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Effects of Cytochalasin B on Sperm–Egg Interactions

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The effects of cytochalasin B (CB) on the interaction of sea urchin (*Arbacia*), molluscan (*Spisula*), and mammalian (*Mus*) gametes have been examined. Despite the absence of sperm incorporation and gamete membrane fusion, CB-treated *Arbacia* and *Spisula* eggs (1–10 μg/ml for 1–10 min) mixed with sperm activated. Unlike the situation observed in *Arbacia* and *Spisula*, mouse eggs treated with CB (1–50 μg/ml for 1 hr) are capable of sperm incorporation. These data are discussed with reference to possible mechanisms by which sperm may induce eggs to activate.

INTRODUCTION

The cortical granule reaction, increased protein synthesis, and initiation of DNA replication are only a few of the many processes initiated by sea urchin eggs at fertilization (Epel, 1975). These events are generally looked upon as a part of egg activation, a phenomenon brought about by the interaction of the gametes or by parthenogenetic agents. The mechanism by which spermatozoa induce eggs to activate is unknown. Activation of the ovum is presumably a result of attachment of the sperm to the surface of the egg or fusion of the plasma membranes of the gametes (gamete fusion) soon after sperm binding (Epel, 1978).

Recent investigations by Jaffe (1976) indicate that a significant change occurs in the membrane potential of the ovum within 3–30 sec after adding sperm to suspensions of eggs. This interaction has no apparent effect on sperm behavior, which is not altered until 20 sec later, presumably at the time when the gametes fuse (Epel *et al.*, 1977). Fusion of ova and sperm has been observed in specimens fixed 30 sec after insemination (Franklin, 1965; Summers and Hylander, 1974; F. J. Longo, personal observations). Some of the events occurring during the brief interval from the addition of sperm to gamete fusion have been examined ultrastructurally (Summers and Hylander, 1975; Summers *et al.*, 1975) but their relation to egg activation has not been established. Epel (1978) has suggested that the membrane potential change reported by Jaffe (1976) may be triggered when the fertilizing spermatozoon interacts with components at the surface of the egg prior to fusion of the gametes.

Related to the means by which sperm induce eggs to activate is the question of the presence of receptors on the surface of eggs which are capable of responding in a specific manner to sperm adhesion (Aketa, 1973; Schmell *et al.*, 1977). Such a response might bring about the activation of the ovum and the subsequent incorporation of the spermatozoon. Hence, such receptors might be analogous to hormone or neurosecretory receptors reported for some somatic cells (Cuatrecasas, 1974).

Isolation of sperm fractions which by themselves activate eggs has not been reported. However, antibodies to eggs act as parthenogenetic agents (Baxandall *et al.*, 1964), suggesting that perturbation of surface components might indeed lead to activation of the ovum.

Recent investigations have demonstrated that echiurid and echinoid eggs treated

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with cytochalasin B and mixed with sperm undergo an activation response but do not incorporate sperm (Byrd *et al.*, 1977; Gould-Somero *et al.*, 1977; Longo, 1977). In this communication, observations concerning the effects of cytochalasin B on the interaction of echinoid, molluscan, and mammalian gametes are reported. Results of these studies provide insights into possible mechanisms by which sperm may induce eggs to activate.

MATERIALS AND METHODS

Experiments employing *Spisula* and *Arbacia* gametes were carried out at the Marine Biological Laboratory, Woods Hole, Mass., and the University of Iowa, Iowa City. Animals supplied by Gulf Specimen, Panacea, Fla. (*Arbacia*), or the Marine Biological Laboratory (*Arbacia* and *Spisula*), and maintained in an aquarium containing artificial sea water (Instant Ocean; Aquarium Systems, Inc., Eastlake, Ohio), were employed for the investigations conducted at the University of Iowa. Cytochalasin B (CB; Aldrich Chemical Co., Milwaukee, Wis.) was dissolved in dimethyl sulfoxide (DMSO; 10 mg/ml) and was added to the gametes with swirling to ensure rapid mixing. Gametes of *Arbacia* and *Spisula* were obtained according to methods outlined by Costello *et al.* (1957). Unfertilized eggs were recovered from superovulated virgin, random-bred Swiss mice (10 to 12 weeks old) as previously described (Inoue and Wolf, 1975). Mouse eggs were transferred directly to microdishes having 200 μl of a modified Krebs–Ringer bicarbonate medium (Inoue and Wolf, 1975) containing CB (1 to 50 μg/ml) and covered with silicon oil. The eggs were mixed with approximately 10^5 sperm/ml (Wolf and Inoue, 1976) and incubated at 37°C in the presence of 5% CO_2 in air. Eggs, recovered at 2-hr intervals for 12 hr, were fixed and processed for electron microscopy as described by Anderson *et al.* (1975).

Unfertilized *Arbacia* and *Spisula* eggs were suspended in various concentrations (0.001 to 10 μg/ml) of CB for 1 to 30 min and examined live with Nomarski optics or prepared for light and electron microscopy according to Longo and Anderson (1972). On occasion, sea urchin eggs treated with CB were also incubated in nicotine [Matheson, Coleman and Bell, Norwood, Ohio; 0.1% (v/v)] and handled as described above. Controls included ova that were incubated in 0.1% DMSO or 0.1% DMSO and 0.1% nicotine and manipulated in the same manner as CB-treated eggs.

Activation of *Arbacia* eggs was determined microscopically by the formation of a fertilization membrane and the discharge of cortical granules. *Spisula* eggs were considered activated if germinal vesicle breakdown and meiotic spindle formation occurred. Eggs are referred to as inseminated or fertilized only when sperm or male pronuclei were observed within them.

The effects of CB on the interaction of *Spisula* and *Arbacia* gametes was assessed by: (1) incubating eggs in various concentrations of CB (0.001 to 10 μg/ml) and mixing with sperm or (2) incubating sperm in 10 μg/ml of CB and mixing with a suspension of eggs. Samples were taken at 15-sec intervals for 4 min after mixing the gametes and prepared for light and electron microscopy. Eggs were examined to determine the percentage activated (i.e., having elevated fertilization membranes or undergoing germinal vesicle breakdown) and containing incorporated sperm.

As a means of verifying our structural observations that despite the absence of sperm incorporation, CB-treated eggs mixed with sperm activated, the percentage incorporation of [^{3}H]thymidine was analyzed. *Arbacia* eggs were suspended in 10 μg/ml of CB for 1 min, then incubated in [^{3}H]thymidine (1 μCi/ml) 1 min after mixing with sperm. Eggs were also (1) left untreated or (2) incubated in 10 μg/ml of CB following sperm incorporation (i.e., 1 min after mixing of the gametes). Forty minutes after mixing with sperm, eggs were washed in sea water (4°C) and treated with

10% trichloroacetic acid (TCA). TCA-insoluble material was digested with tissue solubilizer (Eastman, Rochester, N.Y.) and mixed with 10 ml of Scintiverse (Fisher, St. Louis, Mo.); a portion of the TCA-soluble material was also mixed with the scintillation cocktail. Samples were counted in a Beckman LS-3133 liquid scintillation counter.

RESULTS

Effects of CB on Unfertilized Arbacia Eggs

In all groups examined, the microvilli of unfertilized eggs treated with concentrations of CB from 1 to 10 μg/ml for 1 to 10 min were enlarged and seen to contain a dense aggregation of filamentous material (Figs. 1 and 2). In some eggs, particularly those that were activated spontaneously by CB, the vitelline layer separated from the bases of microvilli (Fig. 3). Similar morphological changes were not observed in the microvilli or vitelline layer of eggs treated in 0.1% DMSO or in 0.1% DMSO and 0.1% nicotine.

In most instances, eggs were not activated when incubated with CB. However, certain groups of eggs treated with CB did exhibit a cortical granule reaction (Table 1). The basis for this difference in the response of some groups of ova to CB was not determined, due, in part, to its sporadic occurrence. Groups of eggs demonstrating spontaneous activation by CB comprised less than 10% of all of those that were studied during the course of this investigation. With increasing time in CB, greater numbers of eggs demonstrated a cortical granule reaction. With a 30-min incubation in 10 μg/ml of CB, almost 50% of the eggs examined possessed fertilization membranes. Similar results were also obtained when eggs were treated with 1 μg/ml of CB.

It was also observed that CB-treated eggs incubated in nicotine, an agent that induces surface changes in cells and has been used to promote polyspermy in sea urchins (Brinson, 1974; Clark, 1936; Johnson and Epel, 1975; Carroll, 1975; Longo and Anderson, 1970; Schwartz, 1976; Werle and Schievelbein, 1965; Westfall and Brasted, 1972), decreased threefold the number of eggs activated by CB (Table 1). Since CB occasionally induced the cortical granule reaction in *Arbacia* eggs, all experiments were monitored for spontaneous activation.

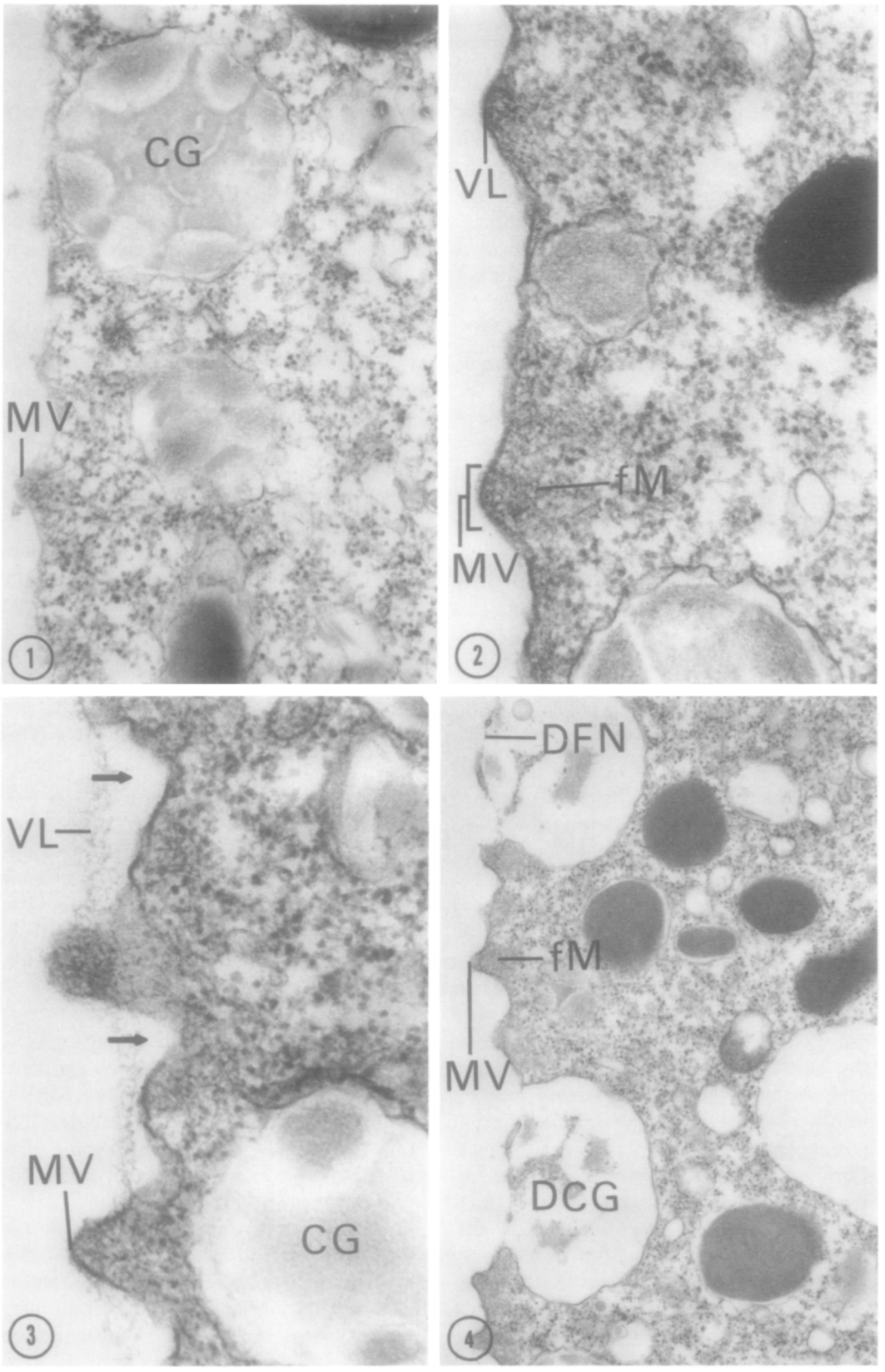
Effects of CB on Arbacia Sperm

Spermatozoa were incubated in 10 μg/ml of CB for 10 min and mixed with untreated ova. Due to dilution upon mixing of the gametes, the concentration of CB was reduced 1000-fold to 10 ng/ml. All eggs examined were inseminated 1 min after mixing of the gametes with no apparent adverse effect on fertilization. When compared to untreated specimens, sperm motility and the acrosome reaction appeared unaffected (cf. also Gould-Somero *et al.*, 1977; Sanger and Sanger, 1975; Brunhouse *et al.*, 1972; Everhart and Rubin, 1974; Niemierko, 1975).

Interaction of CB-Treated Arbacia Ova and Spermatozoa

Time required to activate CB-treated eggs. When CB-treated eggs were incubated with moderately high concentrations of sperm (5×10^7 sperm/ml), all ova developed fertilization membranes by 4 min after mixing of the gametes (Table 2). Controls, eggs not treated with CB, formed fertilization membranes by 1 min after mixing of the gametes. Light and electron microscopy of sectioned specimens indicated that the delay in activation of CB-treated eggs was due to a retarded initiation of the cortical granule reaction. Once initiated, development of the fertilization membrane progressed at the same rate as in untreated ova.

Inasmuch as sperm appeared to be unaffected by CB, it was reasoned that this agent influenced either gamete interaction or the actual mechanism(s) by which eggs are activated. Moreover, in those groups showing spontaneous activation, all eggs treated for a total of 8 min in CB (4 min



FIGS. 1–4

TABLE 1

ACTIVATION OF *Arbacia* EGGS INCUBATED IN CYTOCHALASIN B[a]

Length of incubation in 10 μg/ml of cytochalasin B (min)	Activation (%)	
	−Nicotine	+Nicotine [0.1% (v/v)]
5	13 ± 2	6 ± 2
10	24 ± 5	8 ± 1
15	43 ± 1	11 ± 4
30	48 ± 1	14 ± 5

[a] Only those groups of eggs containing ova which were spontaneously activated by CB are included. Samples of eggs were taken at the times indicated, fixed, and observed microscopically for the percentage having elevated a fertilization membrane.

before and 4 min after mixing with sperm) formed fertilization membranes (Table 2), while only 24% were activated when incubated in the presence of CB alone for 10 min (Table 1). Consequently, at least 76% of the ova from these groups, when mixed with sperm, are activated by virtue of their association with spermatozoa and not by CB. When groups of eggs not spontaneously activated by CB were mixed with sufficient sperm, all activated by virtue of their interaction with spermatozoa.

The delay in activation of CB-treated eggs can be overcome by the addition of nicotine, in that 97% of the ova are activated by sperm 1 min after mixing of the gametes (Table 2). The action of nicotine in this case was not determined.

Effects of various concentrations of sperm on CB-treated eggs. Ova were treated with 10 μg/ml of CB, inseminated with various concentrations of sperm, and fixed 5 min after mixing of the gametes. As demonstrated in Table 3, much higher sperm concentrations, about 50-fold greater than those in controls, were required to activate approximately 100% of the CB-treated ova.

TABLE 2

ACTIVATION OF CYTOCHALASIN B-TREATED *Arbacia* EGGS BY SPERM

Time after mixing of gametes (min)[a]	Activation (%)		
	−Cytochalasin B	+Cytochalasin B (−nicotine)	+Cytochalasin B [+nicotine; 0.1 (v/v)]
0	0	4	3
0.5	81	10	31
1.0	100	32	97
2.0	100	56	100
4.0	100	100	100

[a] Eggs were preincubated in 10 μg/ml of CB for 4 min, then mixed with 5×10^7 sperm/ml. Samples of eggs were taken at the times indicated, fixed, and observed microscopically for the percentage having elevated a fertilization membrane.

TABLE 3

ACTIVATION OF CYTOCHALASIN B-TREATED *Arbacia* EGGS BY DIFFERENT SPERM CONCENTRATIONS[a]

Sperm concentration (number of sperm/ml)	Activation (%)	
	−Cytochalasin B	+Cytochalasin B (10 μg/ml for 10 min)
4×10^5	61	23
1×10^6	98	42
6×10^6	100	64
3×10^7	100[b]	88
5×10^7	100[b]	96

[a] Eggs (15,000/ml) were fixed 5 min after mixing with sperm and observed microscopically for the percentage having elevated a fertilization membrane.

[b] Suspension contained polyspermic ova.

Effects of various concentrations of CB on fertilization. Eggs were treated with CB at concentrations of 0.001–10 μg/ml for 1–10 min and mixed with sperm (Table 4 and Figs. 9A–C). Samples, taken 5 min after mixing of the gametes, were examined for

FIG. 1. Surface of an unfertilized *Arbacia* egg. CG, cortical granule; MV, microvilli. × 55,000.

FIG. 2. Surface of an *Arbacia* egg treated with 10 μg/ml of CB for 10 min, showing microvilli (MV) containing filamentous material (fM). CG, cortical granule; VL, vitelline layer. × 70,000.

FIG. 3. CB-treated *Arbacia* egg in which the vitelline layer (VL) has separated from a portion of the surface of the egg (arrows) while remaining attached to the apices of the microvilli (MV). CG, cortical granule. × 70,000.

FIG. 4. Portion of a CB-treated *Arbacia* egg which was mixed with sperm and is undergoing the cortical granule reaction. Note the microvilli (MV) containing filamentous material (fM) that persist in those regions located between dehiscing cortical granules. DFM, developing fertilization membrane. × 27,000.

TABLE 4

EFFECT OF MIXING SPERM WITH EGGS TREATED IN DIFFERENT CONCENTRATIONS OF CYTOCHALASIN B[a]

Concentration of cytochalasin B (μg/ml)	Eggs activated (% having fertilization membranes)	Eggs fertilized (% containing incorporated sperm nuclei)
0	100	100
0.001	100	100
0.01	100	100
0.1	100	47
1.0	100	0
10.0	100	0

[a] Eggs were incubated in cytochalasin B 10 min prior to mixing with sperm. Samples were taken 5 min after mixing with sperm, fixed, and observed microscopically for the percentage having elevated fertilization membranes (activated) and the percentage containing incorporated sperm nuclei (fertilized).

the percentage of eggs having elevated fertilization membranes and containing incorporated sperm. Ova from all groups exhibited a cortical granule reaction and the elevation of a fertilization membrane. However, sperm incorporation failed to occur in eggs treated with 1–10 μg/ml of CB for 1–10 min. Approximately 50% of the eggs treated with 0.1 μg/ml of CB and all of the eggs treated with concentrations of CB less than 0.1 μg/ml contained incorporated sperm nuclei (Table 4). When CB (10 μg/ml) was added to egg suspensions 1 min after mixing with sperm, all were inseminated. To define more precisely the time required to prevent sperm incorporation, CB (10 μg/ml) was added to eggs at 15-sec intervals, from 1 min before to 1 min after mixing with sperm. Eggs containing incorporated sperm nuclei were first observed in suspensions mixed with sperm 30 sec before CB treatment. Because of this rapid effect upon incorporation, eggs were incubated in CB for at least 1 min prior to mixing with sperm (Tables 2–4).

If eggs were treated for 10 min with 10 μg/ml of CB, washed with sea water (three times with a total of 1 liter), and immediately mixed with sperm, ova activated but did not incorporate sperm. Similar results were also obtained when ova were treated with trypsin to remove the vitelline layer (0.02% trypsin for 10 min or until a fertilization membrane failed to form upon insemination), incubated in 10 μg/ml of CB, and mixed with sperm.

Ultrastructural Observations of CB-Treated Arbacia Eggs Activated by Sperm

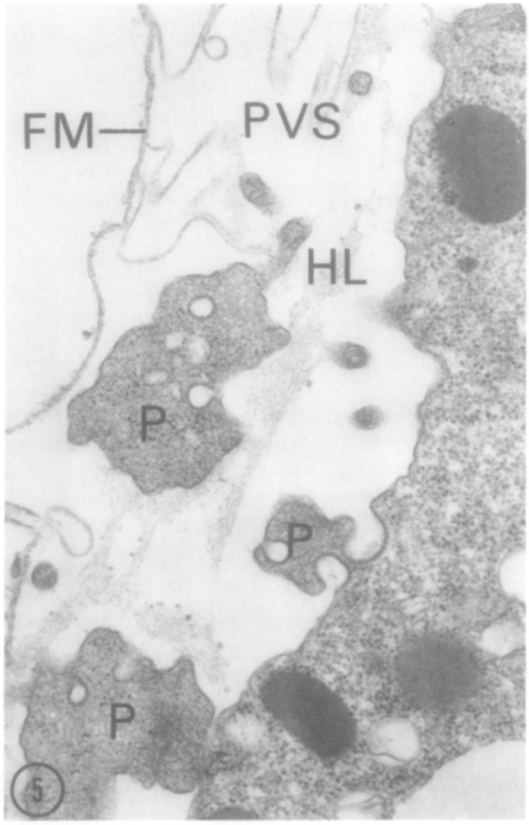
Cortical changes of CB-treated eggs mixed with sperm. The dehiscence of cortical granules in a CB-treated egg is shown in Fig. 4. Cytoplasmic regions located between dehiscing cortical granules persisted and eventually filled with filamentous material (Figs. 4, 12, and 13). By 5 min postactivation, numerous irregular projections containing filamentous material emanated from the surface of the egg and occupied much of the perivitelline space (Figs. 5 and 6). Frequently, activated eggs were observed containing many undehisced cortical granules. In such cases, the cortical granules were surrounded by a dense aggregation of filamentous material (Fig. 8).

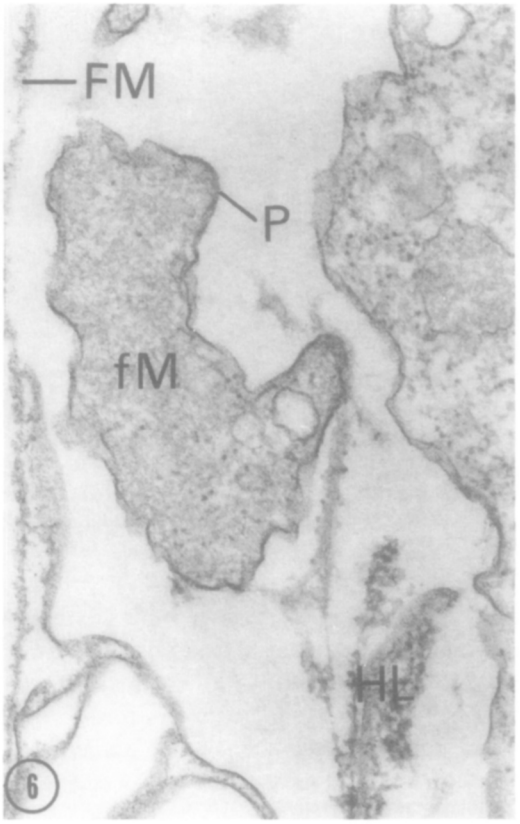
Eggs treated with 0.01 μg/ml of CB for

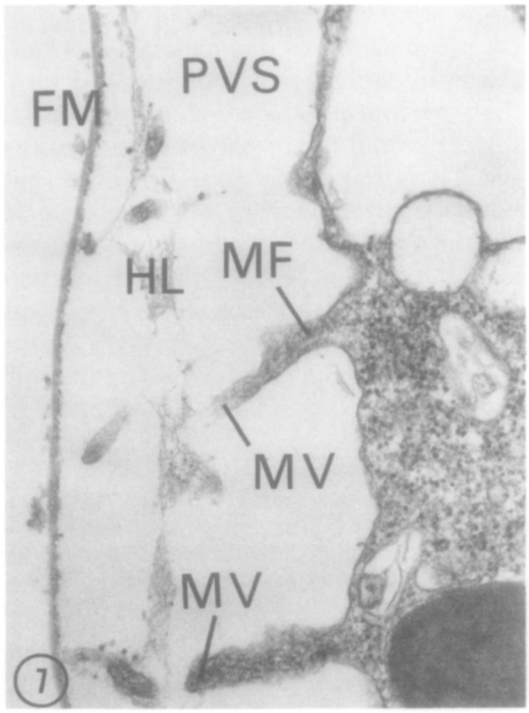
FIGS. 5 AND 6. Surface of CB-treated *Arbacia* eggs (10 μg/ml for 10 min) that have been activated with sperm (5 min after mixing of the gametes). The surface of the activated ova is reflected into numerous projections (P) containing a dense aggregation of filamentous material (fM). FM, fertilization membrane; HL, hyaline layer. Fig. 5, × 18,000; Fig. 6, × 35,000.

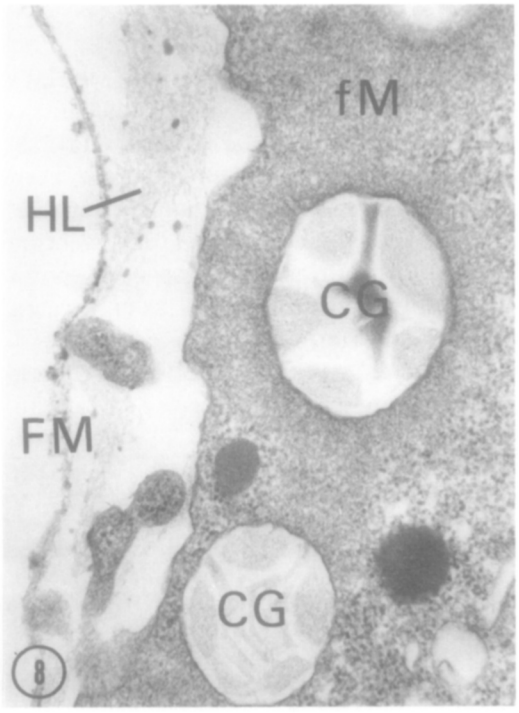
FIG. 7. Surface of an *Arbacia* egg treated with 0.01 μg/ml of CB for 10 min and then inseminated (5 min postinsemination) which is morphologically similar to untreated ova. Note the long microvilli (MV) which project into the perivitelline space (PVS) and the absence of large irregular extensions of the egg cortex, filled with filamentous material, characteristic of eggs treated with higher concentrations of CB. FM, fertilization membrane; HL, hyaline layer; MF, microfilaments; PVS, perivitelline space. × 45,000.

FIG. 8. Portion of a CB-treated *Arbacia* egg (10 μg/ml for 10 min) activated with sperm which contains undehisced cortical granules (CG) surrounded by filamentous material (fM). FM, fertilization membrane; HL, hyaline layer. × 55,000.









FIGS. 5–8

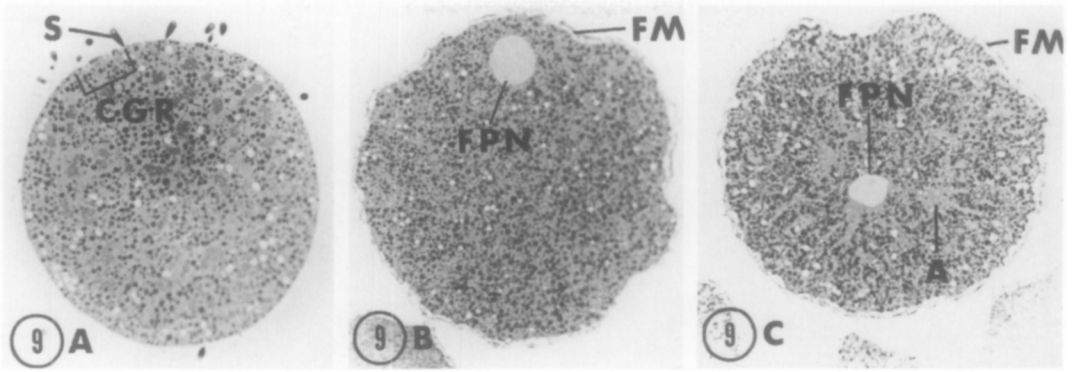


FIG. 9. *Arbacia* eggs treated with 10 μg/ml of CB for 1 min and then mixed with sperm. (A) One minute after mixing of the gametes. Sperm (S) are "attached" to the egg which is initiating cortical granule breakdown (CGR). (B) Activated, CB-treated ovum 4 min after mixing of the gametes. No signs of sperm incorporation were observed in such specimens. FM, fertilization membrane; FPN, female pronucleus. (C) Activated, CB-treated ovum containing a centrally placed female pronucleus (FPN) and asters (A), 12 min after mixing of the gametes. × 500.

10 min before insemination were structurally similar to untreated specimens (Fig. 7). These eggs lacked the large cortical projections filled with filamentous material typical of eggs treated with higher concentrations of CB and possessed long microvilli characteristic of untreated specimens (Fig. 7).

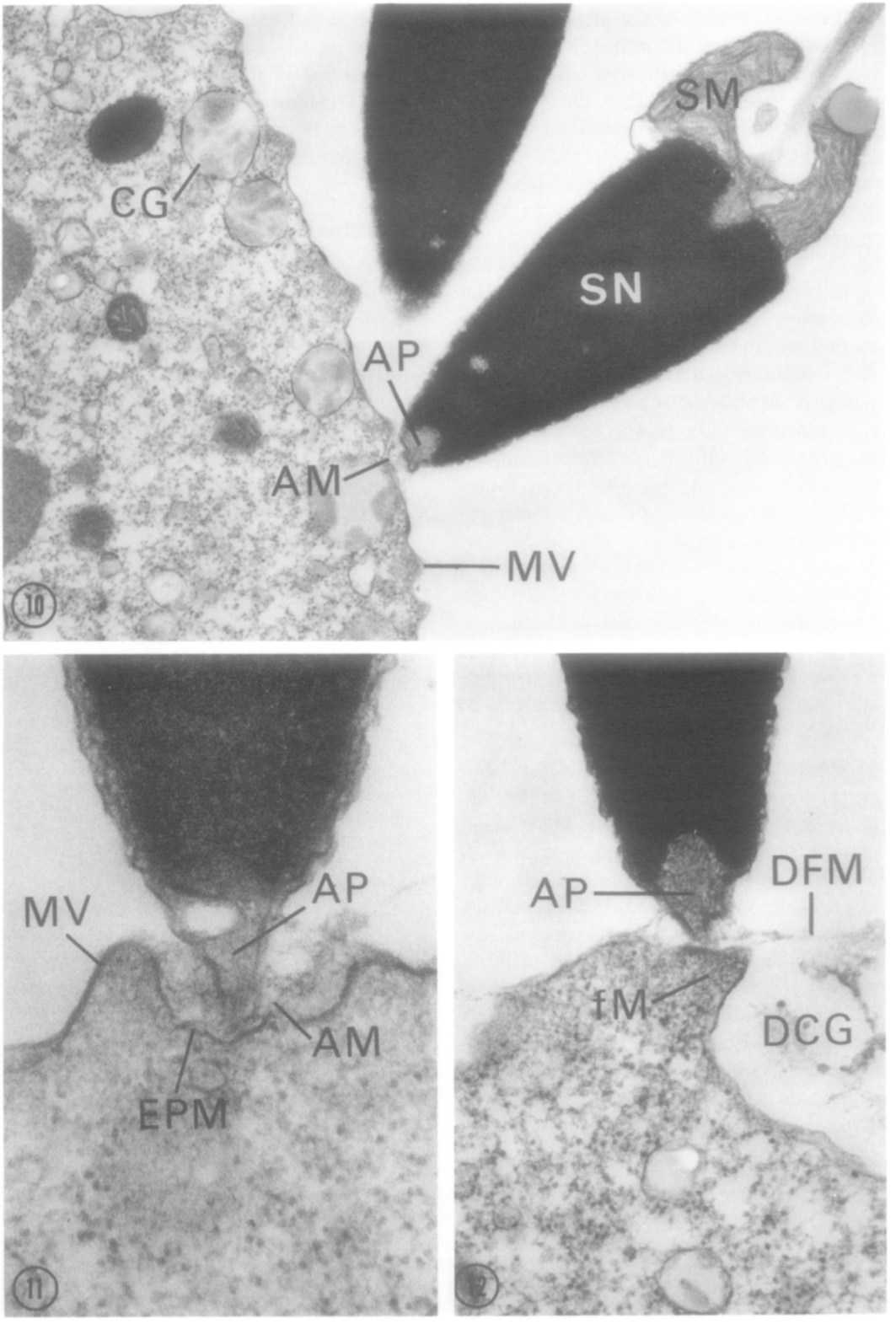
Interaction of CB-treated eggs and sperm. As sperm were not incorporated into eggs treated with appropriate concentrations of CB, attempts were made to fertilize such ova, by either incubation in high sperm concentrations (greater than 10^8 sperm/ml) or the addition of 0.1% nicotine to eggs 5 min prior to mixing with sperm (Longo and Anderson, 1970). In both cases, sperm incorporation failed to occur in CB-treated ova. Morphologically, CB-treated eggs incubated in the presence or the absence of nicotine and subsequently mixed with sperm did not differ.

Sperm were observed associated with CB-treated eggs 15 to 30 sec after mixing of the gametes (Figs. 10 and 11). The acrosomal process of the spermatozoon was usually positioned along the base of or in regions separating microvilli of the egg, in close proximity to the oolemma. Amorphous material, presumably derived from the acrosome and the vitelline layer, joined the acrosomal tubule to the surface of the egg. There was no evidence suggesting that fusion occurred between the membrane of the acrosomal process and the egg plasma membrane. The cortical granule reaction was observed in specimens fixed as early as 30 sec after the addition of sperm.

Sperm, surrounded by dehiscing cortical granules, were "attached" to the surface of

FIGS. 10 AND 11. CB-treated *Arbacia* eggs (10 μg/ml for 10 min) 30 s after mixing with sperm. Sperm are observed closely associated with the cortex of the egg in Fig. 10. Figure 11 depicts a higher magnification of a spermatozoon associated with the cortex of a CB-treated egg. The tip of the acrosomal process (AP) is in close proximity to the plasma membrane (EPM) of the egg and is embedded in some amorphous material (AM; see also Fig. 10). SN, sperm nucleus; MV, microvilli, CG, cortical granules; SM, sperm mitochondria. Fig. 10, × 28,000; Fig. 11 × 70,000.

FIG. 12. CB-treated *Arbacia* egg (10 μg/ml for 10 min) 45 sec after mixing with sperm that has initiated cortical granule breakdown in the region associated with an "attached" spermatozoon. DCG, dehiscing cortical granule; DFM, developing fertilization membrane; AM, amorphous material associated with the acrosomal process and surface of the egg; fM, filamentous material; AP, acrosomal process. × 66,000.



FIGS. 10–12

eggs fixed at 30 and 45 sec after mixing of the gametes (Figs. 12 and 13). Although these images suggested that such sperm may have been responsible for initiating egg activation, we were unable to demonstrate this conclusively. As the cortical granule reaction ensued, spermatozoa were lifted from the surface of the egg with the separation of the vitelline layer (Figs. 14 and 15). By 1 min after mixing the gametes, few if any sperm were associated with the fertilization membrane of activated eggs (see figs. 9B and C).

CB-treated eggs activated by sperm were examined at periodic intervals for up to 1 hr. In many specimens, the female pronucleus migrated centrad and the cytoplasm contained asters (Fig. 9C) which were found to possess aggregations of endoplasmic reticulum and microtubules when examined ultrastructurally. Activated specimens failed to undergo mitosis and cleavage even after prolonged washing with sea water.

Incorporation of [^{3}H]Thymidine into CB-Treated Arbacia Eggs Activated by Sperm

Previous experiments (Estensen, 1971; Estensen and Plagemann, 1972; Graff *et al.*, 1977; Plagemann and Estensen, 1972) demonstrated inhibition of amino acid and nucleoside transport into cells treated with CB. Similar results were also observed during the course of this investigation (Table 5). The percentage incorporation of [^{3}H]-thymidine into eggs incubated in 10 μg/ml of CB 1 min before the addition of sperm was approximately 50% of that observed in untreated specimens or in eggs incubated in CB 1 min after mixing with sperm (Table 5). The reduction of [^{3}H]thymidine incorporation in CB-treated eggs by 50% would be expected if sperm failed to enter the ovum. This result is consistent with microscopic observations indicating that sperm are not incorporated into CB-treated ova.

Effects of CB on Mouse and Surf Clam (Spisula) Eggs

Activation and morphological alterations in the surface of *Spisula* eggs were not observed when treated with 10 μg/ml of CB for as long as 30 min. Although CB-treated *Spisula* ova were activated by sperm, no evidence was obtained to suggest that gamete fusion or sperm incorporation occurred (Figs. 16A–C).

Mouse eggs incubated in medium containing from 1 to 50 μg/ml of CB for 1–3 hr

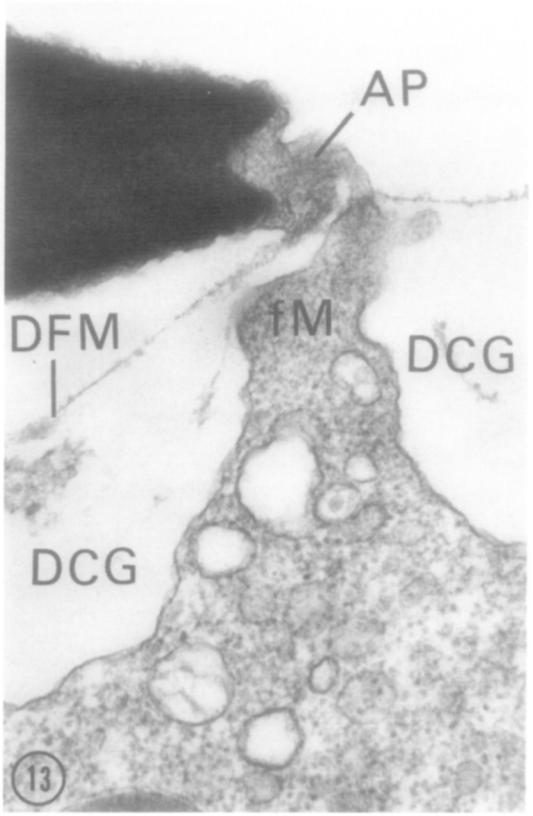
TABLE 5

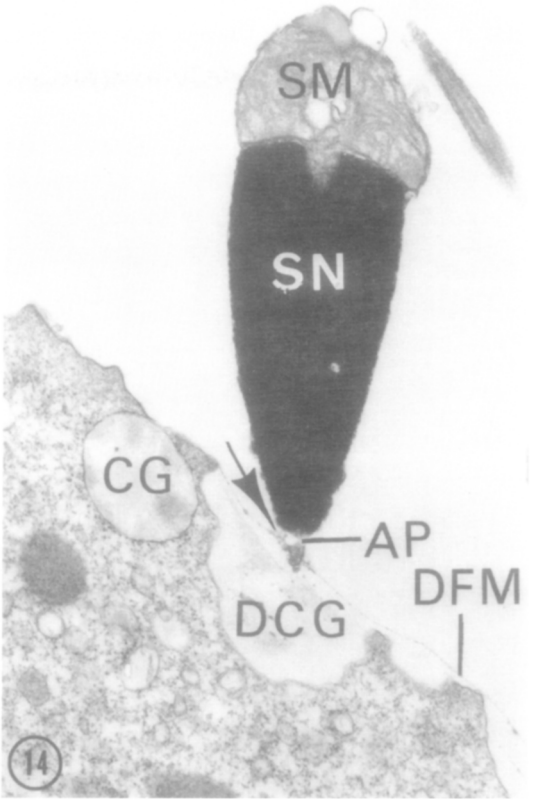
[^{3}H]THYMIDINE INCORPORATION INTO CYTOCHALASIN B-TREATED *Arbacia* EGGS ACTIVATED BY SPERM[a]

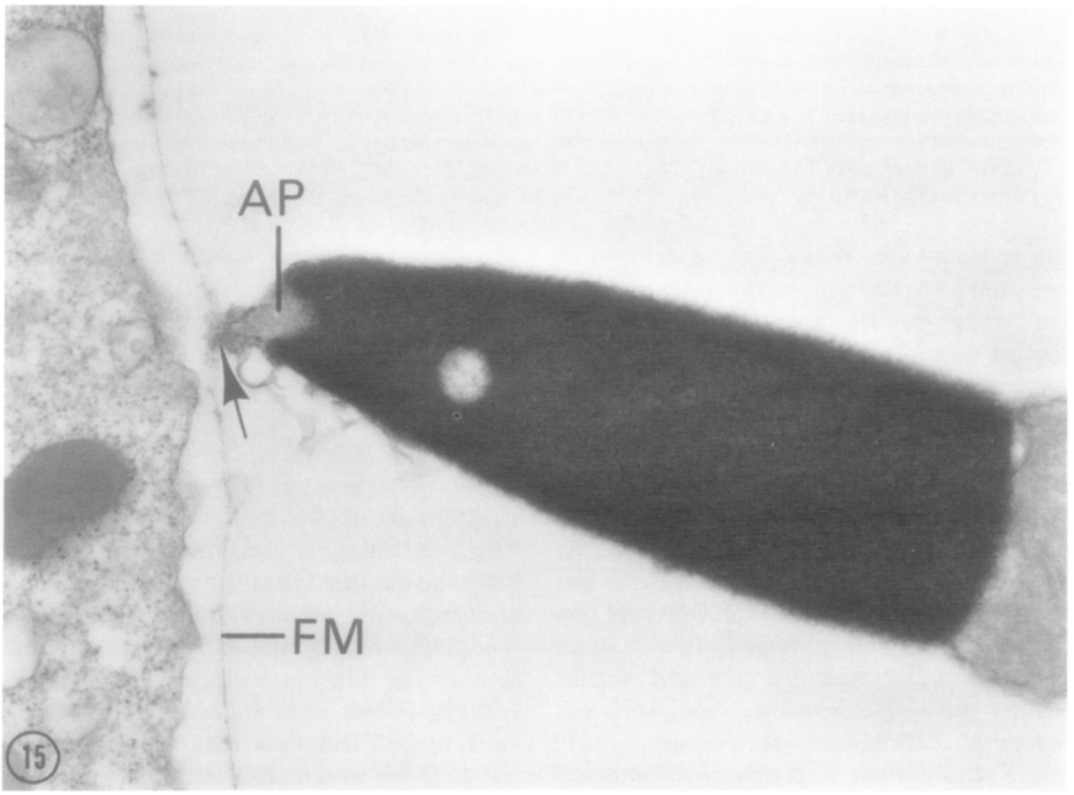
Treatment	(A) cpm in TCA-insoluble material	(B) cpm in TCA-soluble and -insoluble material	Percentage incorporation (A/B × 100)
+CB (applied 1 min prior to mixing of gametes	315	27,120	1.2
+CB (applied 1 min after mixing of gametes)	599	27,254	2.2
−CB	996	39,926	2.5

[a] *Arbacia* eggs (~15,000/ml) were: (1) suspended in 10 μg/ml of CB for 1 min, then incubated in [^{3}H]-thymidine (1 μCi/ml) 1 min after mixing with sperm; (2) incubated in 10 μg/ml of CB following sperm incorporation (i.e., 1 min after mixing of the gametes; or (3) left untreated. Forty minutes after mixing with sperm, eggs were washed in sea water (4°C) and treated with 10% trichloroacetic acid (TCA).

FIGS. 13–15. CB-treated eggs (10 μg/ml for 10 min) 60 sec after mixing with sperm. The vitelline layer is separating from the egg plasma membrane to form the fertilization membrane (DFM) and carries with it attached spermatozoa. Figure 13 depicts a spermatozoon attached to a region between two dehiscing cortical granules (DCG). Note that the attached acrosomal process (AP) has not fused with the plasma membrane of the egg. The arrows in Figs. 14 and 15 point to sites where sperm are attached to the developing fertilization membrane (DFM) of activated, CB-treated eggs. CG, cortical granule; DCG, dehiscing cortical granule; SN, sperm nucleus; SM, sperm mitochondrion; fM, filamentous material. Fig. 13, × 63,000; Fig. 14, × 28,000; Fig. 15, × 35,000.







FIGS. 13–15

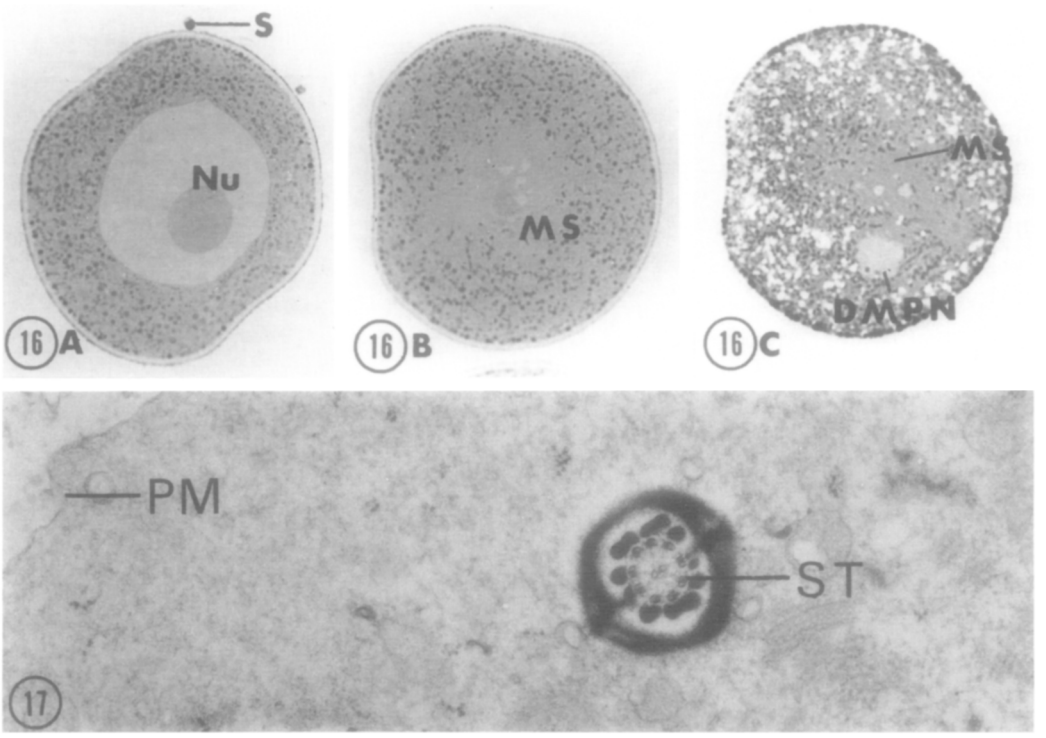


FIG. 16. (A, B) Surf clam (*Spisula*) eggs treated with 10 μg/ml of CB for 10 min and mixed with sperm. (A) Sperm associated with a CB-treated egg 4 min after mixing of the gametes. GV, germinal vesicle; Nu, nucleolus. (B) CB-treated egg activated by sperm (30 min after mixing of the gametes). Note the absence of a germinal vesicle and the presence of a portion of the meiotic spindle (MS). (C) Fertilized *Spisula* egg containing a developing male pronucleus (DMPN) and a portion of the meiotic spindle (MS), 25 min postinsemination. × 700.

FIG. 17. Portion of an inseminated, CB-treated mouse egg (20 μg/ml of CB for 1 hr prior to mixing with sperm) containing an incorporated sperm tail (ST). EPM, plasma membrane of the egg. × 45,000.

did not activate. When CB-treated mouse eggs were mixed with sperm, activated ova, containing incorporated sperm, were observed (Fig. 17). Furthermore, a second polar body failed to form in fertilized, CB-treated mouse eggs.

DISCUSSION

The investigation presented herein demonstrates that: (1) CB-treated *Arbacia* and *Spisula* eggs mixed with sperm activate but do not incorporate sperm; and (2) CB disrupts the normal morphogenesis of the surface of *Arbacia* ova at activation. Similar results have also been obtained with gametes of *Urechis* (Gould-Somero *et al.*, 1977), *Lytechinus*, and *Strongylocentrotus* (Byrd *et al.*, 1977). Although the present observations do not establish unequivocally the means by which sperm induce activation of *Arbacia* and *Spisula* eggs, they do permit some insight into this critical event of development.

The Trigger of Egg Activation: Attachment or Fusion of the Gametes?

Fusion? Since earlier studies demonstrated that sperm incorporation was not necessary for egg activation (Loeb, 1913), it has been assumed that the responses of ova at fertilization are induced by (1) the attachment of the gametes or by (2) the fusion of the plasma membranes of sperm and egg (Epel, 1978; Pasteels, 1965; Yanagimachi, 1977). In both cases, interaction of the gametes would result in a perturbation of the surface of the egg. Although there was no evidence of gamete fusion when CB-

treated eggs of *Arbacia* and *Spisula* were mixed with sperm, it is possible that: (1) Fusion occurred but was not detected, or (2) there was a transient fusion of sperm and CB-treated eggs. Because of technical limitations, the first possibility cannot be entirely dismissed. However, the consistent observation of fused sperm and eggs in controls suggests that gamete fusion does not exist in CB-treated ova. The possibility of a transient fusion of sperm and CB-treated eggs lacks experimental evidence and is beset with numerous assumptions. If such a process did occur, it might encompass a rapid sequence of events involving the fusion of the lipoprotein layers of the sperm and the egg. This may or may not give rise to a confluence of the sperm and egg followed by the separation of their membranes. Consequently, if the gametes were examined following their separation, they would appear to have never fused. Considering the limitations of the techniques employed in this study, and given that such a process may be very rapid, it is possible that such a series of events could go undetected. Moreover, since there is no reliable assay for fusion other than direct observation with the electron microscope, the possible effects of CB on sperm–egg membrane fusion are difficult to determine. Gould-Somero *et al.* (1977) have also suggested that if gamete membrane fusion were in fact inhibited by CB, their results with *Urechis* would demonstrate that fusion is not required for egg activation.

Attachment? Alternatively, it is possible that at the time of or immediately following gamete binding, just prior to gamete fusion, sperm interact with components at the surface of the egg which trigger activation. Evidence for such a scheme is rudimentary; however, a number of observations indicate that it is worthy of consideration. For example, CB-treated eggs from a number of animals are capable of sperm-induced activation without sperm incorporation, sperm do bind to CB-treated eggs, and there is an apparent absence of gamete fusion. Moreover, fused sea urchin gametes are not observed until 30 sec after mixing of the gametes (Franklin, 1965; Summers *et al.*, 1975; F. J. Longo, personal observations), while a change in membrane potential of the ovum occurs 3 to 30 sec after the addition of sperm to egg suspensions (Jaffe, 1976).

The proposition that egg activation is initiated as a result of an interaction between the spermatozoon and the surface of the ovum, prior to gamete fusion, has a number of correlates in other biological systems, e.g., the mechanism of action of some hormones, lectins, and neurosecretory agents (Cuatrecasas, 1974; Edelman, 1976). In these instances, there is an attachment of the agent to a receptor on the surface of the cell and an alteration of membrane function. Moreover, the above proposal is similar to that established for the mating reaction in *Chlamydomonas* by Goodenough and Weiss (1975), who demonstrated that flagellar agglutination, involving surface membrane components, is necessary to trigger the subsequent events of fusion in wild-type cells. Glutaraldehyde-fixed gametes of one mating type will elicit agglutination in the opposite mating type, giving rise to a signal which is passed from the flagellum to its target site(s) in the cell body of the living partner to affect activation of mating structures. The homology between this system and that of fertilization in sea urchins makes the suggestion that eggs are activated by a specific contact of the spermatozoon rather compelling (cf. Metz, 1954).

The apparent absence of gamete fusion in CB-treated eggs mixed with sperm is perplexing. There is evidence that membrane fusion per se is not inhibited by CB, e.g., the acrosomal reaction and cortical granule discharge occur (cf. also Gould-Somero *et al.*, 1977; Sanger and Sanger, 1975). As pointed out by Gould-Somero *et al.* (1977), these observations suggest that merging of the phospholipid bilayers as required for fusion may be unaffected by CB.

However, a specific orientation of surface-associated macromolecules, which could be affected by CB in some instances and not in others, may have to occur before membranes fuse. Similar proposals have been made concerning the effects of CB on exocytotic processes in other cells (Rohlich, 1975; Williams, 1977).

Surface Alterations of CB-Treated Arbacia Eggs

Arbacia eggs incubated in CB (1 μg/ml and higher) accumulate filamentous material within their microvilli and on rare occasions spontaneously activate. Incubation in CB did not promote spontaneous activation of unfertilized *Spisula* and mouse eggs. Similar findings have been previously reported for ova of the same and different organisms (Balakier and Tarkowski, 1976; Eager *et al.*, 1976; Gould-Somero *et al.*, 1977; Peaucellier *et al.*, 1974).

CB also affects the reorganization of the surface of activated *Arbacia* eggs by an apparent inhibition of microvilli formation (Eddy and Shapiro, 1976). Instead of elongate microvilli, activated eggs, incubated in CB, produce large projections containing a filamentous substance. The manner in which CB disrupts the reorganization of the surface of *Arbacia* eggs was not ascertained, however, the effects that are produced may be related to its action on cytoplasmic filaments (Goldman, 1972; Schroeder, 1975; Wessells *et al.*, 1971). It has been proposed that the surface reorganization at fertilization in sea urchin eggs may be due, in part, to actin polymerization (Burgess and Schroeder, 1977; Byrd *et al.*, 1977; Epel. 1978; Schatten and Mazia, 1976). Actin has been demonstrated in unfertilized sea urchin eggs (Hatano *et al.*, 1969), and Burgess and Schroeder (1977) have shown that actin filaments are present in the microvilli of sea urchin zygotes. They suggested that these structures may be involved in the development of the long microvilli characteristic of activated eggs and "take up" the membrane added to the surface of the ovum as a result of the cortical granule reaction (Eddy and Shapiro, 1976). The effects of CB on the surface of many somatic cells has been reported (cf. Godman *et al.*, 1975; Goldman, 1972); Davies and Stossel (1977) have shown that the projections that form on CB-treated macrophages are intact sacs of plasma membrane enclosing contractile protein.

Effects of CB on Eggs and Sperm and their Interactions

The presence of structural changes in the cortex of *Arbacia* eggs and the absence of significant alterations in sperm function indicate that the major effects of CB are on the egg and/or gamete interaction and not the sperm per se. Although Gould-Somero *et al.* (1977) demonstrated that removal of the surface coat in *Urechis* may render the egg less susceptible to CB, the sensitivity of *Arbacia* eggs to CB is not destroyed by trypsinization of the vitelline layer. These results are in agreement with investigations by Lin and Spudich (1974) and Tannenbaum *et al.* (1975), who showed that proteolytic enzymes did not decrease the specific binding activity of cytochalasins to cultured cells. Delay of the cortical granule reaction and elevation of the fertilization membrane described herein have been previously reported (Brunhouse *et al.*, 1972; Sanger and Sanger, 1975) and may be due to reduced sperm binding to CB-treated ova rather than a retardation of the process itself or to an effect on the spermatozoon.

The observation that CB prevents sperm incorporation in *Spisula* eggs is in conflict with the results of Ziomek and Epel (1975), who indicated that CB (0.5 to 5 μg/ml) inhibits development of a block to polyspermy in this organism and has no effect upon sperm penetration. This apparent conflict may be resolved if one considers that CB (1–10 μg/ml) inhibits the formation of polar bodies in *Spisula* (Longo, 1972) and the chromosomes normally taken into these structures remain within the egg and develop into pronuclei. Hence, it is possible

that female rather than male pronuclei were scored by Ziomek and Epel (1975).

The basis for CB failing to inhibit sperm incorporation into mouse ova is unknown (cf. also Niemerko and Komar, 1976); however, it may be related to the manner of sperm–egg interaction in mammals (Austin, 1975; Noda and Yanagimachi, 1976). It should be noted that the technique employed to culture mouse eggs in CB during the present study, i.e., covering the medium with oil, may have affected the amount of CB in the medium. A portion of the CB dissolved in the medium may have partitioned into the oil layer. Nevertheless, the concentration of CB present in the medium was sufficient to retard polar body formation (Balakier and Tardowski, 1976; Longo, 1972; Niemierko, 1975; Peaucellier *et al.*, 1974).

The effects of CB are multiple and its mode of action on cells is not fully understood. Numerous studies have demonstrated that CB can interact directly with actin and/or myosin (Lin and Spudich, 1974; Spudich, 1973; Spudich and Lin, 1972) and exposure of cells to CB can lead to the disappearance or rearrangement of cytoplasmic filaments, which are thought to consist of actin (Goldman, 1972; Schroeder, 1975; Wessells *et al.*, 1971). Furthermore, there is evidence suggesting that CB affects microfilaments only indirectly (Hartwig and Stossel, 1976; Weber *et al.*, 1976; Weihing, 1976), while others have indicated that CB acts primarily at the level of cell membranes (Estensen and Plagemann, 1972; Everhart and Rubin, 1974; Mayhew and Maslow, 1974; Miranda *et al.*, 1974; Plagemann and Estensen, 1972; Plagemann *et al.*, 1976; Tannenbaum *et al.*, 1975; Van Obberghen *et al.*, 1976). The finding that labeled CB interacts with lipid monolayers (Mayhew *et al.*, 1974) and induces morphological effects of the plasma membrane (dePetris, 1974; Scott *et al.*, 1977) strengthens the idea that cellular membranes are directly affected by CB. The precise relationship between these observations and the diverse biological effects of CB presented here is not clear.

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REFERENCES

AKETA, K. (1973). Physiological studies on the sperm surface component responsible for sperm-egg bonding in sea urchin fertilization. *Exp. Cell Res.* **80,** 439–441.

ANDERSON, E. (1968). Oocyte differentiation in the sea urchin, *Arbacia punctulata*, with particular reference to the origin of cortical granules and their participation in the cortical reaction. *J. Cell Biol.* **37,** 514–539.

ANDERSON, E., HOPPE, P. C., WHITTEN, W. K., AND LEE, G. S. (1975). *In vitro* fertilization and early embryogenesis: A cytological analysis. *J. Ultrastruct. Res.* **50,** 231–252.

AUSTIN, C. R. (1975). Membrane fusion events in fertilization. *J. Reprod. Fertil.* **44,** 155–166.

BAKER, P. F., AND PRESLEY, R. (1969). Kinetic evidence for an intermediate stage in the fertilization of the sea urchin egg. *Nature (London)* **221,** 488–490.

BALAKIER, H., AND TARKOWSKI, A. K. (1976). Diploid parthenogenetic mouse embryos produced by heat-shock and cytochalasin B. *J. Embryol. Exp. Morphol.* **35,** 25–39.

BAXANDALL, J., PERLMANN, P., AND AFZELIUS, B. A. (1964). Immuno-electron microscopy analysis of the surface of layers of the unfertilized sea urchin egg. I. Effects of the antisera on the cell ultrastructure. *J. Cell Biol.* **23,** 609–628.

BRINSON, N. (1974). Effect of nicotine on human blood platelet aggregation. *Atherosclerosis* **20,** 137–140.

BRUNHOUSE, R. F., MOUNTS, P., AND BARRETT, D. (1972). Delay on fertilization in *Arbacia* by cytochalasin B treatment. *Biol. Bull.* **143,** 456.

BURGESS, D. R., AND SCHROEDER, T. E. (1977). Polarized bundles of actin filaments within microvilli of fertilized sea urchin eggs. *J. Cell Biol.* **74,** 1032–1037.

BYRD, W., PERRY, G., AND WEIDNER, E. (1977). Role of the egg cortex and actin in fertilization of the sea urchin egg. *J. Cell Biol.* **75,** 267a.

CARROL, E. J. (1975). Nicotine induces polyspermy in sea urchin eggs by inhibiting the cortical granule protease. *Amer. Zool.* **15,** 780.

CLARK, J. M. (1936). An experimental study of polyspermy. *Biol. Bull.* **70,** 361.

COSTELLO, D. P., DAVIDSON, M. E., EGGERS, A., FOX, M. H., AND HENLEY, C. (1957). "Methods for Obtaining and Handling Marine Eggs and Embryos." Lancaster Press, Lancaster, Pa.

CUATRECASAS, P. (1974). Membrane receptors. *Annu. Rev. Biochem.* **43,** 169–214.

DAVIES, W. A., AND STOSSEL, T. P. (1977). Peripheral

hyaline blebs (podosomes) of macrophages. *J. Cell Biol.* **75,** 941–955.

DEPETRIS, S. (1974). Inhibition and reversal of capping to cytochalasin B, vinblastine, and colchicine. *Nature (London)* **250,** 54–56.

EAGER, D. D., JOHNSON, M. H., AND THURLEY, K. W. (1976). Ultrastructural studies on the surface membrane of the mouse egg. *J. Cell Sci.* **22,** 345–353.

EDDY, E. M., AND SHAPIRO, B. M. (1976). Changes in the topography of the sea urchin egg after fertilization. *J. Cell Biol.* **71,** 35–48.

EDELMAN, G. M. (1976). Surface modulation in cell recognition and cell growth. *Science* **192,** 218–226.

EPEL, D. (1975). The program of and mechanisms of fertilization in the echinoderm egg. *Amer. Zool.* **15,** 507–522.

EPEL, D. (1978). Mechanisms of activation of sperm and egg during fertilization of sea urchin gametes. *Curr. Top. Develop. Biol.* **12,** 186–246.

EPEL, D., CROSS, N. L., AND EPEL, N. (1977). Flagellar motility is not involved in the incorporation of the sperm into egg at fertilization. *Develop. Growth Different.* **19,** 15–21.

ESTENSEN, R. D. (1971). Cytochalasin B. I. Effect on cytokinesis of Novikoff hepatoma cells. *Proc. Soc. Exp. Biol. Med.* **136,** 1256–1260.

ESTENSEN, R. D., AND PLAGEMANN, P. G. W. (1972). Cytochalasin B. Inhibition of glucose and glucosomine transport. *Proc. Nat. Acad. Sci. USA* **69,** 1430–1434.

EVERHART, L. P., AND RUBIN, R. W. (1974). Cyclic changes in the cell surface. I. Changes in thymidine transport and its inhibition by cytochalasin B in Chinese hamster ovary cells. *J. Cell Biol.* **60,** 431–441.

FRANKLIN, L. (1965). Morphology of gamete membrane fusion and sperm entry into oocytes of the sea urchin. *J. Cell Biol.* **25,** 81.

GODMAN, G. C., MIRANDA, A. F., DEITCH, A. D., AND TANENBAUM, S. W. (1975). Action of cytochalasin D on cells of established lines. III. Zeiosis and movements at the cell surface. *J. Cell Biol.* **64,** 644–667.

GOLDMAN, R. D. (1972). The effects of cytochalasin B on the microfilaments of baby hamster kidney (BHK-21) cells. *J. Cell Biol.* **52,** 246–254.

GOODENOUGH, U. W., AND WEISS, R. L. (1975). Gametic differentiation in *Chlamydomonas reinhardtii*. *J. Cell Biol.* **67,** 623–637.

GOULD-SOMERO, M., HOLLAND, L., AND PAUL, M. (1977). Cytochalasin B inhibits sperm penetration into eggs of *Urechis caupo* (Echiura). *Develop. Biol.* **58,** 11–22.

GRAFF, J. C., WOHLHUETER, R. M., AND PLAGEMANN, P. G. W. (1977). Effect of temperature and of cytochalasin B and persantin on the nonmediated permeation of non-electrolytes into cultured Novikoff rat hepatoma cells. *J. Biol. Chem.* **252,** 4185–4190.

HARTWIG, J. H., AND STOSSEL, T. P. (1976). Interactions of actin, myosin, and an actin binding protein of rabbit pulmonary macrophages. III. Effects of cytochalasin B. *J. Cell Biol.* **71,** 295–303.

HATANO, S., KONDO, H., AND MIKI-NOUMURA, T. (1969). Purification of sea urchin egg actin. *Exp. Cell Res.* **55,** 275–277.

INOUE, M., AND WOLF, D. P. (1975). Fertilization-associated changes in the murine zona pellucida: A time sequence study. *Biol. Reprod.* **13,** 546–551.

ITO, S. (1969). Structure and function of the glycocalyx. *Fed. Proc.* **28,** 12–25.

JAFFE, L. (1976). Fast block to polyspermy in sea urchin eggs is electrically mediated. *Nature (London)* **261,** 68–74.

JOHNSON, J. D., AND EPEL, D. (1975). Relationship between release of surface proteins and metabolic activation of sea urchin eggs at fertilization. *Proc. Nat. Acad. Sci. USA* **72,** 4474–4478.

LIN, S., AND SPUDICH, J. A. (1974). On the molecular basis of action of cytochalasin B. *J. Supramol. Struct.* **2,** 728–736.

LEOB, J. (1913). "Artificial Parthenogenesis and Fertilization." University of Chicago Press, Chicago.

LONGO, F. J. (1972). The effects of cytochalasin B on the events of fertilization in the surf clam, *Spisula solidissima*. *J. Exp. Zool.* **182,** 321–344.

LONGO, F. J. (1973). Fertilization: A comparative review. *Biol. Reprod.* **9,** 149–215.

LONGO, F. J. (1977). Cytochalasin B inhibits sperm incorporation without inhibiting sperm-induced egg activation. *J. Cell Biol.* **75,** 44a.

LONGO, F. J., AND ANDERSON, E. (1970). The effects of nicotine on fertilization in the sea urchin, *Arbacia punctulata*. *J. Cell Biol.* **46,** 308–325.

LONGO, F. J., AND ANDERSON, E. (1972). Procedures for handling of eggs and embryos for light and electron microscopy. *J. Microsc.* **96,** 255–258.

MAYHEW, E., AND MASLOW, D. E. (1974). Cytochalasin B and the sialic acids of Ehrlich ascites cells. *Exp. Cell Res.* **83,** 255–260.

MAYHEW, E., POSTE, G., GOWDEN, M., TOLSON, N., AND MASLOW, D. (1974). Cellular binding of 3-H cytochalasin B. *J. Cell. Physiol.* **84,** 373–382.

METZ, C. B. (1954). Mating substances and the physiology of fertilization in ciliates. *In*: "Sex in Microorganisms" (D. H. Wenrich, ed.), pp. 284–334. AAAS, Washington, D.C.

MILLONIG, G. (1969). Fine structure analysis of the cortical reaction in the sea urchin egg: After normal fertilization and after electric induction. *J. Submicrosc. Cytol.* **1,** 69–84.

MIRANDA, A. F., GODMAN, G. C. AND TANENBAUM, S. W. (1974). Action of cytochalasin D on cells of established lines. II. Cortex and microfilaments. *J. Cell Biol.* **62,** 406–423.

NIEMIERKO, A. (1975). Induction of triploidy in the mouse by cytochalasin B. *J. Embryol. Exp. Morphol.* **34,** 279–289.

NIEMIERKO, A., AND KOMAR, A. (1976). Cytochalasin B-induced triploidy in mouse oocytes fertilized *in*

vitro. J. Reprod. Fertil. **48,** 279–284.

NODA, Y. D., AND YANAGIMACHI, R. (1976). Electron microscopy observations of guinea pig spermatozoa penetrating eggs *in vitro. Develop. Growth Different.* **18,** 15–23.

PASTEELS, J. J. (1965). Etude au microscop electronique de la reaction corticale. *J. Embryol. Exp. Morphol.* **13,** 327–339.

PEAUCELLIER, G., GUERRIER, P., AND BERGERARD, J. (1974). Effects of cytochalasin B on meiosis and development of fertilized and activated eggs of *Sabellaria alveolata* L. (Polychaete Annelid). *J. Embryol. Exp. Morphol.* **31,** 61–74.

PLAGEMANN, P. G. W., AND ESTENSEN, R. D. (1972). Cytochalasin B. IV. Competitive inhibition of nucleoside transport by cultured Novicoff rat hepatoma cells. *J. Cell Biol.* **55,** 179–185.

PLAGEMANN, P. G. W., GRAFF, J. C., AND WOHLHUETER, R. M. (1976). Binding of [^{3}H]cytochalasin B and its relationship to inhibition of hexose transport in Novikoff rat hepatoma cells. *J. Biol. Chem.* **252,** 4191–4201.

PRESLEY, R., AND BAKER, P. F. (1970). Kinetics of fertilization in the sea urchin: A comparison of methods. *J. Exp. Biol.* **52,** 455–468.

ROHLICH, P. (1975). Membrane-associated actin filaments in the cortical cytoplasm of the rat mast cell. *Exp. Cell Res.* **93,** 293–298.

SANGER, J. W., AND SANGER, J. M. (1975). Polymerization of sperm actin in the presence of cytochalasin-B. *J. Exp. Zool.* **193,** 441–447.

SCHATTEN, G., AND MAZIA, D. (1976). The surface events at fertilization: The movements of the spermatozoon through the sea urchin egg surface and the roles of the surface layers. *J. Supramol. Struct.* **5,** 343–369.

SCHMELL, E., EARLES, B. J., BREAUX, C., AND LENNARZ, W. J. (1977). Identification of a sperm receptor on the surface of the eggs of the sea urchin *Arbacia punctulata. J. Cell Biol.* **72,** 35–46.

SCHROEDER, T. E. (1975). Dynamics of the contractile ring. *In* "Molecules and Cell Movements" (S. Inoue and R. E. Stephens, eds.), pp. 305–334. Raven Press, New York.

SCHWARTZ, S. L. (1976). Interaction of nicotine and other amines with the endocytoic and exocytic functions of macrophages. *Fed. Proc.* **35,** 85–88.

SCOTT, R. E., MAERCKLEIN, P. B., AND FURCHT, L. T. (1977). Plasma membrane intramembranous particle topography in 3T3 and SV-3T3 cells: The effect of cytochalasin B. *J. Cell Sci.* **23,** 173–192.

SPUDICH, J. A. (1973). Effects of cytochalasin B on actin filaments. *Cold Spring Harbor Symp. Quant. Biol.* **37,** 585–593.

SPUDICH, J. A., AND LIN, S. (1972). Cytochalasin B, its interaction with actin and actomyosin from muscle. *Proc. Nat. Acad. Sci. USA* **69,** 442–446.

SUMMERS, R. G., AND HYLANDER, B. L. (1974). An ultrastructural analysis of early fertilization in the sand dollar, *Echinarachnius parma. Cell Tissue Res.* **150,** 343–368.

SUMMERS, R. G., HYLANDER, B. L., COLWIN, L. H., AND COLWIN, A. L. (1975). The functional anatomy of the echinoderm spermatozoon and its interaction with the egg at fertilization. *Amer. Zool.* **15,** 523–551.

TANNENBAUM, J., TANEBAUM, S. W., LO, L. W., GODMAN, G. C., AND MIRANDA, A. F. (1975). Binding and subcellular localization of tritiated cytochalasin D. *Exp. Cell Res.* **91,** 47–56.

VAN OBBERGHEN, E., MEYTS, P. DE. AND ROTH, J. (1976). Cell surface receptors for insulin and human growth hormone. *J. Biol. Chem.* **251,** 6844–6851.

WEBER, K., RATHKE, P. C., OSBORN, M., AND FRANKE, W. W. (1976). Distribution of actin and tubulin in cells and in glycerinated cell models after treatment with cytochalasin B (CB). *Exp. Cell Res.* **102,** 285–297.

WEIHING, R. R. (1976). Cytochalasin B inhibits actin-related gelation of *HeLa* cell extracts. *J. Cell Biol.* **71,** 303–307.

WERLE, E., AND SCHIEVELBEIN, H. (1965). Activity of nicotine and inactivity of kallikein and kallian in aggregation of blood platelets. *Nature (London)* **207,** 871.

WESSELLS, N. K., SPOONER, B. S., ASH, J. F., BRADLEY, M. O., LUDUENA, M. A., TAYLOR, E. L., WRENN, J. T., AND YAMADA, K. M. (1971). Microfilaments in cellular and developmental processes. *Science* **171,** 135–143.

WESTFALL, T. C., AND BRASTED, M. (1972). The mechanism of action of nicotine on adrenergic neurons in the perfused guinea pig heart. *J. Pharmacol. Exp. Ther.* **182,** 409–418.

WILLIAMS, J. A. (1977). Effects of cytochalasin B on pancreatic acinar cell structure and secretion. *Cell Tissue Res.* **179,** 453–466.

WOLF, D. P., AND INOUE, M. (1976). Sperm concentration dependency in the fertilization and zonae sperm binding properties of mouse eggs inseminated *in vitro. J. Exp. Zool.* **196,** 27–38.

YANAGIMACHI, R. (1977). Specificity of sperm-egg interaction. *In* "Immunobiology of Gametes" (M. Edidin and M. H. Johnson, eds.), pp. 255–295. Cambridge University Press, Cambridge.

ZIOMEK, C. A., AND EPEL, D. (1975). Polyspermy block of *Spisula* eggs is prevented by cytochalasin B. *Science* **189,** 139–141.