of intact eggs, is evident.

To provide further documentation that this layer and the membrane to which the granules are attached are derived from the cell surface of the egg, a method to label surface components of intact eggs was devised. Using chloramine-T and $Na^{125}I$, high levels of ^{125}I could be incorporated into egg proteins without impairment of the ability

TABLE VI

Effect of Water- or Ca^{2+}-Induced Lysis on the Solubility of Components of Plasma Membrane-Associated Cortical Granules

Component	Treatment with Ca^{2+}	Treatment with H_2O
	%	%
Soluble protein	20	70
Particulate protein	80	30
Soluble ^{125}I-protein	3	7
Particulate ^{125}I-protein	97	93
Soluble proteoesterase	75	90
Particulate proteoesterase	25	10

Membrane-associated cortical granules were treated with 10 vol of H_2O or with 10 vol of isotonic, Ca^{2+}-containing seawater, centrifuged at 100,000 g, and then both the supernate and pellet were analyzed for protein.

of the egg to be fertilized. Several lines of evidence established that the label was incorporated into protein components of the cell surface; no labeling of cytoplasmic proteins was detected. Since completion of these experiments and publication of a short report (38), it has been shown by others that under appropriate conditions chloramine-T can be used to label the surface lipids of vesicular stomatitis virus (30).

Using ^{125}I-labeled eggs, membrane-associated cortical granules were prepared. The resulting labeled preparation had a specific radioactivity that was 40-fold greater than the crude homogenate, thus providing strong evidence that surface components are associated with the cortical granules. Although the total recovery of label in the cortical granule preparation was only 17–25%, most of the losses can be ascribed to generation of small fragments that are not sedimentable at low centrifugal forces (see Results). Furthermore, this recovery of a surface marker compares very favorably with the best recoveries (19%) of surface marker enzymes isolated in other systems (33). In addition, it should be noted that this 40-fold increase in specific radioactivity is consistent with the extent of purification expected based on the yield of membrane-associated cortical granules (0.5–2.0 mg of protein/100 mg of egg protein).

Several approaches were taken to characterize the membrane-associated cortical granules. Examination of the protein composition by SDS-polyacrylamide gel electrophoresis revealed the

presence of at least six separable polypeptides. The major polypeptide, based on its staining with Coomassie Blue, has an apparent mol wt of 100,000, and is rich in carbohydrates. The majority of the ^{125}I label is found in this glycoprotein, but it is unclear whether this indicates preferential labeling or merely the fact that it is the predominant protein. Compositional analysis of the cortical granule preparation revealed the presence of significant amounts of neutral hexoses and hexosamine. Interestingly, uronic acid, a component of many mucopolysaccharides, is absent. On the basis of chemical and histochemical data it is believed that mucopolysaccharides are present in the cortical granules (21, 34, 41). This conclusion is consistent with our observation (data not shown) that the membrane-associated cortical granules are stained by ruthenium red (26). Because uronic acid is a component of all known mucopolysaccharides except keratan sulfate, the absence of uronic acid indicates that mucopolysaccharides of the granule are not of uronic acid type. These observations are consistent with studies (23) indicating that sea urchin embryos contain novel mucopolysaccharides that differ from those found in higher organisms.

Efforts to determine the presence of enzymes associated with the cortical granule preparation were directed toward measurement of two enzymes known to be associated with the cell surface membrane of higher eukaryotes, oubain-sensitive ATPase, and 5'-nucleotidase (32), and two enzymes believed to be released from the cortical granules at fertilization, proteoesterase, and β-glucanase. Negligible levels of oubain-sensitive, Na^+- and K^+-dependent ATPase or a 5'-nucleotidase were detected (data not shown). Thus, these enzymes are either absent from the surface membranes of *A. punctulata* eggs, or they are inactivated during isolation of the preparation. With regard to the two enzymes believed to be released from the cortical granule at fertilization, convincing evidence was obtained that the proteoesterase is indeed specifically localized in the cortical granule. The specific activity of this enzyme in the purified membrane-associated cortical granules was 32-fold greater than that in the crude homogenate. This value for enrichment is in excellent agreement with the 40-fold purification based on recovery of ^{125}I-labeled surface protein. In contrast, no enrichment of β-glucanase in the cortical granule was detected. Although a β-1,3-glucanohydrolase activity has been suggested as a cortical granule marker (12, 40), Epel et al. (12) has shown that 60% of the activity remains in the egg after fertilization. Therefore, it is not surprising that we found that this enzyme is not specifically enriched in the cortical granules of *A. punctulata*.

To gain further insight into the properties of the cortical granule preparation and its components, the effect of hypotonicity and of Ca^{2+} ions under isotonic conditions were studied. Treatment of the preparation with water demonstrated that the cortical granules are osmotically fragile. Moreover, such treatment resulted in apparent solubilization of the electron-dense core material seen in intact granules. After resuspension and centrifugation of water-lysed, membrane-associated cortical granules, the pellet was found to consist almost exclusively of vesiculated membrane material. Concomitantly, over 70% of the total protein and 80% of the proteoesterase is released in soluble form. Under isotonic conditions the presence of Ca^{2+} causes rupture of the cortical granule. In view of the known role of Ca^{2+} in the cortical reaction (45), and the demonstration by Vacquier (47, 48) that cortical granules attached to immobilized plasma membranes rupture upon addition of Ca^{2+}, this finding was not unexpected. Of interest, however, was the finding that, unlike water lysis, Ca^{2+}-induced lysis does not result in solubilization of the electron-dense core material, and only a small proportion of the total protein (20%) is solubilized. Despite this difference in release of core material by the two treatments, both lysis procedures release at least 75% of the proteoesterase in soluble form. These findings suggest that the proteoesterase is an internal component of the granule rather than a membrane component, as it is unlikely that such dissimilar lytic conditions would both result in its detachment from membranes.

Finally, the effect of the two treatments on the solubility of the major labeled glycoprotein was investigated. Both treatments had the same effect, the majority of the labeled glycoprotein was found in the insoluble, membranous fraction. This finding, coupled with the observation that water-induced lysis released the internal electron-dense core material in soluble form, strongly suggests that the major, labeled glycoprotein resides in either the plasma membrane or the associated vitelline layer. Further studies are in progress on

the localization and function of this glycoprotein and its possible relationship to a glycoprotein reported to be released from eggs by ammonia treatment (22).

In this study we have developed a simple procedure to isolate suspensions of cortical granules adherent to the plasma membrane and its associated vitelline layer. Now that some of the basic ultrastructural and chemical properties of this preparation have been characterized, it may be feasible to investigate systematically the fusion of the membrane of the cortical granule with the plasma membrane in vitro. In addition, it should be possible to isolate and chemically characterize the components of the granule, and to define their role in formation of the hyaline layer and the fertilization envelope.

We are indebted to Ms. Ann Fuhr for her assistance in the preparation of this manuscript.

This work was supported by grants awarded to William J. Lennarz from the National Institutes of Health (HD08357-02) and the Rockefeller Foundation (GA HS 7512). Nancy K. Detering was the recipient of a special fellowship from the Rockefeller Foundation and a postdoctoral fellowship from the National Institutes of Health. Eli D. Schnell was a predoctoral trainee supported by a National Institutes of Health grant (GM00184-16).

Received for publication 2 May 1977, and in revised form 1 August 1977.

ADDENDUM

Very recently it has been shown that a peroxidase involved in fertilization envelope hardening is released from *S. purpuratus* eggs upon fertilization (Foerder, C. A., and B. M. Shapiro. 1977. Release of ovoperoxidase from sea urchin eggs hardens the fertilization membrane with tyrosine cross-links. *Proc. Natl. Acad. Sci. U. S. A.* **74.** In press.). We have detected ovoperoxidase activity in the membrane-associated cortical granules of *A. punctulata.*

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