

# CHANGES IN THE TOPOGRAPHY OF THE SEA URCHIN EGG AFTER FERTILIZATION

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## ABSTRACT

Changes in the topography of the sea urchin egg after fertilization were studied by scanning and transmission electron microscopy. *Strongylocentrotus purpuratus* eggs were treated with dithiothreitol to modify the vitelline layer and to prevent formation of a fertilization membrane. Dithiothreitol treatment caused the microvilli to become more irregular in shape, length, and diameter than those of untreated eggs. The microvilli were similarly modified by trypsin treatment. This effect did not appear to be due to disruption of cytoskeletal elements beneath the plasma membrane, for neither colchicine nor cytochalasin B altered microvillar morphology. Thus, it appears that the vitelline layer may act in the maintenance of surface form of unfertilized eggs.

Since dithiothreitol-treated eggs did not elevate a fertilization membrane, scanning electron microscopy could be used to directly observe modifications in the egg plasma membrane after fertilization. The wave of cortical granule exocytosis initiated at the point of attachment of the fertilizing sperm was characterized by the appearance of pits that subsequently opened, releasing the cortical granule contents and leaving depressions upon the egg surface. The perigranular membranes inserted during exocytosis were seen as smooth patches between the microvillous patches remaining from the original egg surface. This produced a mosaic surface with more than double the amount of membrane of unfertilized eggs. The mosaic surface subsequently reorganized to accommodate the inserted membrane material by elongation of microvilli. Blebs and membranous whorls present before reorganization suggested the existence of an unstable intermediate state of plasma membrane reorganization. Exocytosis and mosaic membrane formation were not blocked by colchicine or cytochalasin B, but microvillar elongation was blocked by cytochalasin B treatment.

The cortical reaction occurring at fertilization leads to a profound alteration of the sea urchin egg surface. Within a few seconds of sperm attachment, a rapid change occurs in the membrane potential of the egg accompanied by a partial block to polyspermy (reviewed in references 15,

17, 30, 32). These changes are immediately followed by a massive exocytosis of the cortical granule contents that is rapidly propagated from the point of sperm attachment over the surface of the egg (10, 11, 25). The vitelline layer is altered by materials released from the cortical granules and is

detached from the plasma membrane (6, 7). The cortical granule material reacts with vitelline layer components to produce the rigid structure of the fertilization membrane (3, 5, 19, 22, 26, 35). Although these phenomena are well described, the mechanisms and biochemical events involved are not clear.

In the present study, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to observe changes at the surface of *Strongylocentrotus purpuratus* eggs occurring with fertilization. The eggs were treated with dithiothreitol (DTT) to prevent formation of a fertilization membrane (13), leaving the egg surface unobscured during and after the cortical reaction. Using the fertilizing sperm as the marker for the site of initiation, it has been possible to examine the wave of cortical granule release and attendant insertion of cortical granule membrane into the plasma membrane after fertilization (11, 25). Although cortical granule exocytosis follows the general pattern of stimulus-induced exocytosis (1, 2, 27), it is a particularly fascinating example because thousands of granules fuse with the surface of the egg during a brief interval. With the release of a large number of granules, there is a substantial increase in the amount of plasma membrane and an appreciable alteration in the nature of the egg surface. The approach used in this study allowed direct observation of some aspects of the cortical reaction, confirmed earlier observations on this process and provided new information on egg surface changes after fertilization.

## MATERIALS AND METHODS

### *Chemicals*

Ruthenium red was obtained from K and K Laboratories, Plainfield, N. J. Pure glutaraldehyde (50% solution) was purchased from Electron Microscopy Sciences, Port Washington, Pa., and cytochalasin B (batch Ph 2622173-1) from Aldrich Chemical Co., Milwaukee, Wisc. Colchicine was obtained from Sigma Chemical Co., St. Louis, Mo., and tosylphenylalanylchloromethyl ketone (TPCK)-treated trypsin from Worthington Biochemical Corp., Freehold, N. J. All other chemicals were of the highest quality commercially available.

### *Handling of Gametes*

*S. purpuratus* were collected subtidally and intertidally in the Strait of Juan de Fuca and stored in the aquarium facilities of the Departments of Biochemistry and Zoology. Gametes were obtained by injection of 1–3 ml of 0.5 M KCl intracoelomically. The eggs were washed

extensively after filtration through cheesecloth. Sperm were stored dry (as collected) and diluted immediately before adding to eggs (insemination). All handling of gametes, until after fixation for microscopy or other experimentation was completed, occurred in an environmental room at 12°C. The sea water used in all the experiments was passed through 0.45  $\mu\text{m}$  Millipore filters before use and is referred to as MSW.

### *Removal of the Jelly Coat*

The jelly coat was removed by suspending eggs in MSW carefully adjusted to pH 4.5 with 0.1 N HCl (16). The eggs were allowed to sit in this acidic sea water for 2 min, after which they were diluted into a much larger volume of MSW and allowed to settle. After several washings in MSW, the eggs were employed as dejellied eggs.

### *Alteration of the Vitelline Layer*

Two different techniques were employed for altering the vitelline layer such that fertilization membranes did not form upon subsequent activation of the eggs. One technique consisted of washing the eggs with DTT as described by Epel et al. (13). Eggs were suspended in 5 mM DTT in MSW at pH 9. After sitting for 3 min in this alkaline DTT solution, they were decanted into a large excess of MSW and allowed to settle. After several washings in MSW, the eggs were used for further experiments.

A second method for altering the vitelline layer and preventing fertilization membrane formation involved treatment with trypsin as described by Runnström (31) and most recently modified by Epel (12). Unfertilized eggs were incubated in TPCK-trypsin in MSW at a final concentration of trypsin of 2.5  $\mu\text{g}/\text{ml}$ . After sitting for 20 min, the eggs were washed two times with MSW and used in further experiments. With both treatments, the eggs could be fertilized, as determined by their ability to divide and to develop normally. However, in neither case did fertilization membranes form.

### *Preparation for Electron Microscopy*

Eggs were fixed for electron microscopy at 12°C by adding 0.5 ml of eggs suspended in MSW to 0.5 ml of a solution of 4% glutaraldehyde, 1.0 mg/ml ruthenium red and 0.01 M cacodylate buffer (pH 7.8), in 80% MSW. After 1 h, the eggs were rinsed at room temperature in cacodylate-buffered MSW, diluted 4 to 1 with distilled water containing 0.5 mg/ml ruthenium red, and then fixed at room temperature for 2–3 h in a solution of one part 4% osmium tetroxide and three parts MSW, which contained 0.5 mg/ml ruthenium red and 0.01 M cacodylate buffer. After this, the eggs were rinsed repeatedly in distilled water, dehydrated in ethyl alcohol, and then split into samples for SEM and TEM. Eggs to be examined by SEM were placed in 20- $\mu\text{m}$  mesh Nytex containers in Freon TF, transferred to the pressure chamber

of a critical point drying apparatus (The Bomar Co., Tacoma, Wash.), infiltrated with Freon 13 and dried. The dried eggs were attached to stubs with silver conducting paste, coated with gold-palladium and viewed in an ETEC SEM. Eggs to be examined by TEM were embedded in Epon by the method of Luft (23), stained on section with uranyl acetate and lead citrate, and viewed in a Philips 300 TEM.

## RESULTS

### *Effect of Vitelline Layer Alteration on Microvillar Morphology*

When eggs were treated with DTT to alter the vitelline layer and to prevent formation of the fertilization membrane, there were changes in the surface morphology that were apparent by SEM. Microvilli of untreated eggs were quite regular in form, length, and distribution (Fig. 1). They were approximately 0.5  $\mu\text{m}$  long, 0.1–0.15  $\mu\text{m}$  in diameter, and about 10 were present per square micrometer of egg surface. The microvilli of eggs treated with DTT were somewhat irregular in length and diameter (Fig. 2) and appeared to be more elongate and tortuous than those of untreated eggs. The degree of alteration in microvillar morphology varied between batches of eggs, but the elongation and tortuosity were always present to some degree. These changes were apparent by TEM as well (data not shown). Microvilli of eggs treated with trypsin to alter the vitelline layer were similarly affected (Fig. 3), with some variation occurring between eggs in the same batch and between batches of eggs. Thus, the sulfhydryl reagent, DTT, and the proteolytic enzyme, trypsin, with quite different chemical mechanisms prevent fertilization membrane formation and lead to similar alterations in microvillar morphology. With both, blebs formed on 1–5% of the eggs.

To examine whether the effects of DTT and trypsin could be mimicked by inhibitors of cytoskeletal components, the effects of colchicine and cytochalasin B on microvillar morphology were determined. The microvilli of unfertilized eggs with an intact vitelline layer were not altered noticeably by treatment with either drug (Figs. 4 and 5). Eggs pretreated with  $1\text{--}5 \times 10^{-4}$  M colchicine for 15 min elevated a fertilization membrane after insemination, but 95–100% of the eggs failed to divide. Likewise, eggs treated with 0.5–5  $\mu\text{g/ml}$  cytochalasin B for 15 min elevated a fertilization membrane and failed to divide after fertilization.

Some unfertilized eggs treated with cytochalasin B developed surface blebs after 15 min. There was no effect of dimethyl sulfoxide (DMSO), used to dissolve cytochalasin B, on egg morphology or normal development.

### *Topography of the Cortical Reaction*

Eggs which had been treated with DTT were fixed at various intervals after addition of sperm. Those fixed 20–30 s after insemination had modifications of their surface that occurred with the initial part of the cortical reaction. A type of pattern seen on some eggs was one in which cortical granule contents were being released onto a region of the egg surface immediately surrounding the anterior tip of a sperm (Fig. 6). The egg surface in such an area had pits with small openings, broad depressions holding spherical masses approximately 1.2–1.3  $\mu\text{m}$  in diameter and smooth areas lacking microvilli. Other eggs were seen which had a larger zone of surface alteration around the anterior tip of a sperm. The surface near the sperm had patches of smooth membrane 2–2.5  $\mu\text{m}$  in diameter separated by irregularly shaped patches of microvilli (Fig. 7). A region of pits with small openings and of depressions holding spherical masses surrounded this zone. These features were numerous toward the sperm but decreased in frequency away from the sperm (Fig. 8). A region with some surface blebs was usually seen on the eggs peripheral to the area of most active exocytosis. TEM of thin sections of eggs at this stage revealed whorls of membranes in some of the surface pits (Figs. 9 and 10). These whorls were not seen in sections of unfertilized eggs or of eggs fixed 3 min after fertilization.

Eggs fixed 60–90 s after insemination showed the surface morphology present when the cortical reaction had ended. The eggs were covered by relatively smooth patches, 2–2.5  $\mu\text{m}$  in diameter, separated by smaller, irregularly shaped patches bearing microvilli (Fig. 11). In many of the eggs examined, microvilli were knobby and contorted and appeared to lie in a loose tangle on the surface. A few pits remained in the surface of eggs at this time.

Eggs fixed 3 min after fertilization or later showed a quite different pattern of surface morphology. The microvilli were elongate, erect, and often interconnected laterally into groups to form lamellae. The microvilli appeared to occupy much of the egg surface, but small patches of smooth

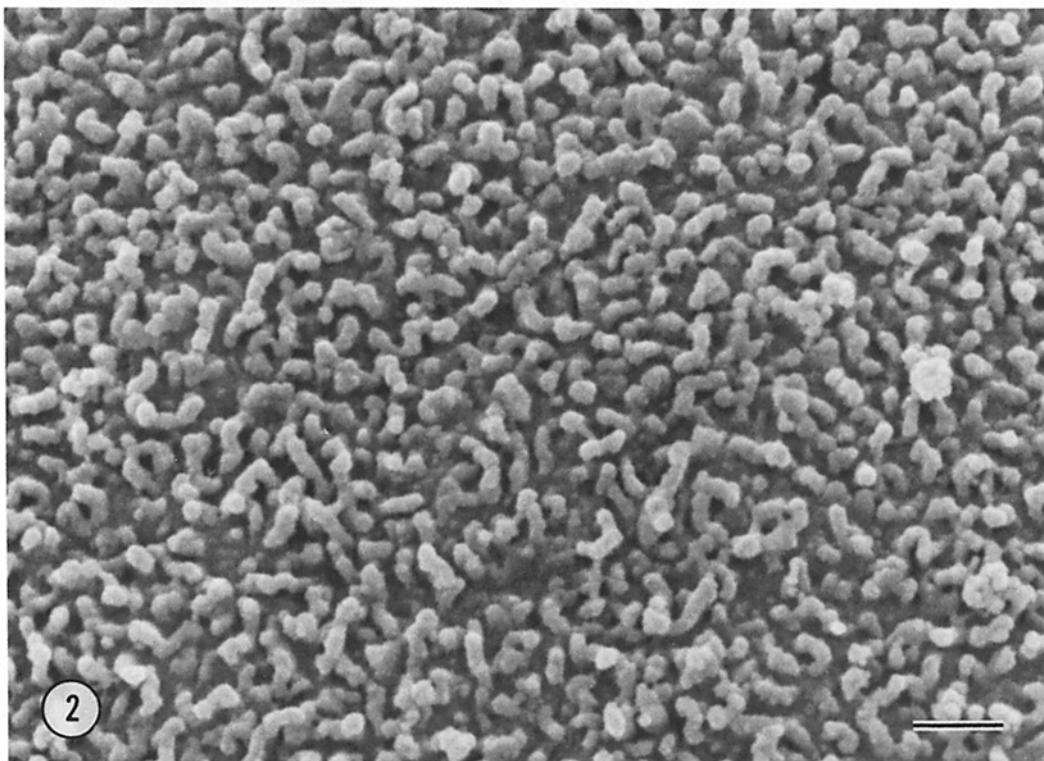
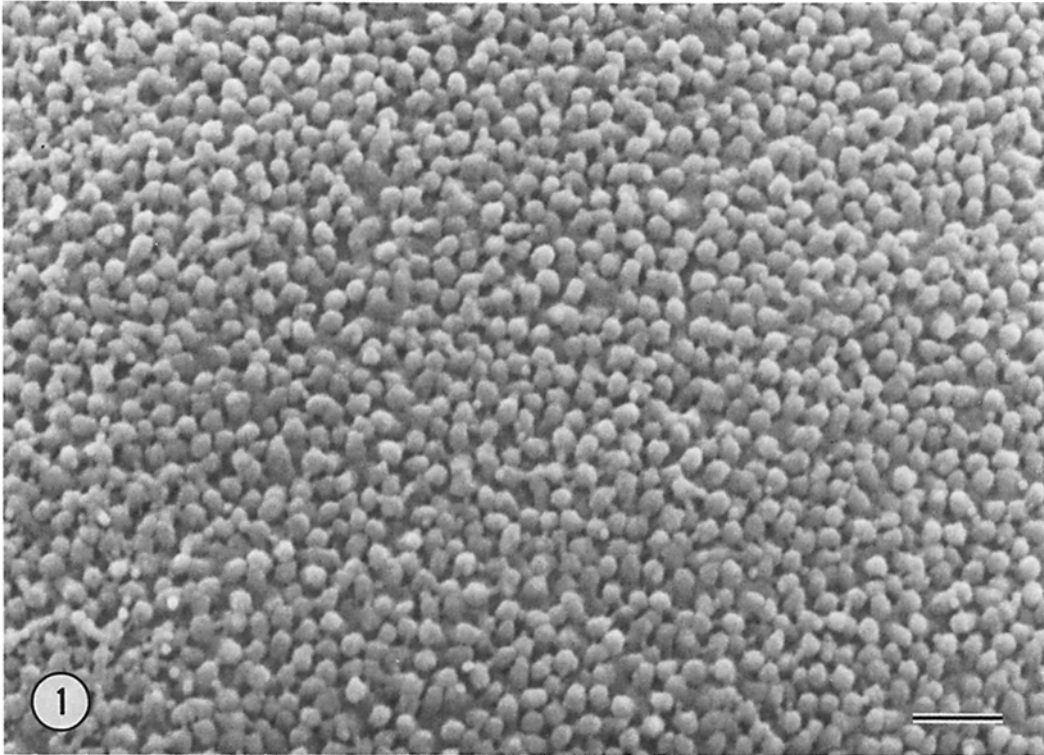


FIGURE 1 Microvilli on the surface of an unfertilized *S. purpuratus* egg. Bar equals 1  $\mu\text{m}$ .  $\times 12,000$ .

FIGURE 2 Microvilli of an unfertilized egg treated with 5 mM DTT in MSW at pH 9 for 3 min. Bar equals 1  $\mu\text{m}$ .  $\times 12,000$ .

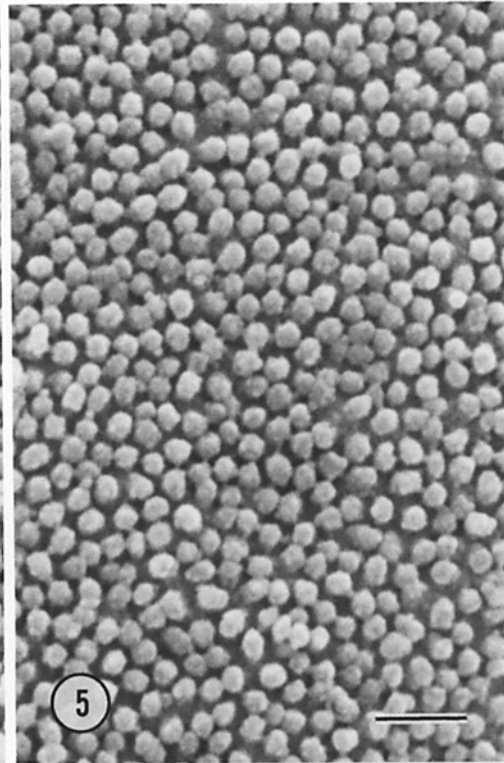
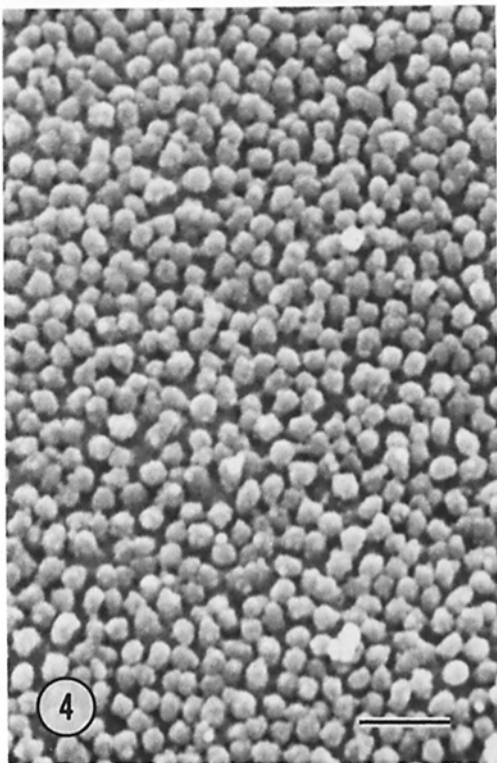
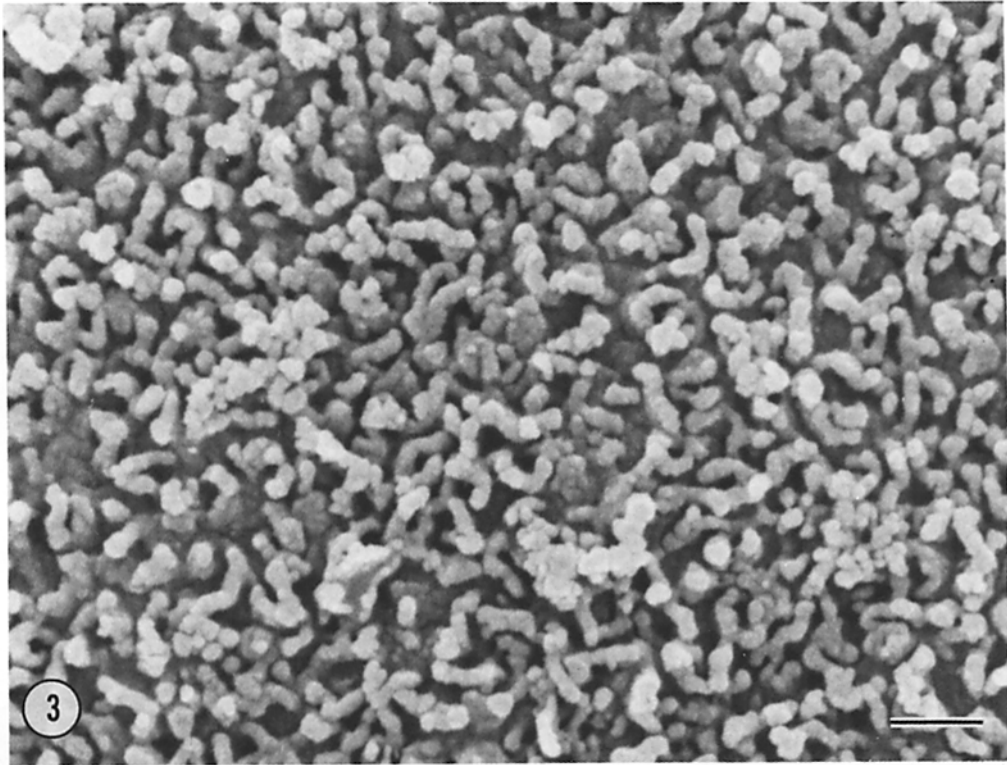


FIGURE 3 Microvilli of an unfertilized egg treated with 2.5  $\mu\text{g/ml}$  trypsin in MSW for 20 min. Bar equals 1  $\mu\text{m}$ .  $\times 12,000$ .

FIGURE 4. Microvilli of an unfertilized egg treated with  $5 \times 10^{-4}$  M colchicine in MSW for 15 min. Bar equals 1  $\mu\text{m}$ .  $\times 12,000$ .

FIGURE 5 Microvilli of an unfertilized egg treated with 2.5  $\mu\text{g/ml}$  cytochalasin B in MSW for 15 min. Bar equals 1  $\mu\text{m}$ .  $\times 12,000$ .

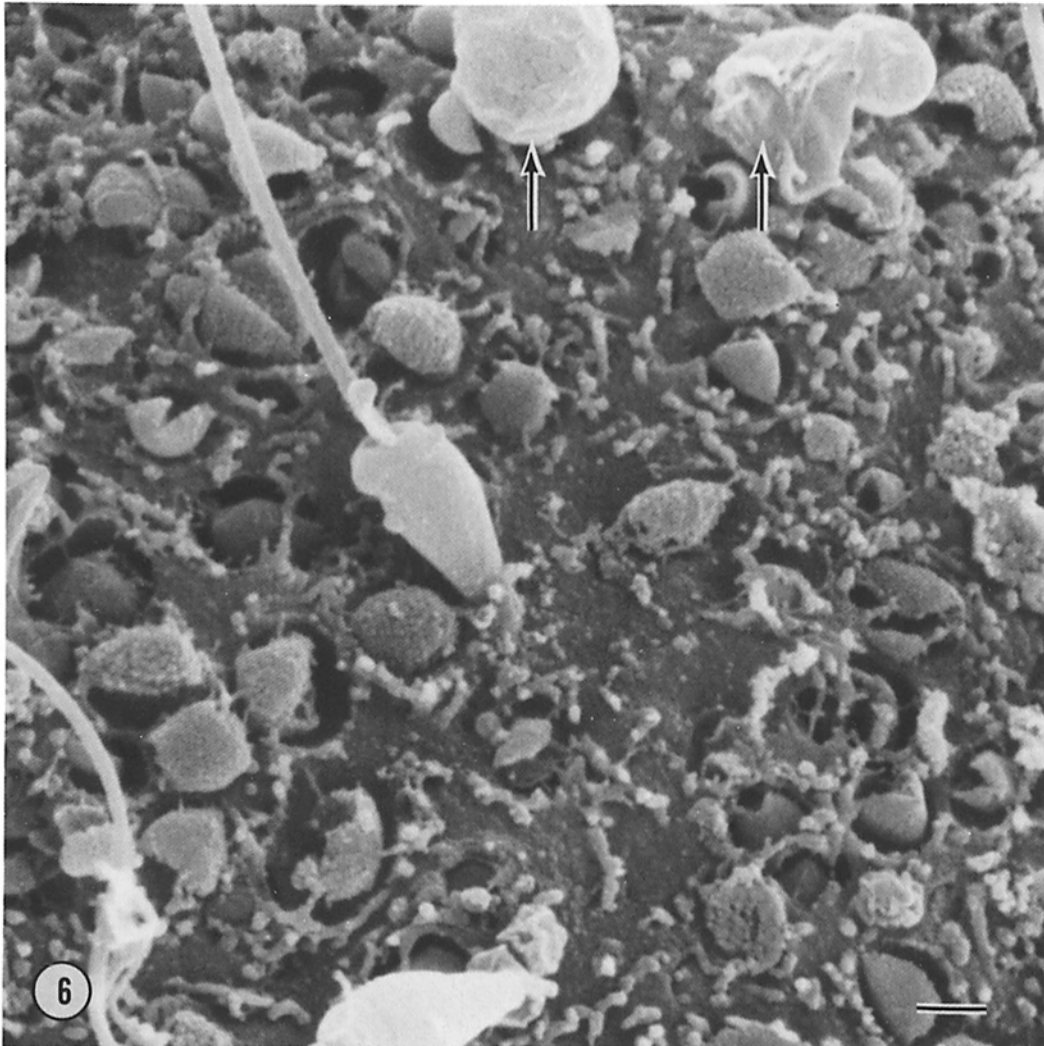


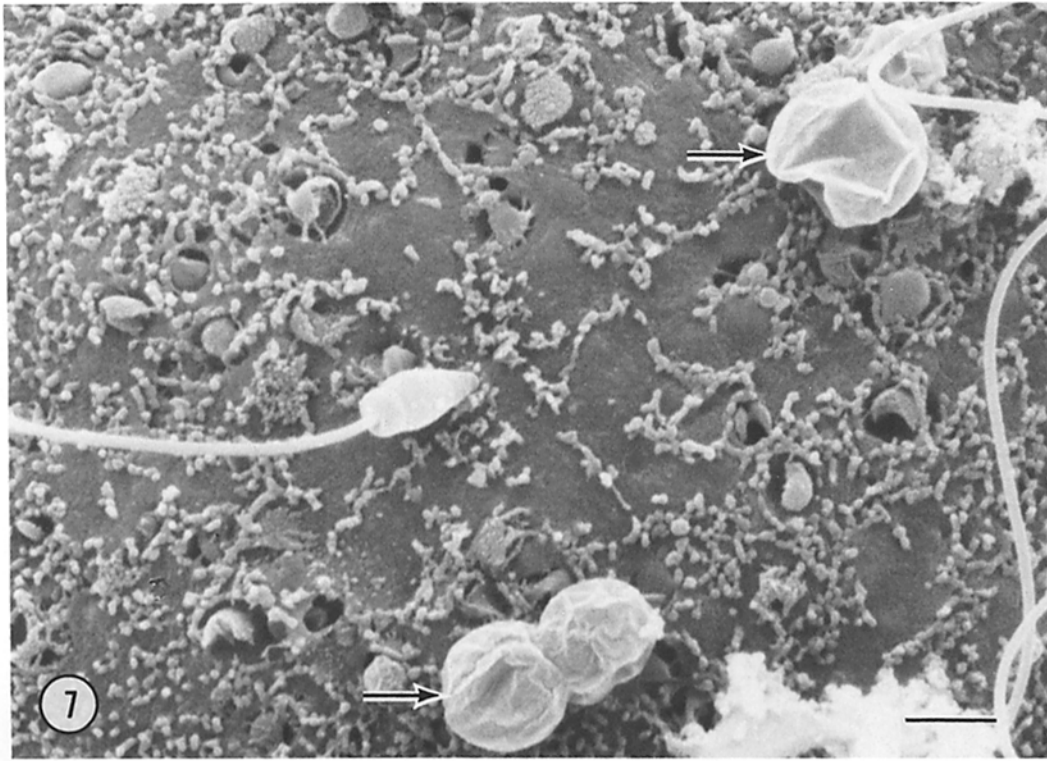
FIGURE 6 Cortical granule exocytosis on a DTT-treated egg fixed 25 s after insemination. Blebs are indicated by arrows. Bar equals 1  $\mu\text{m}$ .  $\times 9,600$ .

membrane approximately 1  $\mu\text{m}$  in diameter were present between the patches of microvilli (Fig. 12).

In order to see whether DTT treatment was responsible for the topographic changes seen with fertilization, the surfaces of eggs on which a fertilization membrane had risen normally were examined. Eggs which had not been exposed to DTT were fixed 1–5 min after insemination, and the fertilization membrane was dissected away with a micromanipulator while being observed in the SEM. The surface of dissected eggs and of eggs which had lost their fertilization membrane during

processing for SEM was comparable to that of eggs fertilized after DTT treatment (Fig. 13).

Eggs which had not been exposed to DTT were treated with colchicine or cytochalasin B and examined as described above. Eggs exposed to  $1 \times 10^{-4}$  M colchicine for 15 min, fertilized in colchicine and fixed 3 min later had elongate, erect microvilli that were interconnected to occupy much of the surface (Fig. 14). Eggs exposed to 2.3  $\mu\text{g}$  per ml cytochalasin B for 15 min before fertilization, and fertilized in cytochalasin B and fixed 3 min later had irregularly shaped microvilli that appeared to collapse onto the egg surface (Fig.



**FIGURE 7** Mosaic surface on a DTT-treated egg fixed 25 s after insemination. The fertilizing sperm is surrounded by patches of smooth membrane separated by patches of microvilli, and exocytosis is occurring peripheral to this zone. Blebs are indicated by arrows. Bar equals 1  $\mu\text{m}$ .  $\times 12,000$ .

**FIGURE 8** Spread of the cortical wave over the surface of a DTT-treated egg fixed 25 s after fertilization. Frequency of exocytosis decreases distal to the fertilizing sperm (indicated by arrow). Bar equals 1  $\mu\text{m}$ .  $\times 3,000$ .

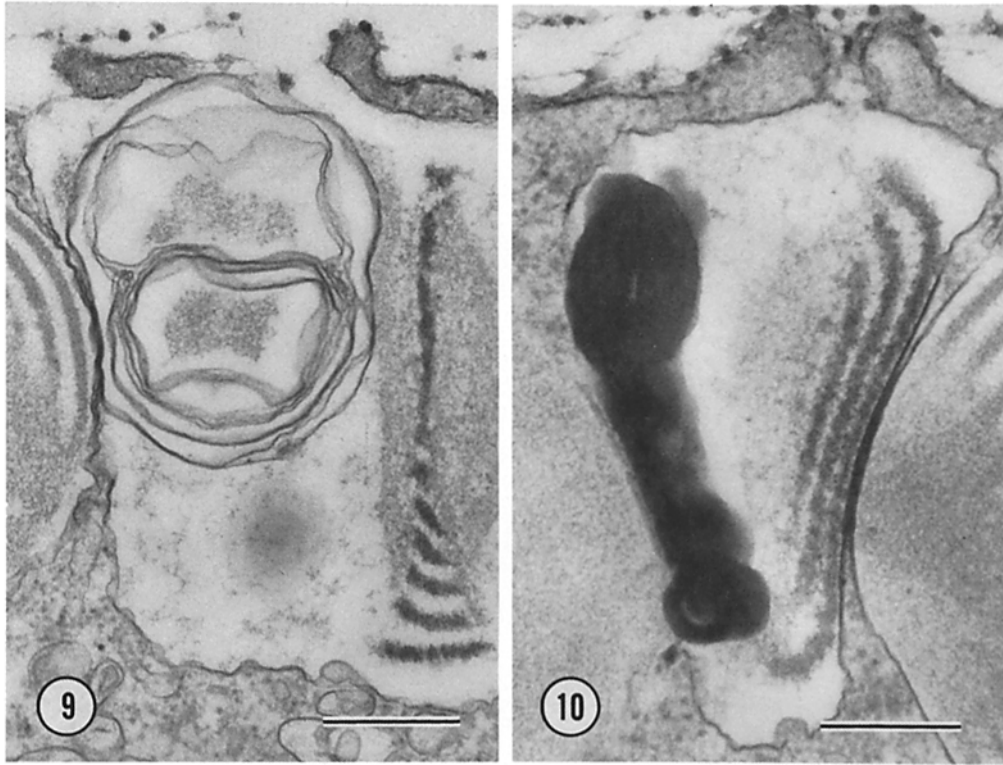


FIGURE 9 Membrane whorl in a surface pit of an egg fixed 35 s after insemination. The whorl is associated with membrane derived from a cortical granule which has become continuous with the plasma membrane. The egg was not dejellied or DTT-treated before fixation. Bar equals  $0.5 \mu\text{m}$ .  $\times 36,000$ .

FIGURE 10 Membrane whorl on a perigranular membrane during exocytosis. This micrograph appears to show an early stage of cortical granule release with only a small opening between the cortical granule and the egg surface. Egg was treated and fixed as in Fig. 9. Bar equals  $0.5 \mu\text{m}$ .  $\times 36,000$ .

15). Patches of smooth membrane,  $2\text{--}2.5 \mu\text{m}$  in diameter, were present between the patches of microvilli. DMSO, used as the solvent for cytochalasin B, had no effect on the eggs, even at  $0.4\%$  concentration, a level 10 times that used with cytochalasin B.

#### DISCUSSION

Removal or alteration of the vitelline layer of unfertilized *S. purpuratus* eggs with DTT or trypsin resulted in changes in microvillar morphology. It was recently reported that DTT did not alter the microvilli of *Lytechinus* eggs, but mechanical removal of the vitelline layer or treatment with ammonia caused microvillar elongation (24). Vacquier et al. (38) found that the microvilli of *S. purpuratus* eggs exposed to fertilization product (known to have protease activity) became disarranged and adhered to each other. Also, it was

shown that, when the vitelline layer was removed from *Spisula* eggs, the eggs lost their rigidity and became flaccid (39). These observations suggest that the vitelline layer may serve a role in the maintenance of egg surface form. However, any treatment which alters the vitelline layer may also lead to secondary alterations in other cellular components. To test the possible role of cytoskeletal elements, eggs with intact vitelline layers were exposed to colchicine or cytochalasin B, agents presumed to disrupt microtubules or microfilaments. Although these agents blocked division after fertilization, the microvillar morphology of unfertilized eggs was not altered.

This study has shown that when DTT is used to prevent formation of the fertilization membrane, dramatic changes may be observed at the surface of sea urchin eggs after fertilization. The spread of the cortical reaction in a wave from the initiation



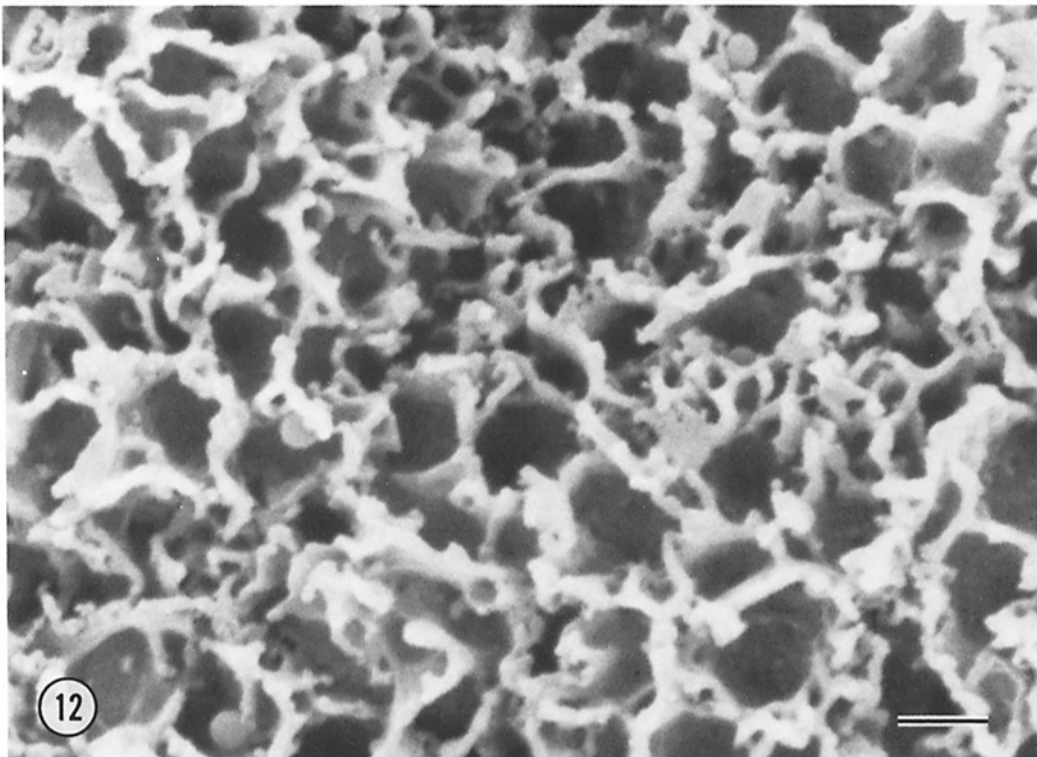
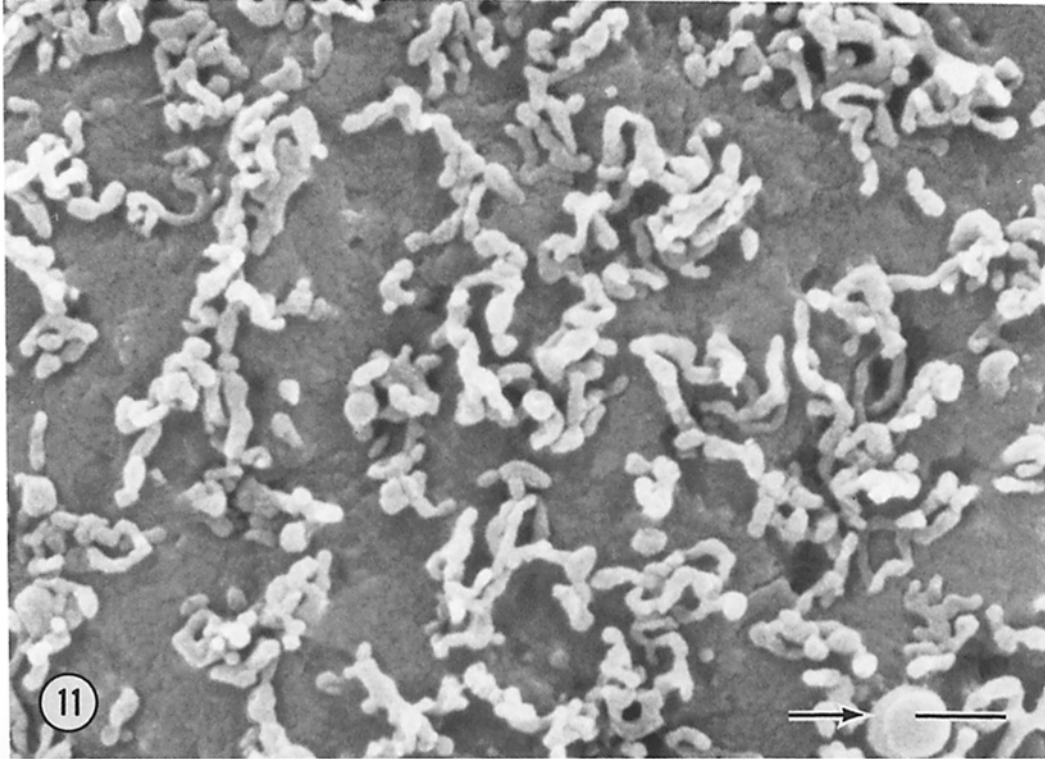


FIGURE 11 Mosaic plasma membrane of a DTT-treated egg fixed 60 s after insemination. Patches of smooth membrane lie between patches of microvilli. The microvilli are knobby and contorted and lie in a loose tangle on the surface of the egg. Pits and an occasional bleb (arrow) are present. Bar equals 1  $\mu\text{m}$ .  $\times 12,000$ .

FIGURE 12 Topography of a DTT-treated egg fixed 8 min after fertilization. The microvilli are quite long, erect and interconnected to form lamellae. Small patches of smooth membrane are present between the microvilli, preserving the mosaic pattern. Bar equals 1  $\mu\text{m}$ .  $\times 12,000$ .

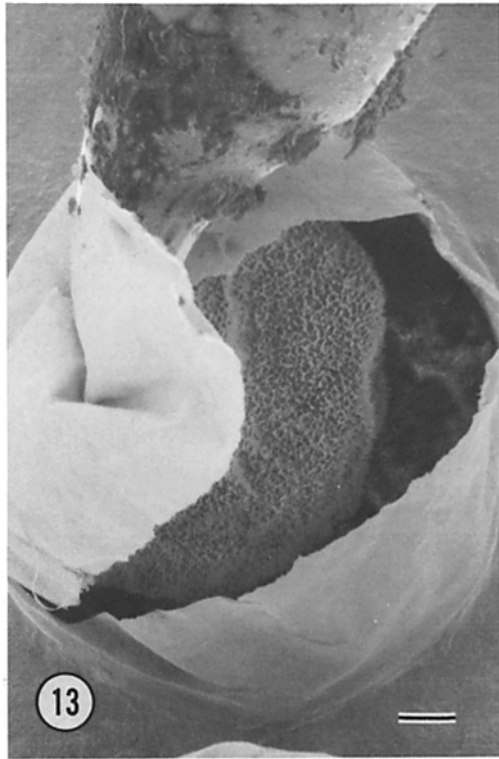


FIGURE 13 Removal of the fertilization membrane with a micromanipulator in the SEM. The egg was fixed 5 min after fertilization. Topography is comparable to that of eggs fertilized after DTT treatment. Bar equals  $10\ \mu\text{m}$ .  $\times 750$ .

site marked by a sperm was accompanied by sequential alterations in surface morphology. Many of the changes seen were what would be expected from previous studies of the cortical reaction (3, 10, 11, 15, 22, 25, 32) and are interpreted in light of these previous data. Exocytosis of cortical granules occurs by fusion of perigranular membrane with plasma membrane to form pits in the egg surface (Figs. 6-8). These pits open to become

depressions that contain the cortical granule contents. As the cortical wave spreads from the sperm, the cortical granule contents detach from the egg surface, leaving smooth patches. This produces a topographically mosaic egg plasma membrane, composed of smooth patches, derived from perigranular membrane, and of microvillous patches, derived from the original plasma membrane (Figs. 11-13). This mosaic surface is then reorganized by the elongation of microvilli and by the reduction in size of the smooth patches. Thus, the changes at the surface of sea urchin eggs after fertilization are the consequences of exocytosis of cortical granules, formation of a topographically mosaic surface, and elongation of microvilli.

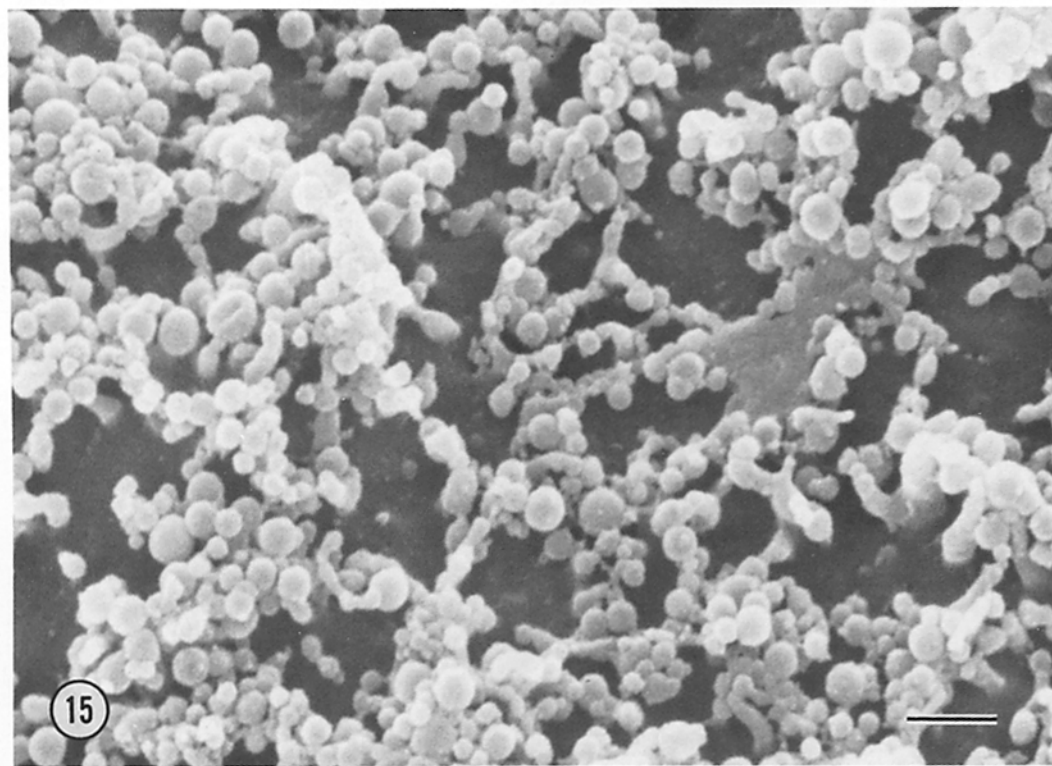
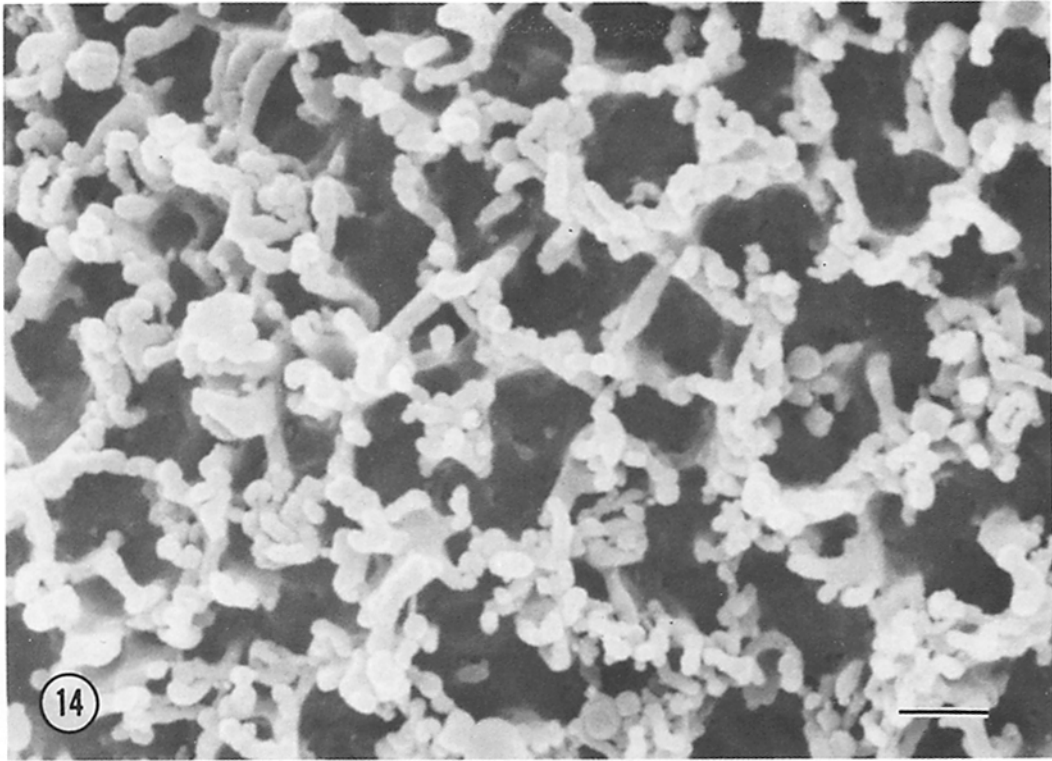
This study of sea urchin fertilization provided the opportunity for direct observation of some aspects of exocytosis. The focal site of initiation is marked by the fertilizing sperm, and exocytosis spreads over the surface of the egg in a wave from that site. The perigranular membrane appears to be elevated to the level of the plasma membrane during this process. The mechanisms responsible for cortical granule release are not known, but colchicine and cytochalasin B, agents known to disrupt exocytosis in other systems (1, 2), did not block elevation of the fertilization membrane in this study, or calcium-induced cortical granule break-down in a previous study (37).

The mosaic surface produced by the cortical reaction was reduced, but still recognizable, after microvillar elongation. The smooth patches and microvillous patches did not migrate together to intermix before microvillar elongation, indicating that there is restricted mobility of topographical features of fertilized eggs. This restriction may be due either to heterogeneity of the plasma membrane, or to structural organization of the underlying cytoplasm. In the first case, the perigranular membrane may be of sufficiently different composition to remain as patches within the plasma

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FIGURE 14 Microvilli of an egg treated with  $1 \times 10^{-4}$  M colchicine in MSW for 15 min before insemination and until fixed 3 min after insemination. The microvilli which develop beneath the fertilization membrane are elongate and erect and separated by small patches of smooth membrane. Bar equals  $1\ \mu\text{m}$ .  $\times 12,000$ .

FIGURE 15 Microvilli of an egg treated with  $2.3\ \mu\text{g/ml}$  cytochalasin B in MSW for 15 min and until fixed 3 min after fertilization. The microvilli which develop beneath the fertilization membrane are irregular and contorted and collapse onto the egg surface. Smooth patches of surface membrane are present between the microvilli. Bar equals  $1\ \mu\text{m}$ .  $\times 12,000$ .



membrane. Although proteins and lipids are usually thought to undergo rapid lateral diffusion in membranes (4, 9, 14, 33), lateral phase separation of lipids may occur (28, 29). In the second case, there is evidence for a structural network in the cortex of sea urchin eggs that may be responsible for some mechanical properties (18, 37). Cytoskeletal elements have been implicated in cell surface events, including exocytosis and endocytosis (1, 2), phagocytosis (36), and the restriction of lectin receptor mobility (8), because of antagonistic effects of colchicine and cytochalasin B on these activities. In the present study, the formation of a mosaic surface after fertilization was not blocked by treatment with colchicine or cytochalasin B for 15 min, although microvillar elongation was disturbed by the latter drug. The absence of inhibition of mosaic membrane production is not sufficient evidence to eliminate the involvement of cytoskeletal elements of course, and further work is required to determine what restricts lateral mobility on the surface of the fertilized egg.

Cortical granule exocytosis results in an increase in the amount of plasma membrane (11, 25, 37). It has been reported previously that the *S. purpuratus* egg (80  $\mu\text{m}$  in diameter) has a total surface area of  $2 \times 10^4 \mu\text{m}^2$  (37). However, the egg is not a smooth sphere, but has about  $2 \times 10^5$  microvilli on its surface which serve to increase the surface area. The total surface area of the plasma membrane of the egg is probably more on the order of  $6 \times 10^4 \mu\text{m}^2$ . The cortical granules have an average surface area of about  $5 \mu\text{m}^2$  and there are about 15,000 cortical granules per egg (37). Thus, when the cortical reaction occurs and the mosaic membrane forms, the egg surface receives approximately  $7.5 \times 10^4 \mu\text{m}^2$  of new membrane, more than doubling the egg surface. This correlates well with recent findings that the number of low-affinity concanavalin A binding sites on the sea urchin egg surface doubles after fertilization (Veron and Shapiro, manuscript submitted for publication). Furthermore, the observations in this study suggest that the eggs accommodate the increase in surface membrane after fertilization by elongation of microvilli. This process was blocked by cytochalasin B, but not by colchicine, indicating that microfilaments may be involved. In addition, microfilaments have recently been observed in association with elongating microvilli of *Lytechinus* eggs (20).

In the period before microvillar elongation,

blebs formed on the egg surface and membranous whorls appeared in the cortical granule pits. These structures may have reflected an unstable intermediate state of plasma membrane organization that occurred during formation of the mosaic plasma membrane. This does not necessarily mean that the structures seen after fixation were present in the living state; the blebs and whorls may have been artifacts. Nevertheless, they were not present on unfertilized eggs or on eggs fixed 3 min after fertilization, suggesting that these unusual membranous features were indicative of a transient membrane lability.

It is somewhat unusual that the sea urchin egg accommodates new plasma membrane by microvillar elongation. Augmentation of surface membrane due to exocytosis in other systems is often handled by endocytosis in order to recycle the membrane material (reviewed in references 1, 2, 27, 29). However, it is of interest that features similar to those which were interpreted as unstable intermediate membrane forms on sea urchin eggs have been seen both during exocytosis by mast cells (21) and in new membrane formation after injury to the plasma membrane of *Amoeba proteus* (34). In the latter case, not only were membranous figures present at the cell surface during membrane replacement, but they were demonstrated only when ruthenium-containing fixatives were employed. Labile membrane features were also best seen on sea urchin eggs with ruthenium-containing fixatives. These results suggest that, although the sea urchin eggs eventually accommodate new plasma membrane differently than do most cells, the unstable intermediate membrane state which they experience may be a more general phenomenon.

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