Bulk Membrane Fluidity Increases after Fertilization or Partial Activation of Sea Urchin Eggs*

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Membrane fluidity increases within 10 min after fertilization of Strongylocentrotus purpuratus and Lytechinus pictus eggs as detected by a decrease in order parameter of the spin label fatty acid 5-doxylstearate. The magnitude of this change is proportional to the fraction of fertilized eggs present. The order parameter decreases when unfertilized eggs are partially activated by ammonia treatment but does not change when the extracellular coats are removed. The increase in membrane fluidity, therefore, appears to be a "late" event in the fertilization program. The increase in fluidity is confined to the polar head group region of the membrane bilayer. Spectral and autoradiographic analyses indicate that the spin label resides in lipid bilayers throughout the cell. Thus, these measurements apply to bulk cell membranes. Detectable changes in lipid composition do not occur shortly after fertilization.

The fluid properties of biological membranes may play an important role in regulating membrane function (1). The activities of membrane proteins (2) and the coupling of hormone receptors to adenylyl cyclase (3) respond to changes in the fluidity of their environments. The fluidity of a membrane may dictate the orientation of inserted proteins (4) and the accessibility or function of receptors and antigens (5-7).

Both prokaryotes and eukaryotes hold membrane fluidity constant in the face of environmental pressures such as temperature changes and the availability of fatty acids (8-10). Failure to maintain membrane fluidity may be fatal (11). Control of fluidity in these cases occurs by altering the degree of saturation of phospholipid acyl chains.

On the other hand, a cell's response to an external stimulus can be a change in membrane fluidity. Some examples of stimuli that elicit changes in membrane fluidity are the binding of prostaglandin E to the human erythrocyte (12) and the binding of mitogenic lectins to lymphocytes (13, 14). Changes in membrane fluidity have also been documented for cells experiencing a physiological change of state, such as the fusion of myoblasts to form multinucleate myotubes (15, 16), the expansion of tetracythmena nuclei in preparation for DNA synthesis (17), and the malignant transformation of lymphocytes (18). In these examples little is known about how the change in fluidity is effected or its physiological role.

The unfertilized egg responds dramatically to the external stimulus of a fertilizing spermatozoon. Fertilization transforms a metabolically quiescent ovum into an actively dividing embryo. A well-defined sequence of events follows fertilization of the sea urchin egg (19). The first changes protect the egg from polyspermy. Metabolic processes increase sharply a few minutes later. The egg may be partially activated in the absence of sperm so that most of the early events do not occur.

Here we show that activation of the ovum results in an increase in membrane fluidity. This fluidity change is not a direct response to the fertilizing sperm; rather, it appears to be characteristic of the activated state. The fluidity change is a late event in the fertilization sequence (19).

MATERIALS AND METHODS

Handling of the Gametes—Gametes of Lytechinus pictus and Strongylocentrotus purpuratus were obtained by injecting 0.5 mM KCl into the body cavities. Eggs were washed at least three times with artificial sea water (Instant Ocean, Aquarium Systems, Inc., Eastlake, Ohio), pH 8.0, sedimented 1 min at 40,000 X g, and maintained at 15°C. Sperm were stored undiluted at 4°C.

Eggs were fertilized by adding dilute suspensions of sperm in sea water. Fertilization was scored by estimating under phase contrast microscopy the fraction of eggs with elevated fertilization membranes. For all animals used, more than 95% of the eggs were fertilizable.

When indicated, the jelly coat was removed by titrating a 10% (v/v) suspension of unfertilized eggs in sea water to pH 5.0 with 0.5 N HCl, gently swirling for 2 min, then adding 1 mM Trizma base (Sigma Chemical Co., St. Louis, Mo.) to a final pH of 8.0. The eggs were allowed to settle and then were washed (twice with sea water. Vitelline layers were disrupted by incubating a 10% (v/v) suspension of unfertilized eggs in sea water containing 10 mM dithiothreitol (Sigma), pH 9.1, for 10 min at 15°C (20). Alternatively, vitelline layers were removed by treating the egg suspension with cortical granule proteases (21) for 40 min at 15°C. The eggs then were washed three times with sea water.

Unfertilized eggs were activated by incubating a 5% (v/v) suspension of eggs for 20 min at 15°C in sea water titrated to pH 9.0 with NH4OH (22, 23). Activation was scored after incubating the eggs for 70 min in sea water, then fixing eggs for 30 min in 30 volumes of Carnoy's solution (60% ethanol, glacial acetic acid (9:1, v/v)) and staining condensed chromosomes with 1% orcein, 75% acetic acid.

Spin Labeling and ESR Measurements—Eggs were labeled by incubating 0.6 ml of a 25% (v/v) suspension of washed eggs for 10 min at 18°C with 0.4 mM of BSA-solubilized 5-, 12-, or 16-doxylstearate (N-oxyl-4',4'-dimethyloxazolidine derivative of 5-, 12-, or 16-keto-stearate, SVVA, Palo Alto, Calif., 1 mg/ml, 5% (w/v) BSA (essentially fatty acid free, Sigma) in sea water. Three washes with 10 ml of sea water removed unincorporated spin label.

Fertilized, labeled eggs grew to blastula, a feeding larval stage reached about 3 days after fertilization, and the labeled eggs were indistinguishable from the unlabeled control sample. Treatment of unfertilized eggs with BSA or BSA/spin label affected the elevation of fertilization membranes upon subsequent fertilization. Fertilization membranes of the affected eggs (approximately 25% of the population) detached incompletely from the egg surface, giving the fertilization membrane a blooby appearance under phase contrast microscopy.

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1 The abbreviation used is: BSA, bovine serum albumin.
Eggs fertilized in the presence of soybean trypsin inhibitor exhibited a similar effect. This effect may be attributable to the titration of endogenous proteases by BSA (24). Eggs were usually labeled after fertilization to avoid this complication.

Spin-labeled samples were examined in 50-μl capillary pipettes (Van Labs, Van Waters and Rogers, New York, N. Y.) after sealing one end of the pipette in a flame. The Van Labs Pipettes sealed more readily than the glass pipettes from several other sources. A plastic adapter fitted to the quartz temperature control insert (25) held the sample tube in the microwave cavity of a Varian E-104A or E-4 ESR spectrometer. Spectra were recorded using a modulation amplitude of 1 to 2 G and 10 milliwatt microwave power. The modulation amplitude was constant within a particular experiment.

A polarity-corrected order parameter, S, was measured from the ESR spectra (25, 26). Percent changes in S were calculated as ΔS/S, where ΔS is the change in order parameter observed and S is the order parameter of the unfertilized egg sample.

Eggs recovered from the sample tubes after recording ESR spectra were 70 to 90% viable as judged by their ability to complete the first cell division after fertilization.

Preparation of Lipid Dispersions—Lipids from dejellied S. purpuratus eggs (7 ml packed volume) were extracted (27) and applied to a column (5 cm diameter × 35 cm) packed with silica (Unisil, Clayadhesive, Clifton, N. J.). The layer was eluted with hexane/petroleum ether (9:1) with 90% (v/v) chloroform. Egg neutral lipids were stored at −20°C in chloroform after removing silica particles by centrifugation.

Sonic dispersions were prepared using bovine phosphatidylserine, dipalmityl glycerol, tristearin, cholesterol, and phosphatidylcholine (Applied Sciences, State College, Pa.), egg phosphatidylcholine (Sigma), mineral oil (E. R. Squibb & Sons, Princeton, N. J.), and sea urchin egg neutral lipids. The lipid was transferred to a glass sonication vessel, and then 10 ml of a 25% (v/v) suspension of chloroform was added and the sample was sonicated for 15 min at 20°C with a Branson model W-350 sonicator to produce a single-phase mixture. Lipid dispersions (0.2 ml) were added and the sample was subjected vigorously for 2 to 3 min and then transferred to a 50-μl capillary pipette for ESR measurements.

 Autoradiography of Eggs Labeled with Radioactive Fatty Acid—Washed unfertilized or fertilized eggs (0.3 ml of a 25% (v/v) suspension) were incubated for 10 min at 15°C in 8% (v/v) BSA/[14]cysteic acid (2.6 mM [5,10-3H]cysteic acid (0.13 mCi/μmol; New England Nuclear, Boston, Mass.) in sea water. After washes with sea water (5 ml each) to remove BSA and unincorporated label, the samples were fixed for 20 min at room temperature in 0.1% osmium tetroxide (Polysciences, Warrington, Pa.), 20 mM sodium cacodylate, pH 7.5, in sea water. Some samples were run through three times with 20 mM sodium cacodylate, pH 7.5, in sea water and postfixed for 60 min at room temperature in 3% glutaraldehyde, 70% sea water. The samples were washed three times with sea water and then dehydrated in a graded (30 to 100%) ethanol series followed by three washes in propylene oxide. The amount of radioactivity remaining after fixation and dehydration was determined by drawing a portion of the sample into a 100-μl capillary pipette and counting the number of eggs under a microscope. The eggs were transferred quantitatively into Triton/toluene scintillation fluid for scintillation counting.

 Autoradiographic preparations were stained and developed slides were examined in a bright-field microscope and photographed using Kodachrome 25 film.

 Kinetics of Label Uptake—Washed eggs (0.6 ml of a 25% (v/v) suspension) were incubated with BSA/5-doxyl stearate at 15°C with shaking. Labeling was terminated by washing the eggs three times with distilled water (10 ml each). The washing procedure was completed within 2 min. The egg pellet was suspended in methanol (1.3 ml) with vigorous mixing. Spin label uptake was measured from the ESR signal amplitude of the methanol extract. Cell protein was measured by a modification of the Lowry method described in Ref. 29, using BSA as a standard.

Cholesterol and Fatty Acid Analyses—Unfertilized and fertilized eggs were collected by centrifugation and homogenized in distilled water (2 ml). Cholesterylstea late served as an internal standard for cholesterol analysis. Lipid extracts (27) were dried in glass tubes under a stream of nitrogen gas. For cholesterol analysis the sample was dissolved in hexanes and analyzed by gas-liquid chromatography on a 1.8-m glass column packed with 3% OV-17 on 100/120 mesh Gas Chrom Q (Applied Sciences) using a Hewlett-Packard 5830 chromatograph at a column temperature of 260°C.

For fatty acid analysis the dry lipid extract was treated for 20 min at 60°C with 10% boron trichloride in methanol (0.5 ml, Applied Sciences). Distilled water (0.5 ml) and hexanes (1 ml) were added and the fatty acid methyl esters were recovered from the upper hexane phase. The mixture was resolved by gas-liquid chromatography on a 1.8-m glass column packed with 12% dithylene glycol succinate on cornosorb P (AW) (Applied Sciences) at 170°C. Identification of peaks was based on retention time comparison to the following standards (Applied Sciences): myristate, palmitate, palmitoleate, oleate, and erucate. Peaks with longer retention times were tentatively identified as eicosenate, arachidonate, and erucate by the method of Jameson (30). The relative amount of each component was calculated from the area under the chromatogram peaks.

Phospholipid Assay—At least 95% of the phosphatase in lipid extracts migrated as phospholipid on silica thin layer chromatography. Lipid phosphatase was measured by a modification of the method of Rouser et al. (31). Dry samples were digested with 70% perchloric acid (0.25 ml) for 20 min at 180°C. The samples were allowed to cool, then distilled water (0.95 ml), 1.25% (w/v) ammonium molybdate (0.4 ml), and 5% (w/v) ascorbate (0.4 ml) were added, and the mixtures were incubated at 100°C for 10 min. The absorbance was measured at 797 nm. NaH_{2}PO_{4}, served as a standard.

RESULTS

The Order Parameter of Eggs Labeled with 5-Doxylstearate Decreases upon Fertilization—5-Doxylstearate carries a paramagnetic N-oxazolidine ring separated from the COOH terminus by three methylene groups. In membranes and phospholipid bilayers the COOH terminus associates with the phospholipid head groups and the hydrocarbon tail extends into the bilayer (32). The ESR spectra of unfertilized and fertilized eggs labeled with 5-doxylstearate reveal rapid, anisotropic motion typical of a fatty acid residing within a phospholipid bilayer (32). The order parameter, S, is derived graphically (see Fig. 1). S equals 1 for a spin label moving rapidly about only one axis and S equals 0 for rapid, isotropic motion (29).

Order parameters were measured for unfertilized and fertilized eggs of two sea urchin species (Table 1). The data in Table 1 are representative of the data obtained in more than 30 experiments. Each batch of eggs represents the gametes from a different animal. For L. pictus eggs the average decrease in

![Fig. 1. ESR spectra of unfertilized and fertilized sea urchin eggs. ESR spectra of S. purpuratus eggs labeled with 5-doxylstearate were recorded at 12°C as described under Materials and Methods. The spectral parameters 27$\theta$ and 27$\theta$ were measured graphically and the order parameter, S, was calculated from these values (see Materials and Methods). The broad high field peak was recorded at a higher gain in order to place its position more accurately.]

\[ S = \frac{1}{2} \left( \frac{A}{B} \right) \]
**Membrane Fluidity of Sea Urchin Eggs**

S after fertilization was 2.08% (standard error = 0.29%). For *S. purpuratus* eggs the average decrease in S was 2.46% (standard error = 0.59%). Differences between multiple measurements of S and ΔS from a single batch of eggs did not exceed 0.5%. The spectral parameter 2T1 always decreased after fertilization while 2T1 remained constant or increased slightly (data not shown).

The change in order parameter is proportional to the fraction of eggs fertilized—the absolute value of S and the magnitude of ΔS varied between egg batches. The sperm/egg ratio needed to obtain maximal fertilization (>95%) also varied with the batch of gametes. A series of samples was prepared containing a constant number of eggs/sample and increasing numbers of sperm. For each sample the fraction of fertilized eggs was estimated. Fig. 2 shows the change in S as a function of relative sperm/egg ratio; 100% maximum ΔS is the largest decrease in S attained for that egg batch. A relative sperm/egg ratio of 1 is the ratio sufficient to yield maximum fertilization. The data were normalized in this way because the absolute sperm/egg ratio varied from one batch of gametes to another and depended on the experimental conditions.

The data of Fig. 2 was also plotted as ΔS, per cent of maximum versus per cent fertilized eggs (plot not shown). The change in S increased linearly with the proportion of fertilized eggs. The slope of this plot calculated by linear regression analysis approached 1 (slope = 0.975) and the correlation coefficient was 0.92. These results suggest that the fertilization-induced decrease in S is due to the fertilized state of the eggs and not the presence of sperm.

**Effect of Partial Activation and Disruption of Egg Surface Layers**—Two sets of biochemical experiments take place prior to the first cell division: early events occur within min and late events commence about 5 min after fertilization (19). Is the observed increase in membrane fluidity (decrease in S) an early or late event? Recording the ESR spectra requires approximately 10 min, so the decrease in S cannot be assigned to either category directly.

Early events include a Na+ influx and a change in membrane potential, a transient increase in free Ca2+ concentration, the Ca2+-induced exocytosis of thousands of cortical granules by fusion with the plasma membrane, and removal of extracellular layers. A jelly coat of glycoprotein (33) and the thin

![Table I](image)

**Effect of fertilization on order parameter of spin-labeled eggs**

Order parameters were measured from ESR spectra of 5-doxylstearate-labeled eggs recorded at 12°C (see "Materials and Methods"). Each batch of eggs represents the gametes from a different animal. The reproducibility of multiple measurements for the same batch of eggs was better than ±0.5%. The mean %ΔS ± standard error was 2.08 ± 0.29 for *L. pictus* and 2.46 ± 0.59 for *S. purpuratus*. By Wilcoxon's signed ranks test the significance levels of these differences are p = 0.003 for the *S. purpuratus* data, p = 0.008 for the *L. pictus* data, and 0.001 < p < 0.005 for the combined data.

![Figure 2](image)

**FIG. 2. Effect of sperm/egg ratio on fertilization-induced change in order parameter.** Eggs were counted using a hemocytometer or capillary pipettes to measure aliquots of egg suspensions. Sperm were counted using an electronic particle counter (Coulter model Z, Coulter Electronics, Hialeah, Fla.). For each batch of eggs the minimum sperm/egg ratio necessary for maximum (>95%) fertilization was defined as a relative sperm/egg ratio = 1. From 30 to 50% of the number of sperm necessary to achieve this ratio was added to eggs. The order parameter and percent fertilization were measured as described under "Materials and Methods."

**TABLE II**

Effect of various treatments on order parameter of spin-labeled eggs

| Experiment                         | Treatment | %ΔS   |
|-----------------------------------|-----------|-------|
| 1. Fertilization                  |           | -2.6  |
| 2. Ammonia activation             |           | -1.5  |
| 3. Sea water titrated to pH 9 with NaOH |           | +0.1  |
| 4. Jelly removal                  |           | -0.1  |
| 5. Vitelline layer removal with dithiothreitol |       | -0.1  |
| 6. Vitelline layer removal with dithiothreitol |   | +0.3  |
| 7. Fertilization                  |           | -2.6  |
| 8. Fertilization                  |           | -1.6  |
| 9. Vitelline layer removal with protease |           | -0.3  |

Vitelline layer near the plasma membrane (34) surround the unfertilized egg. A protease released from the cortical granules detaches the vitelline layer from the plasma membrane (24). This layer then rises, displacing the jelly coat, and becomes the "fertilization membrane" (34). The vitelline layer can be removed by treating unfertilized eggs with dithiothreitol or the purified cortical granule protease (see "Materials and Methods"). Removal of the jelly coat by washing unfertilized eggs in acidic sea water did not affect S (Table II). Removal of the vitelline layer by two different methods also had no effect on S (Table II).

Late events beginning about 5 min after fertilization include increased rates of protein synthesis, nutrient transport, increased K+ permeability, the initiation of DNA synthesis, and chromosome condensation (19). The intracellular pH rises between 1 and 4 min after fertilization (35, 36). Incubation of unfertilized eggs in ammonia-containing sea water (see "Materials and Methods") increases the intracellular pH (35). This treatment partially activates eggs in that they commence DNA and protein synthesis. None of the early changes take
place and the eggs fail to complete the first cell division, although chromosome condensation occurs (37). The change in $S$ following ammonia activation was comparable to the fertilization-induced change (Table III). The average decrease in $S$ was 1.65% (standard error = 0.20%) for $L.\text{pictus}$ eggs and 1.68% (standard error = 0.48%) for $S.\text{purpuratus}$ eggs. Condensed chromosomes were visible in eggs examined 90 min after exposure to ammonia/sea water. Unfertilized eggs incubated for 20 min in sea water titrated to pH 9.0 with NaOH, a treatment that mimics the ammonia activation procedure with respect to external pH without activating eggs, did not exhibit a change in $S$ (Table II). Chromosome condensation did not occur in NaOH-treated eggs. These results suggest that the decrease in $S$ is a late postfertilization event.

**Localization of the Spin Label Fatty Acid in Phospholipid Bilayers Distributed throughout the Egg** — The above results show that a decrease in $S$ measured with 5-doxylstearate accompanies metabolic activation of sea urchin eggs. The interpretation of this result depends upon where the spin label resides within the egg. Is the label confined to membranes? Which membranes are labeled?

The spin label fatty acid was introduced into eggs using BSA as a protein carrier. If BSA-bound 5-doxylstearate adheres to unfertilized eggs to a greater extent than to fertilized eggs, the result would be an apparent decrease in $S$ after fertilization. Another spin label fatty acid, 16-doxylstearate, was used to measure the amount of protein-bound probe remaining in unfertilized and fertilized eggs. The ESR spectra of 5- and 16-doxylstearate bound to BSA are very similar, but their spectra differ markedly in membranes because the paramagnetic group of 16-doxylstearate is deep in the hydrophobic interior of the bilayer where the acyl chain segments undergo nearly isotropic motion (38). The spectrum of 16-doxylstearate changes more dramatically than the spectrum of 5-doxylstearate when the label leaves BSA and enters a membrane. The spectrum changes upon adding sea urchin eggs to BSA/16-doxylstearate (Fig. 3). The new spectral features appearing after addition of eggs reveal more rapid motion of the label (39). After washing away unbound label, the remaining spectrum (Fig. 3c) is that of 16-doxylstearate in a bilayer membrane (39). This spectrum should not be analyzed in terms of order parameters because the motion is nearly isotropic. Examination of the low field region of the spectra allows one to estimate that less than 5% of the spin label fatty acid in eggs is bound to protein. From this result it is unlikely that the fertilization-induced change in $S$ is attributable to label binding to protein. The spectra of washed unfertilized and fertilized eggs were indistinguishable. Eggs labeled with 16-doxylstearate also showed no change in spectrum after fertilization. The change in fluidity after fertilization, therefore, is localized near the polar faces of the bilayer.

The sea urchin egg contains large amounts of neutral lipid. This lipid serves as an energy source for the embryo (33, 40-42). Neutral lipids (except cholesterol) are mainly absent from biological membranes and they probably do not form bilayers.

**Table III**

| Species     | Batch | Unfertilized | Ammonia activated | %ΔS |
|-------------|-------|--------------|-------------------|------|
| $L.\text{pictus}$ | 1     | 0.699        | 0.688             | -0.6 |
|             | 2     | 0.715        | 0.702             | -0.8 |
|             | 3     | 0.709        | 0.700             | -1.3 |
|             | 4     | 0.727        | 0.710             | -2.3 |
|             | 5     | 0.718        | 0.708             | -1.4 |
| $S.\text{purpuratus}$ | 1     | 0.736        | 0.729             | -1.0 |
|             | 2     | 0.739        | 0.729             | -1.4 |
|             | 3     | 0.751        | 0.738             | -3.1 |
|             | 4     | 0.730        | 0.721             | -1.2 |

$\%\Delta S \pm$ standard error was 1.65 ± 0.20 for $L.\text{pictus}$ and 1.65 ± 0.48 for $S.\text{purpuratus}$.

$\%\Delta S$ is a late postfertilization event.

For the anisotropic spectrum observed in Sample 11 the isotropic hyperfine splitting constant, $\alpha$, was calculated from the relation (65)

$$\alpha = \left(\frac{1}{2}T_L^2 - \frac{1}{5}T_P^2\right)/3.$$
various neutral lipids to evaluate the likelihood that it partitions into the sea urchin egg oil droplets or neutral lipid stores (Table IV).

The ESR spectrum of a spin label tumbling rapidly and isotropically consists of three sharp peaks. The separation between these peaks measures the polarity of the spin label's environment. Larger splittings correspond to increased polarity; for example, the isotropic splitting of 5-doxylstearate is 15.7 G in water and 14.25 G in toluene.

The spectra of samples containing 5-doxylstearate added to sonicated dispersions of triolein, dipalmitoylglycerol, mineral oil, or cholesteryl palmitate were identical to the spectrum of 5-doxylstearate in buffer (isotropic splittings, approximately 15.7 G) (Table IV). Longer incubation times and higher temperatures did not alter these results. 5-Doxylstearate prefers the aqueous phase to these lipid droplets. Sea urchin egg neutral lipids were dispersed in buffer at concentrations of 1 and 5 mM, based on an average lipid molecular weight of 750. The spectrum of 5-doxylstearate added to the 1 mM dispersion consisted of three sharp peaks with a splitting of 15.6 G. In the presence of the 5 mM dispersion the ESR spectrum exhibited two spectral components. Both spectral components corresponded to rapid, isotropic motion, and two splittings could be measured: 15.5 and 14.25 G. The spin label fatty acid partitions between the lipid phase and the aqueous phase under these conditions. Even though the spin label fatty acid exists predominantly in the ionized form at neutral pH, the uncharged form may reside in the neutral lipid droplets, where it tumbles rapidly and isotropically. Consistent with this interpretation, 5-doxylstearate methyl ester added to the 5 mM egg neutral lipid dispersion yielded a single-component spectrum very similar to the 5-doxylstearate spectral component having a splitting of 14.25 G (Table IV, Sample 10). Adding a small amount of a sonic dispersion of phospholipid to the 5 mM egg neutral lipid dispersion altered the spectrum of 5-doxylstearate to a spectrum showing rapid anisotropic motion of the label (Table IV, Sample 11). Apparently 5-doxylstearate partitions into a phospholipid bilayer in preference to a neutral lipid droplet. The spectrum obtained from sea urchin eggs probably represents label in phospholipid bilayers because only in that environment is the motion of 5-doxylstearate rapid and anisotropic.

A light microscopy autoradiography experiment was performed to ascertain the probable location of the spin label fatty acid in eggs. Saturated lipids may migrate to new sites during autoradiography (43) so [3H]oleate was chosen because its double bond permits chemical fixation to unsaturated lipids within the membrane (28) (see "Discussion"). Eggs were labeled with [3H]oleate under the same conditions used for spin labeling. The eggs then were fixed, imbedded in sectioning medium, thick-sectioned, and autoradiographed (see "Materials and Methods").

Unfertilized and fertilized eggs incorporated [3H]oleate and 5-doxylstearate at approximately the same rate and to approximately the same extent. From the specific activity of [3H]oleate added and the measured uptake of 5-doxylstearate (see below) the levels of [3H]oleate uptake expected were 10 and 8 cpm/egg for unfertilized and fertilized eggs, respectively. The observed values were 12 and 8.1 cpm/egg for unfertilized and fertilized eggs, respectively.

Immediately after labeling the eggs were fixed in osmium tetroxide. After osmium tetroxide treatment, 45% (unfertilized eggs) and 51% (fertilized eggs) of the radioactivity was lost from the eggs by washing. The washed samples then were postfixed with glutaraldehyde. Subsequent washes removed very little radioactivity (2.5% from fertilized eggs, 4.6% from fertilized eggs). Glutaraldehyde fixation probably cross-links amino-containing phosphatides, glycolipids, and membrane proteins (28, 44). This fixation sequence should preserve the position of [3H]oleate within the egg during the subsequent dehydration and imbedding steps. Unfertilized and fertilized eggs lost 25 and 23%, respectively, of their initial radioactivity during dehydration and imbedding.

The imbedded samples were sectioned (1 pm thickness) and the sections were exposed to a photographic emulsion for 28 days. Then the emulsion was developed and examined for exposed silver grains. The distribution of silver grains appeared random over the egg sections (Fig. 4, a and b) except that the nuclear regions showed few if any silver grains.

**Table IV.**
Sections of fertilized and unfertilized eggs appeared indistinguishable. The section shown in Fig. 4b was stained with toluidine blue to identify the nucleus (Fig. 4c). It is clear that silver grains appear throughout the cytoplasm, at the cell surface, cortex, and nuclear periphery but not over the nucleus. These results suggest that \( ^{3}H \) oleate distributes rapidly (within 10 min) throughout the egg.

**Kinetics of 5-Doxylstearate Uptake**—The uptake of 5-doxylstearate by unfertilized and fertilized eggs was measured as described under “Materials and Methods” (Fig. 5). The uptake level after a 10-min labeling period varied from 1.1 to 1.6 mol % (relative to egg phospholipid) for unfertilized eggs and from 1.0 to 1.3 mol % for fertilized eggs. No evidence of spin-spin interaction was seen in the ESR spectra of eggs and spin-spin interactions should not occur at these spin label concentrations. This effect is not likely to play a role here because the label concentrations are low and the concentrations are nearly the same in fertilized and unfertilized eggs.

**Characterization of Egg Lipids**—Changes in membrane cholesterol level and fatty acid composition affect fluidity measured by spin labels (47). The cholesterol level in *S. purpuratus* eggs was measured (Fig. 6). Eggs from three

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**Table V**

| Component | Composition at following times after fertilization |
|-----------|----------------------------------------------------|
|           | 0 min  | 10 min | 30 min | 90 min | 120 min |
| 14:0      | 12.9   | 12.1   | 13.1   | 12.1   | 12.7    |
| 16:0      | 15.3   | 15.9   | 16.3   | 15.0   | 16.9    |
| 16:1      | 4.5    | 4.4    | 4.6    | 4.6    | 5.1     |
| 18:1      | 5.9    | 5.3    | 5.8    | 5.8    | 6.1     |
| 20:0      | 12.8   | 13.6   | 12.7   | 13.6   | 13.2    |
| 20:1      | 8.7    | 9.3    | 9.7    | 9.7    | 9.9     |
| 20:4      | 15.7   | 15.7   | 13.6   | 13.6   | 14.2    |
| 22:1      | 11.8   | 11.9   | 11.1   | 11.1   | 11.2    |
| Total C16| 19.8   | 20.3   | 20.9   | 19.6   | 21.1    |
| Total C18| 37.2   | 38.6   | 36.0   | 36.9   | 37.3    |
| Total saturated| 41.0 | 41.6 | 42.1 | 40.7 | 41.9 |
| Total unsaturated| 46.6 | 46.6 | 47.1 | 44.8 | 46.5 |

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**Fig. 5.** Uptake of 5-doxylstearate by eggs. *S. purpuratus* eggs were labeled with 5-doxylstearate for varying times. Labeling was terminated and the spin label extracted by the addition of methanol (see “Materials and Methods”). Spin label uptake was measured from the ESR signal amplitude of the methanol extract.

**Fig. 6.** Effect of fertilization on the cholesterol/phospholipid ratio of eggs. Cholesterol and phospholipid levels were measured at the indicated times after fertilization of *S. purpuratus* eggs (see “Materials and Methods”).

**Fig. 7.** Temperature dependence of order parameter in eggs labeled with 5-doxylstearate. The temperature was increased from 5 to 18°C in 1°C increments. The *S. purpuratus* eggs were allowed to equilibrate for at least 10 min at each temperature before measurement.

Animals were analyzed for cholesterol and phospholipid content. The cholesterol/phospholipid mole ratio (0.57 ± 0.04) did not change significantly within 2 h after fertilization. Other sea urchin species also show no change in cholesterol level after fertilization (41, 48).

Fatty acid analyses were performed on *S. purpuratus* eggs (Table V). The results shown are averages from three animals. Neither the degree of saturation nor the average chain length of fatty acids changed significantly within 2 h of fertilization.

Other workers failed to detect changes in phospholipid composition after fertilization (48). Our analyses also showed no detectable changes (data not shown). These results suggest
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that changes in lipid composition are not responsible for the increase in membrane fluidity after fertilization.

Temperature Dependence of the Order Parameter—The order parameter of unfertilized and fertilized eggs decreased continuously over the temperature range 4 to 18°C (Fig. 7). The difference in order parameter between unfertilized and fertilized eggs was nearly constant over this range. No abrupt changes in slope were seen, although small changes in slope occurred at temperatures that varied with the batch of eggs.

DISCUSSION

The egg undergoes a remarkable change of state in response to fertilization. The results presented here indicate that an increase in bulk membrane fluidity accompanies the transition from metabolic repression to vigorous proliferation.

The order parameter, S, measures the mean angular deviation from the bilayer normal, \( <\cos^2\theta>\), of the fatty acid chain segment at the position of the oxazolidine ring (49):

\[
S = (3 <\cos^2\theta> - 1)/2
\]

A decrease in S may be interpreted as an increase in fluidity (25) even though S does not directly measure rates of molecular motion (49, 50). For example, a 2% increase in S caused by an increase in plasma membrane cholesterol level decreases the (Na,K)-ATPase activity of Chinese hamster ovary cells by 2- to 3-fold (54). Another way to appraise the change in S is to compare it to the change in S associated with a lipid phase transition. Further definition of the intracellular location of the spin label fatty acids to bilayer perturbations depends on the location of the cholesterol ring along the fatty acid chain.

5-Doxylstearate was introduced into the eggs using BSA as a carrier. The ESR spectrum of 5-doxylstearate in sea urchin eggs reveals rapid (\( T_1 > 10^{-5} \text{ s} \)) anisotropic motion (49). BSA-bound 5-doxylstearate has a different spectrum. Little or no protein-bound label remains in washed eggs, as judged by the spectra of eggs labeled with 16-doxylstearate. Thus, the observed decrease in S after fertilization is not caused by binding of label to BSA or other proteins. L. pictus eggs labeled with ethanol-solubilized 5-doxylstearate also exhibited a decrease in S after fertilization (average, 2.01%). The BSA method was preferred because eggs divided abnormally in the presence of low concentrations of ethanol.

The membrane structural change reported by 5-doxylstearate occurs near the polar head group region of the bilayer since 12-doxylstearate and 16-doxylstearate revealed no differences between fertilized and unfertilized eggs. The response of spin label fatty acids to bilayer perturbations depends on the location of the oxazolidine ring along the fatty acid chain. For example, the condensing effect of cholesterol on phosphatidylcholine bilayers is reported as a change in S of 0.15 for 5-doxylstearate, 0.32 for 12-doxylstearate and 0.07 for 16-doxylstearate (38).

Eggs labeled with 12-doxylstearate lost spin label paramagnetism more rapidly than eggs labeled with 5-doxylstearate or 16-doxylstearate, probably by enzymatic reduction of the label. Fertilized eggs destroyed the ESR signal faster than unfertilized eggs. The active site of the membrane-bound reductase system may be located near the position of the oxazolidine ring in 12-doxylstearate. Reduction of spin label fatty acids has been observed in other biological systems (7, 59).

The spin label fatty acids appear to reside in the lipid phase of sea urchin egg membranes. The line shapes of the spectra of 5-, 12-, and 16-doxylstearate indicate rapid, anisotropic motion typical of a lipid bilayer environment, with a gradient of increasing fluidity toward the center of the bilayer (38, 60, 61). 5-Doxylstearate does not readily partition into neutral lipid droplets in vitro. At high concentrations of egg neutral lipids some label enters the lipid phase but the shape of the spectrum does not indicate a lipid bilayer environment. Adding 10 mol % phospholipid dispersion to this mixture drives the spin label fatty acid into the phospholipid bilayer environment. These spectroscopic experiments seem to eliminate the sizable pool of neutral lipids present in sea urchin eggs as a potential location for 5-doxylstearate.

Further definition of the intracellular location of the spin label fatty acids would be provided by experiments with 5-doxylstearate.

2 J. Elhai, D. Treco, and C. J. Scandella, unpublished results.
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label fatty acid was attempted by autoradiography of [3H]-oleate-labeled sea urchin eggs. The results of this experiment suggest that the spin label fatty acid distributes throughout intracellular membranes. Radioactive 5-doxylstearate was not used for this experiment because saturated lipids may migrate during autoradiography (43, 62). The double bond in [3H]-oleate permits cross-linking to membrane lipids. Cross-linking of [3H]oleate in sea urchin eggs by osmium tetroxide followed by glutaraldehyde should ensure its anchorage within the membrane (63).

5-Doxylstearate resembles an 18-carbon monounsaturated fatty acid in its melting point, chromatographic behavior, and ability to support the growth of cultured fibroblasts (7, 64). The rate and extent of uptake of [3H]oleate were very similar to those of 5-doxylstearate in both fertilized and unfertilized eggs. Despite these similarities it is possible that their cellular distribution differs. A second difficulty with autoradiographic localization is that [3H]oleate may redistribute during the fixation, dehydration, and imbedding steps.

The possibility of redistribution of label also hinders attempts to localize the label by membrane fractionation. Kaplan et al. (59) isolated membrane fractions from intact mammalian cells labeled with 5-doxylstearate. ESR signal was detected in all of the membrane fractions. These authors noted that the label might redistribute upon homogenization because of its appreciable water solubility.

Sections stained with toluidine blue (Fig. 4, b and c) revealed silver grains over regions corresponding to the cell surface, cortex, and nuclear periphery, but not to the nucleus. Despite these similarities it is possible that their cellular localization is that [3H]oleate may redistribute during the fixation, dehydration, and imbedding steps.

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Sections stained with toluidine blue (Fig. 4, b and c) revealed silver grains over regions corresponding to the cell surface, cortex, and nuclear periphery, but not to the nucleus. If the label is confined to a subgroup of intracellular membranes, the subgroup must include surface membranes and probably nuclear membranes. Frequent collisions between paramagnetic molecules distort the ESR spectrum (45). The absence of this distortion in our experiments, together with the levels of 5-doxylstearate present and the autoradiographic results, argues that 5-doxylstearate is diluted throughout most of the cell membranes.

A plasma membrane fraction isolated from fertilized and unfertilized eggs yielded different ESR spectra, compared to whole eggs. In other cells 5-doxylstearate may label the plasma membrane selectively (65). It seems clear that selective labeling of the plasma membrane does not occur in sea urchin eggs.

Our experiments show that (a) the spin label is not bound to protein, (b) the spin label is probably not located in lipid droplets, and (c) exogenously added fatty acid partitions rapidly throughout the egg. From these results we infer that the spin label resides in membranes throughout the cell.

The lipid composition of a membrane affects its fluidity. Lipid analyses revealed no changes in composition after fertilization. The cholesterol content and fatty acid distribution of S. purpuratus eggs remained constant for 2 h after fertilization. Other sea urchin species show a similar constancy (41, 48). Gaffney and Chen (47) report that spin labels detect a 10% change in saturation or a 5% change in cholesterol content of model systems. The magnitude of these changes is close to the error limits for our analyses. The localization of the change in fluidity near the head group region suggests that changes in cholesterol content and acyl chain composition are not involved.

Several other possible mechanisms remain. Changes in intracellular pH and ionic content (66) alter membrane fluidity. The intracellular pH of the sea urchin egg rises approximately 0.4 pH unit after fertilization (35, 36). Higher pH favors the charged forms of phosphatidylethanolamine, resulting in increased mobility, as measured by phosphorus and proton NMR (67). This effect should be more apparent near the polar face of the membrane.

Divalent cations aggregate negatively charged phospholipids (68–70). The intracellular Ca2+ concentration rises after fertilization, but this increase may be confined to the cortex region (71, 72). A direct interaction of Ca2+ with phospholipids cannot account for the fertilization-induced fluidity change because (a) the majority of cell membranes may not experience the increase in Ca2+ concentration, (b) increasing Ca2+ decreases fluidity in model systems, (c) the Ca2+ level needed to produce a measurable effect on fluidity in lipid model systems is 2 to 3 orders of magnitude higher than the peak concentration observed after fertilization, and (d) ammonia activation elicits the increase in fluidity but not the rise in intracellular Ca2+ concentration (73).

Monovalent cation permeabilities change after fertilization (74) and ammonia activation (75). It is not clear that bulk concentrations of these ions change appreciably. Monovalent cations interact with the polar region of membranes (96).

Membrane protein decreases fatty acyl chain flexibility (76–78). Fertilization changes the distribution of intramembrane particles seen in freeze-fracture electron micrographs (79). The formation of protein-free patches within the membrane might increase its fluidity. Proteolysis or phosphorylation of membrane proteins might also alter fluidity.

The complexity of the intact cell makes it difficult to establish the mechanism(s) responsible for the increase in membrane fluidity after fertilization of sea urchin eggs. We have pursued these studies using a plasma membrane fraction isolated from fertilized and unfertilized eggs (80).

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REFERENCES

1. Singer, S. J., and Nicholson, G. L. (1972) Science 175, 720–731
2. Sandermann, H. (1978) Biochim. Biophys. Acta 515, 207–237
3. Rimon, G., Hanski, E., Braun, S., and Levitzki, A. (1978) Nature 276, 394–396
4. Wickner, W. T. (1977) Biochemistry 16, 254–258
5. Horwitz, A. F., Hatten, M. E., and Burger, M. M. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 3115–3119
6. Boronhov, H., and Shintizky, M. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 4526–4530
7. Hatten, M. E., Scandella, C. J., Horwitz, A. F., and Burger, M. M. (1978) J. Biol. Chem. 253, 1527–1532
8. Sinensky, M. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 522–525
9. Martin, C. E., Hirai, M., Kitajima, Y., Nozawa, Y., Skrivar, L., and Thompson, G. A., Jr. (1976) Biochemistry 15, 5218–5227
10. Okuyama, H., Yamada, K., Kameyamani, Y., Ikazeau, N., Aka- matsuy, Y., and Nojima, S. (1977) Biochemistry 16, 2668–2673
11. Eisser, A. F., and Souza, K. A. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 4111–4115
12. Kury, P. G., and McConnell, H. M. (1978) Biochemistry 17, 2983–2986
13. Barnett, R. E., Scott, R. E., Puchet, L. T., and Kersey, J. H. (1974) Nature 249, 465–466
14. Toyoshima, T., and Osawa, T. (1975) J. Biochem. 78, 1655–1660
15. Prives, J., and Shinitzky, M. (1977) Nature 268, 761–763
16. Herman, B. A., and Fernandez, S. M. (1978) J. Cell Biol. 79, 479–490
17. Wunderlich, F., Giese, G., and Bucherer, C. (1978) J. Cell Biol. 79, 479–490
18. Petrou, M., Tuy, F., Rosenfeld, C., Mishal, M., Painrad, M., Jasin, C., Mathe, G., and Inbar, M. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2306–2310

2 J. Campisi and C. J. Scandella, unpublished results.
