

## Structural Changes of the Endoplasmic Reticulum of Sea Urchin Eggs during Fertilization

LAURINDA A. JAFFE AND MARK TERASAKI\*

*Department of Physiology, University of Connecticut Health Center, Farmington, Connecticut 06032; and*

*\*Laboratory of Neurobiology, NINDS, National Institutes of Health, Bethesda, Maryland 20892*

*Accepted October 16, 1992*

The endoplasmic reticulum (ER) of the sea urchin egg includes a system of cisternae, a cortical tubular network, and the nuclear envelope. In previous work (Terasaki and Jaffe, 1991, *J. Cell. Biol.* 114, 929-940) we showed that the long chain fluorescent lipophilic dye DiI spread from an intracellularly injected oil drop into all of these parts of the ER. In this previous study, we observed that in the first minute after fertilization, the ER cisternae became more finely partitioned; the large cisternae then reformed by 5-8 min after fertilization. To investigate whether these changes indicated a breaking up of the ER into discontinuous pieces, we have now examined the ability of DiI to spread in the ER at various times after fertilization. In eggs injected after fixation with aldehydes, we find that DiI spreads through the ER in unfertilized eggs and in eggs at 10 min postinsemination. However, DiI does not spread out of the oil drop in eggs fixed at 1 min postinsemination. We also find that in live eggs, the spreading of DiI is slower in eggs at 1-3 min postinsemination than in unfertilized eggs or in eggs later after fertilization. We interpret these observations to indicate that in the first few minutes after fertilization, the egg's ER is fragmented. This may be functionally significant for the early events of egg activation. © 1993

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### INTRODUCTION

The endoplasmic reticulum (ER) of the sea urchin egg includes a cortical tubular layer, a system of cisternae throughout the interior of the egg, and the nuclear envelope. These structures have been identified by electron microscopy (Longo and Anderson, 1968; Chandler, 1984; Sardet, 1984; Luttmner and Longo, 1985), and by immunocytochemical localization of a calsequestrin-like protein (Henson *et al.*, 1989, 1990). The three-dimensional organization and apparent continuity of these parts of the ER were recently demonstrated in living eggs by confocal microscopy of eggs injected with the lipophilic dye, DiI, dissolved in soybean oil (Terasaki and Jaffe, 1991). DiI spread from the oil drop to label these ER mem-

branes, but not other membrane-bound organelles. The principle of the selective staining of the ER is that this highly hydrophobic molecule can spread only along continuous lipid pathways in contact with the oil drop; DiI does not pass through the aqueous spaces that exist between the oil drop and all of the organelles at any distance away (see Terasaki and Jaffe, 1991, for references).

One question that was left unanswered in our previous study was whether the apparent continuity of the ER network throughout the cytoplasm could be a result of vesicular membrane traffic that occurred between separate ER fragments. We have now resolved this issue by injection of DiI into fixed eggs, where vesicular membrane traffic could not be occurring. In fixed eggs as well, DiI spreads throughout the cytoplasm, demonstrating ER continuity throughout the egg.

The DiI oil drop method also revealed a dramatic change in ER structure at fertilization (Terasaki and Jaffe, 1991). Within 1 min after fertilization, the large cisternae changed to a more finely divided structure, the form of which was not fully characterized. This change began as a wave which passed across the egg starting at the point of sperm entry. Then, starting at about 3 min after fertilization, the large cisternae began to reform, returning to a form similar to that of the unfertilized egg by 5-8 min.

To investigate whether these changes indicated a breaking up of the ER into discontinuous pieces, we have now examined the ability of DiI to spread in the ER at various times after fertilization. As in unfertilized eggs, DiI spreads through the cytoplasm of eggs fixed at 10 min after insemination. However, we find that DiI does not spread when injected into eggs fixed at 1 min after insemination. In live eggs, DiI spreading from the oil drop is slower in eggs at 1-3 min after fertilization, compared to unfertilized eggs or eggs at >3 min after fertilization. These observations indicate that fertilization may result in a transient decrease in the number of

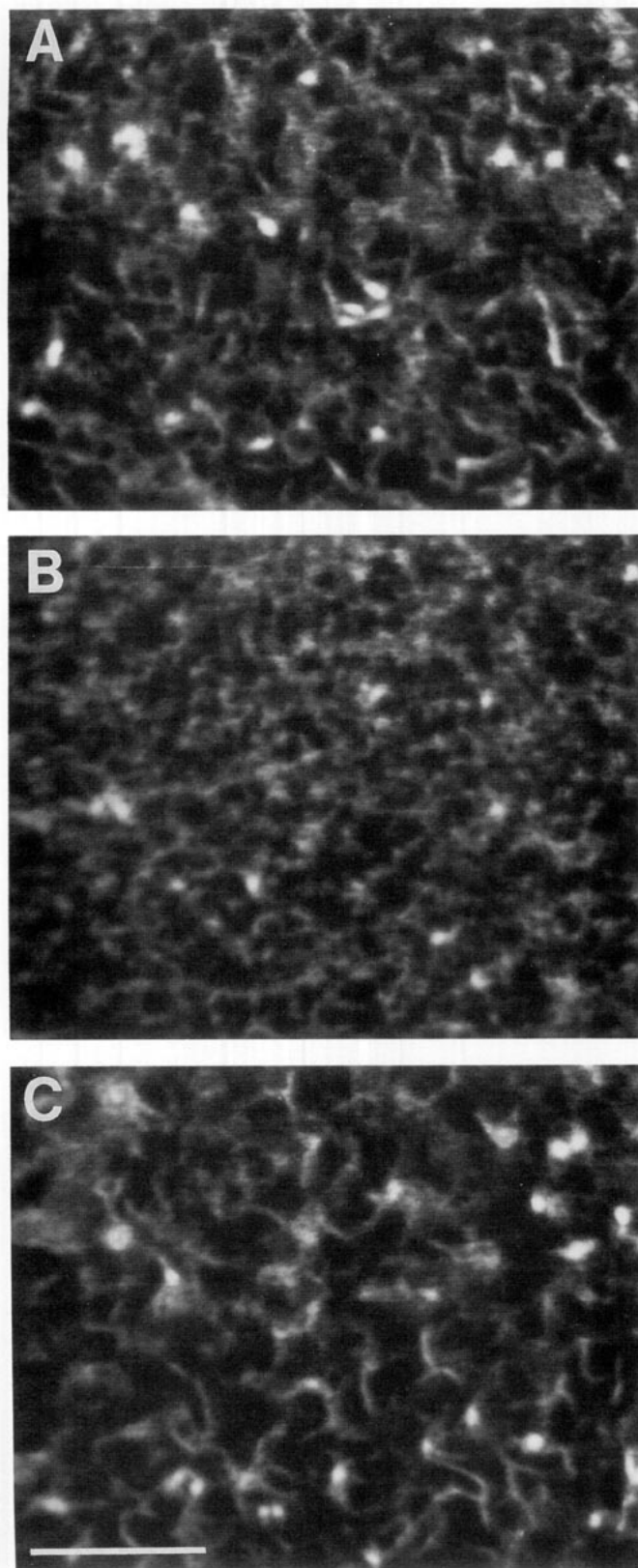


FIG. 1. Change in ER structure at fertilization, visualized in a live egg injected with a DiI-saturated oil drop. Optical sections about  $3\ \mu\text{m}$  below the surface. (A) Unfertilized egg. (B) One minute after the start

connections in the ER network; such a fragmentation of the ER could be functionally significant for regulating early events following fertilization.

#### MATERIALS AND METHODS

Sea urchins (*Lytechinus variegatus*) were obtained from Kingfisher Biological (Durham, NC) and from Susan Decker (Hollywood, FL). Gametes were obtained by injection of  $0.5\ M$  KCl into the coelomic cavity. Experiments were performed at  $22\text{--}24^\circ\text{C}$  in natural seawater (obtained at the Marine Biological Laboratory, Woods Hole, MA). Eggs were loaded into an injection/observation chamber in which they were held between two parallel coverslips (see Terasaki and Jaffe, in press). Inseminations in the injection/observation chamber were made by replacing the seawater in the chamber reservoir with a sperm suspension ( $\sim 1:1000$  dilution). Inseminations in glass vials were made by mixing equal volumes of sperm suspension ( $\sim 1:1000$  dilution) and egg suspension ( $\sim 30,000$  eggs/ml).

DiI<sub>16</sub>(3) (1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) and DiI<sub>18</sub>(3) (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) were obtained from Molecular Probes (Eugene, OR). Similar results were obtained with each; we use the term DiI to refer to either compound. DiI was dissolved in soybean oil (Wesson Oil, Giant Foods, Wheaton, MD) to make a saturated solution, and the DiI solution was microinjected into eggs as described previously (10 to 60 pl per egg, 1.7 to 10% of the egg volume) (see Terasaki and Jaffe, 1991, in press). Injections of these volumes of oil caused no obvious distortions of the eggs and did not inhibit development (see Results).

Observations were made using a laser scanning confocal microscope (Model 600; Bio-Rad Laboratories, Oxford, CA) with a Zeiss Axioplan microscope and Planapo  $63\times$ ,  $1.4\ \text{NA}$  objective lens. The confocal aperture was set at 3–5. The depth of each optical section from the cell surface was determined by focusing at the surface and then focusing into the egg using a calibrated stepper motor. Under these conditions, images taken at successive  $0.36\text{-}\mu\text{m}$  steps differed markedly, giving an indication that the thickness of the optical section is in this range. The best image quality was obtained within  $10\ \mu\text{m}$  of the cell surface; however, as described previously (Terasaki and Jaffe, 1991), the structure of the ER is similar throughout the cell interior.

The video output from the confocal microscope frame store card was run through a Panasonic WJ-810 time

of the ER change. (C) Five minutes after the start of the ER change. Bar,  $10\ \mu\text{m}$ .

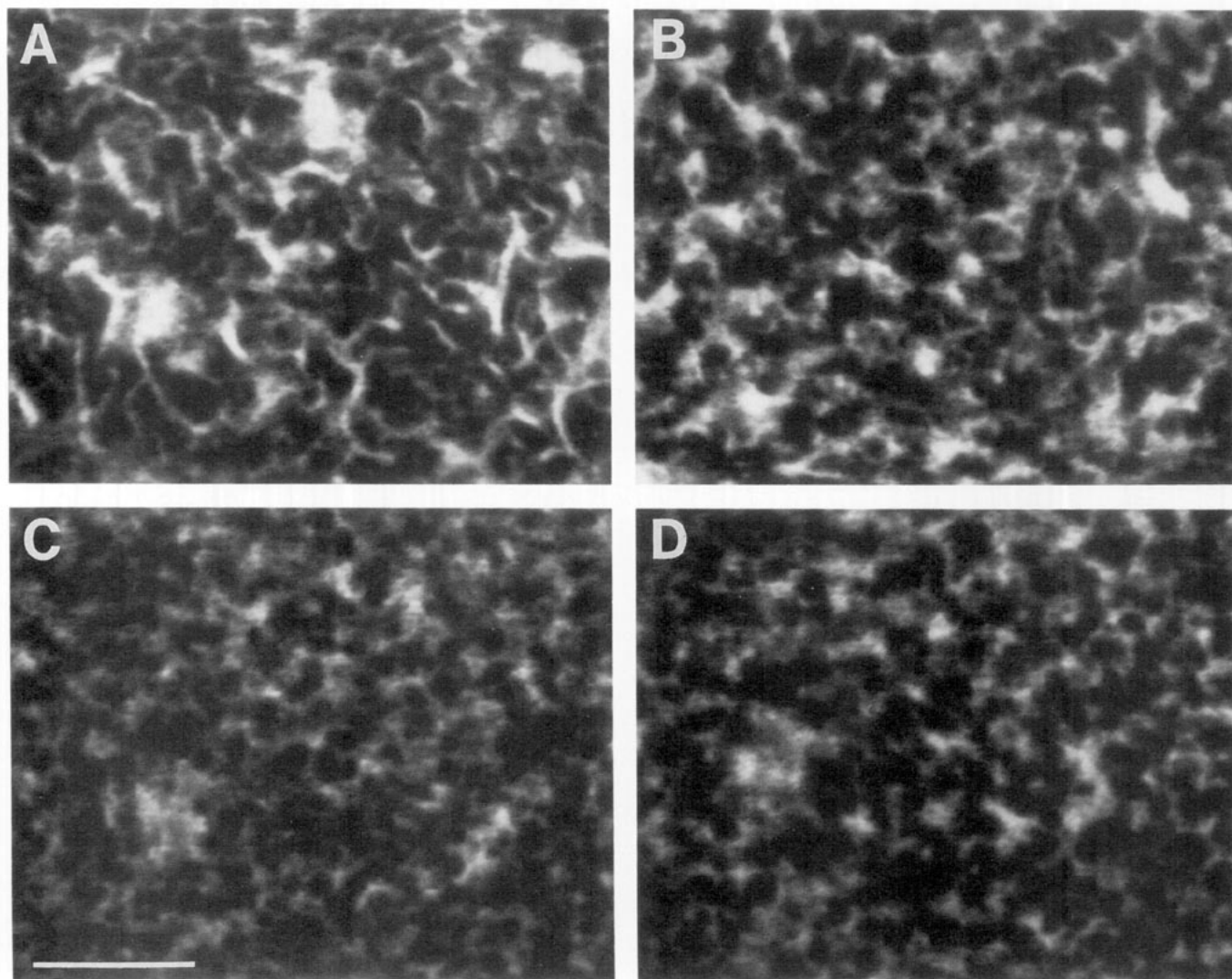


FIG. 2. Effects of fixation on the structure of the endoplasmic reticulum. Eggs were labelled by injection of a DiI-saturated oil drop and then fixed by changing the seawater in the observation chamber to 1% glutaraldehyde and 1% paraformaldehyde in calcium-free seawater. (A) A live unfertilized egg. (B) Same egg as A, 3 min after fixative was applied. (C) A live fertilized egg, 1 min after the ER change was first observed. (D) Same egg as C, 3 min after fixative was applied. Bar, 10  $\mu$ m.

date generator, and images were stored on a Panasonic 3031F optical memory disc recorder in high-resolution mode (Terasaki and Jaffe, 1991, in press). Images were photographed from a video monitor. For measurements of fluorescence intensity, the data stored on the optical memory disc recorder were transferred back to the confocal microscope frame store card through the video input and then were analyzed using Bio-Rad software; a gray scale test image was used to adjust the black level and contrast. The composite images in Fig. 6 were made by transferring data from the optical memory disc recorder to a Macintosh II computer with a Data Translation QuickCapture frame grabber card (Marlboro, MA) and processing the data with Image software (from Wayne Rasband, Research Services Branch, NIH, Be-

thesda, MD). The final image was transferred to film using a Montage FR1 system (Presentation Technologies, Sunnyvale, CA).

The following fixatives were used: 1 or 2% glutaraldehyde in natural seawater, or 1% glutaraldehyde and 1% paraformaldehyde in calcium-free artificial seawater (440 mM NaCl, 9 mM KCl, 23 mM  $MgCl_2$ , 26 mM  $MgSO_4$ , 2 mM  $NaHCO_3$ , 5 mM EGTA, 20 mM Hepes, pH 7.0). We used calcium-free seawater in some experiments to avoid the possibility that calcium entry into the cytoplasm during fixation might cause artifactual changes. Fixatives were applied to eggs in the injection/observation chamber by replacing the seawater from the chamber reservoir with fixative (see Terasaki and Jaffe, in press). For these experiments, the coverslip shelf

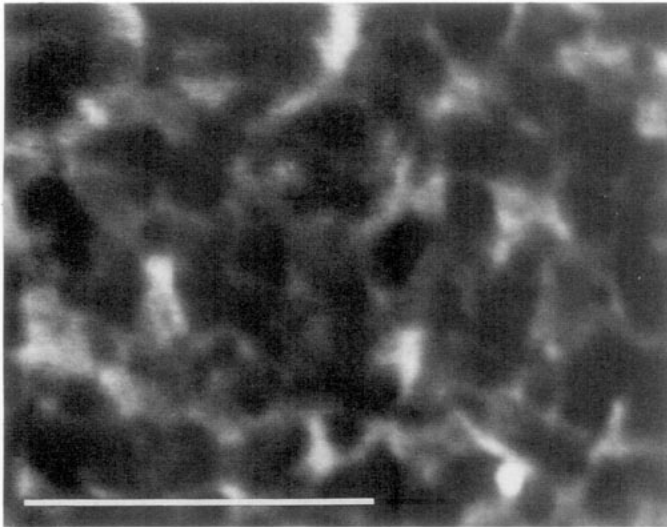


FIG. 3. Endoplasmic reticulum of an unfertilized egg fixed in 1% glutaraldehyde and 1% paraformaldehyde in calcium-free seawater and injected 30 min later with a DiI-saturated oil drop. The egg was imaged 70 min after injection. Bar, 10  $\mu$ m.

supporting the eggs was kept very narrow ( $\sim 150$ – $250$   $\mu$ m) in order to allow rapid exchange of the fixative (see Terasaki and Jaffe, in press). For microinjection of fixed eggs, the eggs were either fixed in the injection/observation chamber, as described above or, in most cases, fixed in glass vials and then loaded into the chambers. To fix eggs in glass vials, 10 vol of fixative were added to 1 vol of egg suspension. Microinjection of these eggs was performed with the eggs in the original fixative or, in some cases, after transfer into calcium-free artificial seawater without fixative. Methods for microinjection of fixed eggs were the same as for live eggs, although the consistency of the cross-linked cytoplasm differed from that of a live egg. By observing noninjected eggs, autofluorescence due to glutaraldehyde fixation was seen to increase with time, but the fluorescence from DiI was always much brighter than autofluorescence.

## RESULTS

### *ER Structure and Its Changes at Fertilization in Lytechinus variegatus*

As a basis for the following studies using *Lytechinus variegatus*, we summarize briefly our observations confirming that properties of the ER in its eggs are similar to those previously described for *Lytechinus pictus* (Terasaki and Jaffe, 1991). As with *L. pictus*, injection of *L. variegatus* eggs with a DiI-saturated oil drop resulted in uniform spreading of the dye throughout the ER in  $\leq 30$  min. The ER consisted of a stationary cortical tubular network and of cisternae throughout the interior (Figs.

1A and 2A) which were in constant motion. The nuclear envelope was also labeled.

When DiI-labeled *L. variegatus* eggs were fertilized, the cisternae appeared to become more finely divided ( $n = 13$  eggs, 4 females) (Figs. 1B and 2C). This change began as a wave passing across the cytoplasm at about the same time as the cortical contraction. For 4/4 eggs that we observed, reformation of large cisternae occurred between 3 and 8 min after fertilization (Fig. 1C), as in *L. pictus*. Fertilized DiI-labeled eggs of *L. variegatus* were observed to form plutei.

### *Effects of Fixation on ER Structure*

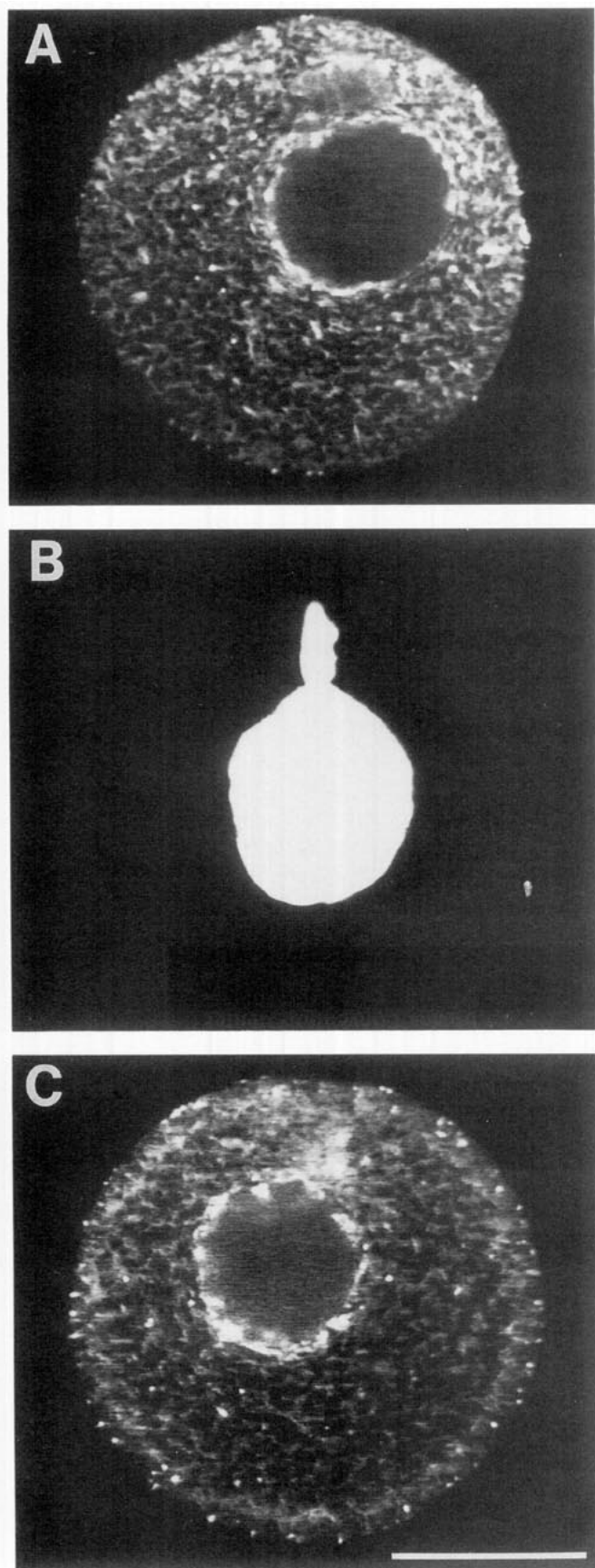
In order to evaluate our subsequent studies on fixed eggs, we observed the egg's ER during the process of fixation. Unfertilized eggs that had been labeled with DiI were fixed in the observation chamber by exchanging the solution in the chamber's reservoir to either 1 or 2% glutaraldehyde in seawater, or 1% glutaraldehyde and 1% paraformaldehyde in calcium-free seawater ( $n = 5$  eggs, 2 females). By 1–2 min after fixative addition, the characteristic movement of the ER had stopped. The structure of the ER of the fixed egg (Fig. 2B), although not identical to that of the unfixed egg (Fig. 2A), retained the basic form of large cisternae. Observations of 4 eggs fixed at  $\sim 1$  min after the initial ER change at fertilization (Figs. 2C and 2D), and 2 eggs fixed at  $\sim 10$  min, also showed structures similar but not identical to those prior to fixation. No obvious differences were seen between eggs treated with the different fixatives.

### *Injection of a DiI-Saturated Oil Drop into Fixed Unfertilized Eggs*

Eggs fixed with either 1% glutaraldehyde or 1% glutaraldehyde and 1% paraformaldehyde were injected, 0.5 to 5 hr after fixation, with a DiI-saturated oil drop (Figs. 3 and 4A). As in the live eggs, the DiI spread throughout the cytoplasm, labeling structures similar to those labeled in the live egg—a cortical network, internal cisternae, and the nuclear envelope. In 23/25 fixed eggs (3 females) that were examined at 1–1.5 hr after injection, the dye had spread completely through the cytoplasm. Observations made at 10–20 min after injection showed that the spreading of the dye occurred somewhat more slowly in the fixed eggs compared to live eggs.

### *Injection of a DiI-Saturated Oil Drop into Eggs Fixed at Various Times after Fertilization*

To determine whether spreading of DiI might be altered in the period just after fertilization when the ER structure appears more finely divided ( $\sim 1$ – $3$  min post-



fertilization), we first injected DiI into fixed eggs. Use of fixed eggs avoided the limitations imposed by the transience of the change in ER structure. We used either 1% glutaraldehyde or 1% glutaraldehyde and 1% paraformaldehyde and obtained the same results with each. Injections of DiI-saturated oil drops were made 0.5–5 hr after fixation.

DiI did not spread in the cytoplasm of eggs fixed at 1 min after insemination. DiI was retained in the original oil drop (Fig. 4B); at high magnification, intensely labeled spots were seen on the surface of the oil drop, probably corresponding to organelles in direct contact with the oil drop (Fig. 5). Similar observations were made for 13 of 14 eggs (3 females) observed at 1–22 hr after injection. This suggested that at 1 min postinsemination the ER network was discontinuous.

In contrast to eggs fixed at 1 min after insemination, in eggs fixed at 10 min, DiI spread throughout the cytoplasm, as in unfertilized eggs (Fig. 4C). Similar observations were made for 15 of 16 eggs (3 females) observed at 0.5–2.5 hr after injection. This indicated that at 10 min postinsemination the ER network was continuous.

In eggs fixed at 2 min after insemination, variable degrees of DiI spreading were observed. Of six eggs observed at 1.5 hours after injection, two showed DiI through most of the cytoplasm, two showed only very local spreading of DiI near the oil drop, and two were intermediate. This suggested that ER continuity might begin to be reestablished as early as 2 min postinsemination, but the timing of the recovery could not be determined exactly since the rate of fixation was unknown.

#### *Measurements of the Rate of DiI Spreading in Live Eggs before and Immediately after Fertilization*

In this series of experiments, we compared the rate of spreading of DiI in live unfertilized eggs and in live eggs within the first several minutes after fertilization. Eggs were injected and then 20–30 sec later the samples to be

FIG. 4. Eggs fixed before and after fertilization and then injected with DiI-saturated oil drops. (A) Unfertilized egg, injected 40 min after fixation and imaged 75 min after injection. The dark circular structure in the interior is the oil drop; DiI has partitioned out of the oil into the ER. (B) Egg fixed at 1 min postinsemination, injected 45 min later, and imaged 75 min after injection. The bright structure is the DiI-saturated oil drop; DiI has not moved into the surrounding cytoplasm. The stem-like extension of oil from the injected drop followed the path of the microinjection pipet and was often seen in eggs that had been fixed before injection. This was probably due to the less effective sealing of the hole made by the pipet in fixed compared to live eggs; it was seen in both unfertilized and fertilized eggs. (C) Egg fixed at 10 min postinsemination, injected 40 min later, and imaged 90 min after injection. DiI has partitioned out of the oil into the ER. The fixative was 1% glutaraldehyde and 1% paraformaldehyde in calcium-free seawater. Bar, 50  $\mu$ m.





FIG. 5. A high magnification view of a region of the DiI-saturated oil drop in an egg fixed at 1 min postinsemination, injected 45 min later, and imaged 2 hr after injection. The intensity of the laser beam was set such that the oil drop appears gray. The white spots probably correspond to intensely stained cytoplasmic structures that are in direct contact with the oil drop. The rest of the cytoplasm is unlabeled and appears black. Bar, 10  $\mu$ m.

observed after fertilization were inseminated. The experimental chamber was constructed to allow immediate contact of many sperm with the eggs, thus ensuring little delay between insemination and fertilization.

Starting at 1.5 min after injection, corresponding to about 1 min after fertilization, the distribution of DiI was observed with the confocal microscope (Fig. 6). In unfertilized eggs, spreading of DiI between 1.5 and 3.5 min after injection was apparent both as a decrease in the fluorescence of the oil drop and as an increase in the extent of the ER showing labeling. By comparison, during the same time period in fertilized eggs, there was less decrease in fluorescence in the oil drop as well as less increase in the labeling of the ER. Between 4.0 and 6.0 min after injection of fertilized eggs, the rate of DiI spreading appeared to increase.

To analyze this difference in the spreading of DiI between unfertilized and fertilized eggs, we measured the time course of the decrease in fluorescence intensity of the oil drop (Fig. 7) in a set of six unfertilized and five fertilized eggs from the same female. Between 1.5 and 3.5 min after injection (corresponding to about 1–3 min after fertilization), the fluorescence intensity of the oil drop decreased less in fertilized eggs ( $25 \pm 3\%$ , SD) than in unfertilized eggs ( $54 \pm 4\%$ ). After this period, however, the rate of DiI spreading from the oil drop in fertilized eggs increased.

#### DISCUSSION

Our previous observations (Terasaki and Jaffe, 1991) of the spreading of DiI from DiI-saturated oil drops in-

jected into living sea urchin eggs provided evidence that the cortical ER network, interior cisternae, and nuclear envelope are part of one membrane that is continuous throughout the egg. This interpretation, based on examination of serial optical sections, as well as the selective spreading of the dye in the ER but not into other organelles, is now strengthened by evidence that DiI can spread through the ER of fixed eggs. No active membrane traffic process, such as vesicle formation and refusion with discontinuous compartments, would occur in a fixed egg. Furthermore, DiI labeling of the plasma membrane of fixed neurons showed no lipid transfer between the plasma membrane and membranes of intracellular organelles (Godemont *et al.*, 1987). In that study, transfer of DiI between plasma membranes of axons and adjacent cells was seen in several instances; it was suggested, however, that this might be occurring at close or specialized contacts between the cells. Since we did not see transfer of dye to cortical granules or yolk platelets, it seems very likely that DiI spreads in the fixed eggs solely by diffusion in a continuous bilayer, providing further evidence for the conclusion that the cortical ER, internal cisternae, and nuclear envelope comprise a continuous ER compartment.

We had previously observed the disappearance of large cisternae in the ER of eggs at 1–3 min postfertilization (Terasaki and Jaffe, 1991). Our new results concerning the change in the ER structure at fertilization are as follows: (1) In live eggs, DiI spread out of the oil drop more slowly during the first few minutes after insemination than in unfertilized eggs. (2) In eggs fixed at 1 min postinsemination, DiI did not spread out of the oil drop.

We consider the results with live eggs first. Three possible explanations for a decrease in the rate of spreading of DiI after fertilization are as follows: (1) A change in the shape of the ER membrane; for example, a conversion of a cisterna to a long convoluted tubule would increase the length of the path that DiI molecules would have to diffuse in order to move the same distance across the cell. (2) A decrease in the number of connections in the continuous ER network; this would result in fewer paths for diffusion and therefore slower DiI spreading. (3) A larger decrease in the number of connections resulting in fragmentation of the ER network; under these circumstances, DiI could not spread across the cell by diffusion alone, but could still spread, at a slower rate, if the fragments were in a dynamic state of forming and breaking connections.

Considering the results with the fixed eggs, the lack of DiI spreading in eggs fixed at 1 min postinsemination is consistent with a complete fragmentation of the ER, rather than with a change in membrane shape or with a

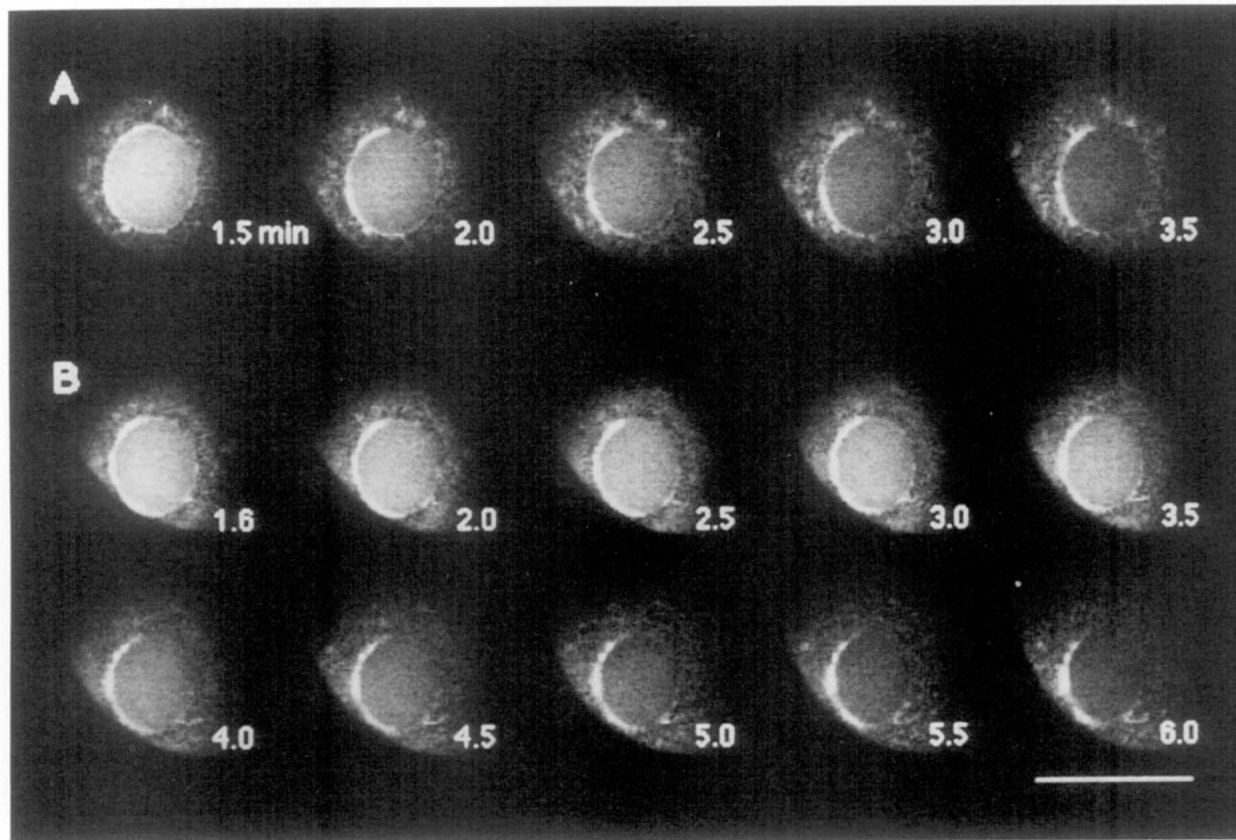


FIG. 6. Time course of DiI spreading from the oil drop into the ER. (A) Unfertilized egg. Images were recorded at the indicated times starting at 1.5 min after injection of a DiI-saturated oil drop. The bright circular structure in the first frame is the oil drop; around the oil drop, DiI staining of the ER can be seen. Between 1.5 and 3.5 min, the oil drop becomes dimmer and the area of fluorescently stained ER increases. (B) Fertilized egg. The egg was inseminated 0.5 min after injection of a DiI-saturated oil drop, and the fertilization envelope was first seen at 0.9 min after injection. Images were recorded at the indicated times starting at 1.6 min after injection. Between 1.6 and 3.5 min, DiI spreading from the oil drop occurs more slowly than in the unfertilized egg. Between 4.0 and 6.0 min, the rate of DiI spreading appears to increase. For these observations, the microscope was focused on the largest diameter of the oil drop. We determined that the focal plane remained approximately the same during the time series by measuring the diameter of the oil drop. (For the 11 eggs investigated, of which 2 typical examples are shown, the oil drop diameter changed by  $5 \pm 5\%$  (SD) over a 10-min period.) Bar, 50  $\mu\text{m}$ .

decrease in the number of connections in a still continuous ER. It cannot be ruled out that the ER at 1 min postfertilization is in a state that is susceptible to fragmentation by fixative; however, fixative did preserve continuity in unfertilized and 10-min-postfertilization eggs. If the fixed egg results are not artifactual, then membrane traffic involving breaking and reforming connections between fragments of ER is the best explanation for the slow spreading of DiI into the ER in live eggs just after fertilization.

One biological significance of a transient loss of ER connections at fertilization could be, as discussed previously (Terasaki and Jaffe, 1991), in relation to the breakdown and reformation of the sperm nuclear envelope. These changes in the nuclear envelope, a specialized region of the ER, occur with a time course similar to that of the cell-wide transitions in ER structure. Thus, one function of the cell-wide ER changes could be to

allow the changes in the sperm nuclear envelope, which are necessary for sperm chromatin condensation.

Another function of loss of connections in the ER membrane meshwork could be to facilitate the initial phases of sperm entry that occur prior to the establishment of the sperm aster, in the first few minutes after fertilization (see Schatten and Schatten, 1981). These processes might be impeded by the meshwork of ER in the unfertilized egg, and breakdown of this meshwork would allow more free movement of the sperm nucleus in the egg cytoplasm.

Evidence that small objects encounter reduced resistance to movement during the approximate period of the apparent fragmentation of the ER has been provided by measurements of the displacement of an iron particle (5–9  $\mu\text{m}$  diameter) in the cytoplasm of a sea urchin egg in response to an applied magnetic field (Hiramoto, 1969a,b). These changes in cytoplasmic vis-

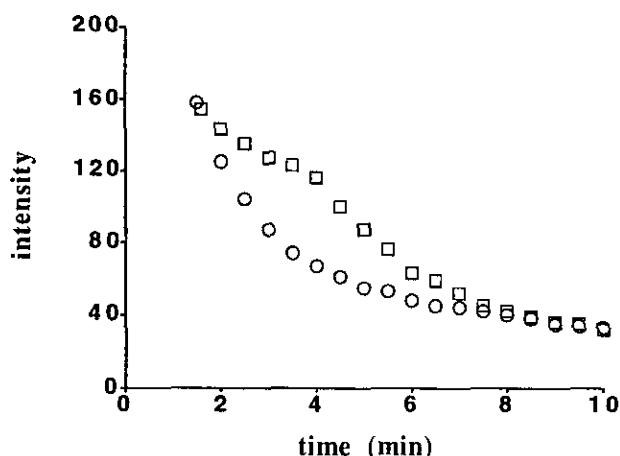


FIG. 7. Time course of the decrease of DiI fluorescence intensity of an oil drop in the cytoplasm of an unfertilized (○) or fertilized (□) egg. Same eggs as shown in Fig. 6. Arbitrary units of intensity. These are typical examples: For the fertilized egg shown in the graph, the fluorescence intensity decreased by 20% between 1.5 and 3.5 min after injection; by comparison the average decrease measured in five eggs was  $25 \pm 3\%$  (SD). For the unfertilized egg shown in the graph, the fluorescence intensity decreased by 49% between 1.5 and 3.5 min after injection; by comparison, the average decrease measured in six eggs was  $54 \pm 4\%$  (SD).

cosity are biphasic, involving an initial increase followed by a decrease and then a return to the original level, and their timing cannot be compared directly with our measurements due to differences in species and temperature. However, the kinetics are consistent with the interpretation that fragmentation of the ER accounts for the measured viscosity decrease. The ER changes may correspond to the changes in an "invisible network structure" as proposed by Hiramoto (1969b) to account for his observed changes in viscosity.

Transient decreases in cytoplasmic viscosity at fertilization have been reported for eggs of several species, including frogs (Elinson, 1983; determined by microdissection) and the marine worms *Urechis* and *Chaetopterus* (Tyler, 1932; Heilbrun and Wilson, 1948; determined by centrifugal stratification of cytoplasmic organelles). Viscosity decreases in these other species might also be accounted for by fragmentation of the ER. This might be a widespread mechanism for facilitating sperm entry and possibly other cytoplasmic rearrangements at fertilization.

This work was conducted in the Laboratory of Neurobiology at NIH; we thank Tom Reese for his support of the work, as well as his interest and advice. We also acknowledge useful discussions with Douglas Chandler and Mu-ming Poo, and thank Fraser Shilling, Bernhard Flucher, and Paul Gallant for review of the manuscript. This work was partially supported by grants from the NSF (DCB-9023987) and NIH (HD 14939) to L.A.J.

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