Turning on of activities in unfertilized sea urchin eggs: Correlation with changes of the surface
(fertilization/deression/cell surface)

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Contributed by Daniel Mazia, August 20, 1975

ABSTRACT Unfertilized sea urchin eggs exposed to low concentrations of ammonia enter into a number of activities which normally appear after fertilization. It is shown that the effects are attributable to ammonia, rather than to NH$_4^+$ ions or elevated pH. The same effects are obtained by exposure to isotonic urea and to glycerol at very low ionic strengths. All treatments which produce these changes (such as the turning on of chromosome replication and condensation in unfertilized eggs) also bring about changes of the outer cell surface which are visible in the scanning electron microscope. The most striking indicator is the elongation of the microvilli which cover the surface of the unfertilized egg. The changes of the surface are interpreted as the dissociation of a component from the outer surface layer. This component is not the “vitelline” sheet as defined morphologically or by the ability of the egg to form a fertilization membrane upon insemination. It is proposed further that this component is a peripheral component of the plasma membrane, whose removal modifies the membrane functionally and leads to the derepression of various processes within the egg.

An earlier series of publications describes the effects of ammonia in initiating, in unfertilized sea urchin eggs, a number of events which normally begin after fertilization. They include: polarization of the membrane by the development of K$^+$-conductance (1); stimulation of protein synthesis (3); turning on of DNA synthesis (3) leading to the condensation of the replicated chromosomes (4, 5); the polyadenylation of cytoplasmic messenger RNA (6). The effects are not equivalent to parthenogenetic activation; the “early” events of activation are bypassed (1, 2) and the egg does not divide or develop.

The present work describes other treatments which arouse these activities in unfertilized eggs. It will be shown that all the effective treatments modify the surface of the egg visibly. Their action will be interpreted as the dissociation of a peripheral component of the membrane from the outer surface.

MATERIALS AND METHODS

Eggs. The gametes of the sea urchin Lytechinus pictus and Strongylocentrotus purpuratus were used. The jelly coats were removed from the eggs by a brief (about 1 min) treatment with sea water acidified to pH 4. The full account of experimental results given below applies to eggs of L. pictus. The basic findings on the ammonia effects, including the surface changes, have been confirmed with eggs of S. purpuratus.

Solutions. “NH$_3$-sea water” was prepared in two ways. One was the titration of sea water to pH 9–9.2 with NH$_4$OH. The other was the addition of known amounts of NH$_4$Cl (0.5–5 mM final concentration) and titration with NaOH to pH 9–9.2. The latter method is convenient for controlling and calculating the concentration of the active component, NH$_3$, at a constant pH.

Observation of Chromosomes. The methods are described by Mazia (5).

Membrane Potential. The techniques used have been described earlier (7).

Scanning Electron Microscopy. After treatments, eggs were glued to polylysine-coated glass plates (8) and fixed with 5% glutaraldehyde in 80% sea water (pH 8.2). The samples were dehydrated in ethanol, dried at the critical point in Freon, and coated with platinum–carbon.

Protamine-Coated Glass Fibers. The glass fibers were prepared by grinding Whatman glass paper (GF/A) in a mortar with a small amount of water. The fibers, a few micrometers thick and of variable (about 20–200 μm) length, were then washed four to five times in sea water to remove unadsorbed protamine.

RESULTS AND INTERPRETATIONS

1. The ammonia effects

(a) Changes in the Outer Cell Surface. With the scanning electron microscope (SEM), it is possible to see changes in the outer aspect of the egg which will be correlated consistently with the ammonia effects. The surface of the normal unfertilized egg is densely papillated with low microvilli, and the vitelline sheet, the precursor to the fertilization coat, overlies the plasma membrane. Fig. 1 shows the surface of normal unfertilized eggs of L. pictus. Fig. 2 shows the surfaces after a treatment with NH$_3$-sea water just sufficient to produce the ammonia effects. The conspicuous change of surface is the elongation and disarrayal of the microvilli, as though they have been released from some ordering restraint. This visible change of the surface will be seen with all treatments which have the same effects as does NH$_3$-sea water.

(b) Variables of the Effects of Ammonia-Sea Water. An aqueous solution of NH$_4$OH contains NH$_4^+$ and OH$^-$ ions and NH$_3$ molecules. The effects of NH$_3$-sea water depend on the concentration of the unionized NH$_3$. Epel et al. (2) found that chromosome condensation could be turned on even at pH 8 in sea water containing a 5 mM or higher total concentration of ammonium salt. In the present work, the same experiment was done at pH 9.2, adding various amounts of NH$_4$Cl to sea water and titrating with NaOH. At this pH, sea water containing 0.5 mM NH$_4$Cl was fully effective (in 20 min) in turning on chromosome condensation; 0.2 mM NH$_4$Cl was insufficient. The concentration of NH$_3$ required to produce the described effects, calculated on the basis of pK = 4.75, is 0.2–0.3 mM.

Sea water brought to pH 9 with NaOH does not have the effects of sea water titrated to the same pH with NH$_4$OH. NaOH-sea water at pH 9 could not turn on chromosome
condensation even with an exposure of 1 hr. Nor did the NaOH-sea water affect the aspect of the surface of the egg as observed with the SEM; the low profile of the microvilli was preserved.

An analog of NH₂OH, ethylamine, has been found to be effective in turning on chromosome replication and condensation in Lytechinus eggs. The solution used was 5 mM ethylamine hydrochloride in sea water, titrated to pH 9.2 with Na₂CO₃, with a 15 min exposure.

2. Treatments with non-electrolytes; “ammonia effects” without ammonia?

The set of changes which have been described as effects of ammonia-sea water can be produced by brief exposure of unfertilized eggs to isotonic solutions of non-electrolytes containing very low concentrations of electrolytes.

In older literature, it was recognized that such media attack the outer surface layers, at least to the extent of removing the vitelline sheet (9).

Sea urchins washed thoroughly in isotonic non-electrolyte solutions undergo spontaneous lysis. Addition of CaCl₂ to 0.1 mM will stabilize them.

Lytechinus eggs are exposed briefly to 1 M urea, containing 0.1 mM Ca and adjusted to pH 8 with NaOH. After a wash in the urea solution to remove residual sea water, the eggs are exposed for 2–3 min, then returned to sea water. The increase in membrane potential sets in rapidly (Fig. 3). The chromosome condensation is seen by around 90 min after the treatment with urea. The surface changes, seen by SEM as the extension and disarray of microvilli, are conspicuous.

The same effects have been obtained with isotonic (1 M) solutions of glycerol and of glucose. The non-electrolyte solutions contained 0.1 mM CaCl₂ and 1 mM Na added as Na₂CO₃; they were adjusted to pH 8. Again the eggs were quickly washed in the medium before the exposures of 5–10 min. The chromosome cycle was turned on; condensed replicated chromosomes were seen in all the eggs by 90 min. The modification of the outer surface is pronounced after these brief treatments with non-electrolyte media (Fig. 4); the vitelline sheet is removed completely, and the microvilli are quite extended and are spaced irregularly.

These results with non-electrolyte solutions undermine the earlier (1, 2) interpretation of the ammonia effects as consequences of the penetration of ammonia into the cell, acting through changes in internal pH.

3. Mechanical removal of surface components

In the results presented so far, all the treatments which caused unfertilized eggs to undergo certain changes typical of fertilized eggs could be interpreted as the removal of
components of the outer surface layer. A test of this interpretation would be the direct mechanical removal of the outer surface layer. This was possible with *Lytechinus* eggs by attaching protamine-coated glass fibers to the outer surface and shaking the egg suspension strongly on a mechanical shaker. The fibers adhered strongly to the outer surface and the turbulence caused them to pull away, carrying with them the surface components to which they were attached (Fig. 5). The effects of this mechanical peeling of regions of the outer surface are seen in Fig. 5 where microvilli extend in regions where an outer sheet has been torn.

The membrane potential of *Lytechinus* eggs so treated was measured. The membrane developed a potential of −60 mV or more, just as do eggs treated with NH$_4$OH or urea. They entered the chromosome cycle and continued to repeat it, as is shown in Fig. 5c.

The success of the mechanical "peeling" of the outer surface of the *Lytechinus* egg could not be repeated with the eggs of *S. purpuratus*. Neither the visible effect on the surface nor the activation of post-fertilization activities could be obtained with fibers coated with protamine.

4. Components of the outer surface; removal of the vitelline sheet

The vitelline sheet is the recognized structure overlying the plasma membrane of the unfertilized egg. It is defined by its function as the precursor to the fertilization membrane and has been described in electron microscopic studies (ref. 10; earlier literature summarized in ref. 11). Various methods have been used to remove it in order to obtain fertilized eggs without fertilization coats. One such method employs dithiothreitol (12). In the present work, we have treated eggs of both *L. pictus* and *S. purpuratus* with dithiothreitol in sea water at concentrations of 5–20 mM and at pH levels up to 9. The vitelline sheets are removed; membranes do not elevate at fertilization.

The removal of the vitelline sheet with dithiothreitol is not followed by the changes observed after treatment with ammonia or urea. The membrane potential is not changed; the chromosome cycle is not turned on. With the SEM, we observe (Fig. 6a) that the microvilli retain their regular arrangement and low profile, as though still restrained. If the exposure to dithiothreitol is followed by exposure to NH$_3$-sea water (or to the non-electrolyte medium) the microvilli extend (Fig. 6b). The changes typical of the ammonia effect follow. Comparing Fig. 6a and 6b, it is seen that some material remains on the outer surface after the dithiothreitol treatment and is then removed by the NH$_3$-sea water. It is the removal of this layer which, we propose, is responsible for the changes in the outer surface which lead to the turning on of various processes in the unfertilized egg.

There is no correlation between the removal of the vitelline sheet and the turning on of activities in the egg. Non-electrolyte solutions remove the vitelline sheet rapidly. Ammonia removes it slowly and the eggs will still raise fertilization coats when inseminated after the minimum effective treatment with ammonia. Ethylamine produces the ammonia effects with still less impairment of the vitelline sheet. In Fig. 7 one sees the surface of the egg through the vitelline sheet; the microvilli have elongated but are pressed flat against the cell surface.

5. Summary of results

Unfertilized sea urchin eggs exposed to NH$_3$-sea water are incited to enter a number of changes which normally follow fertilization. The effects of NH$_3$-sea water are attributed to ammonia (or unionized NH$_4$OH); they have also been obtained with ethylammonium hydroxide. Similar results may be obtained with isotonic urea solutions and with isotonic glycerol solutions at minimal concentrations of ions. The effects are correlated with a conspicuous change of the outer cell surface that can be seen with the SEM; one can predict the behavior of the egg from observations of the surface or predict the appearance of the surface from the behavior of the eggs.

We will interpret these effects as the result of the removal of a component of the outer surface. The component is not the vitelline sheet as defined morphologically or as the pre-
cursor to the fertilization membrane. It is proposed that the component is situated below the vitelline sheet and on the plasma membrane.

DISCUSSION

The interpretation of these findings appeals to fundamental theses of membrane biology. An older one is that changes in the cell membrane can regulate changes in the functions of cells. A newer one is that changes on the outer surface of the cell can modify the functions of the plasma membrane. These propositions have been implicit in the analysis of fertilization for a long time. Fertilization is an extreme case. A rather dormant egg, equipped to do almost everything a cell can do, is aroused to activities of all kinds; it is vividly evident that the changes start at the cell surface. The surface of the egg is very complex, but the complexity may represent only a more explicit display of features found in other cell surfaces.

In interpreting the results, we adapt the concept (13) that the plasma membrane consists of integral components, built into the lipid bilayer, and peripheral components which play a part in the functions of the membrane but can be dissociated from the bilayer. The substances removed by our various treatments are peripheral components; their removal changes the membrane. [We present here no evidence that they are proteins, but the removal of surface proteins by NH$_3$-sea water is shown by Johnson and Epel (14).]

In the interpretive diagram (Fig. 8), the integral components are shown as channels and the removal of the peripheral components is imaged fancifully as the pulling of stoppers. This is only a symbolic figuration of changes in permeabilities which follow fertilization and which also follow the ammonia effects. In both cases, an increase in K$^+$-conductance ensues. There are increases in the transport of thymidine (3) and of amino acids (2), though these come more slowly after the treatment with ammonia than they do after fertilization. After fertilization, there is an increase in passive permeability to water (15) and to non-electrolytes (16). The diagram would be misleading if taken to imply that the changes in various permeability functions following dissociation of the peripheral component are immediate.

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**FIG. 6.** (a) Surface of an L. pictus egg after 15 min in sea water at pH 8.5 containing 20 mM dithiothreitol. The microvilli are low but no longer so closely and regularly arranged as in Fig. 1. On the surface between the microvilli one sees a sprinkling of small particles. After removal of the vitelline sheet with dithiothreitol the egg behaves as a normal unfertilized egg (X10,000). (b) After removal of the vitelline sheet with dithiothreitol eggs were treated with sea water adjusted to pH 9.2 with NH$_3$OH. The microvilli are much longer and are separated by areas of the cell surface which no longer show the small particles observed in (a) (X10,000).

**FIG. 7.** Surface of an L. pictus egg treated with sea water containing 5 mM ethylamine and adjusted to pH 9.2 for 15 min. The disarrayed and elongated microvilli are seen through the remaining vitelline sheet (X10,000).

**FIG. 8.** A schematic interpretation of present results. At the outer surface of the normal unfertilized egg, peripheral components (P) of the plasma membrane are linked to integral components (I), regulating functions of the membrane; components P also are bonded to the vitelline sheet (VS). Removal of the vitelline sheet with dithiothreitol does not affect the peripheral component. Treatments with ammonia-sea water or non-electrolyte solutions dissociate the components P from the membrane; changes which normally follow fertilization are turned on. In normal fertilization, the surface reactions dissociate components P from the membrane as well as elevating the vitelline sheet to form a fertilization coat (FC).
We may include the surface changes at normal fertilization in the scheme shown in Fig. 8. The longest-known and most obvious happening at fertilization is the separation of an outer layer from the surface of the egg, forming a fertilization coat (17). This has seemed to be a dispensable event, since fertilization proceeds normally without the formation of the fertilization coat after various treatments, as with di-thiotreitol. Now it is proposed that normal fertilization does not merely lift up an overlying and dispensable vitelline sheet. It separates from the membrane an outer surface complex which is better regarded as a component of the membrane of the unfertilized egg and which may be responsible for the repression of activities in that egg. The visible sign of this dissociation, the elongation of the microvilli, has been observed in normal fertilization (18).

In fertilization, the egg uses the complex gadgetry of the "cortical reactions"—fusion of secretory vesicles with the membrane and discharge of their contents—to bring about effects which can be imitated by ammonia, etc. The imitation is not a substitution. Fertilization and proper parthenogenetic procedures motivate additional events which are all-important for development.

It is quite in accord with current views about the cell surface that peripheral components of the membrane, on the outer surface, should be responsible for the properties of the membrane of the unfertilized egg and for the repressed state of activity in that egg. The effects of ammonia and of non-electrolytes define conditions of the dissociation of the components from the surface, using visible changes and the turning on of various processes as criteria. It should be possible to identify the components and to reassociate them with the surface; an indication that this may be done is reported by Johnson and Epel (14).

This work has depended on support from the following grants: U.S. Public Health Service GM 13882 to D.M.; National Science Foundation GB 42547 to R.S.; and National Science Foundation GB 38359, which provided a scanning electron microscope to the Electron Microscope Laboratory of this institution.