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The problem of sea urchin egg fertilization and its implications for biological studies

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Summary. The analysis of sea urchin egg fertilization shows that several phenomena common to other biological systems are involved: cell recognition, cell fusion, exocytosis and initiation of mitotic activity. Both the role of calcium ions in cell fusion and exocytosis, and the function of the cell surface in the initiation of mitotic activity appear to have general applicability. The study of fertilization can contribute to the elucidation of these processes and, reciprocally, progress in this field can help to advance our understanding of the mechanisms of fertilization in sea urchins and other organisms.

Fertilization is the fusion of a spermatozoon and an egg, resulting in the formation of the zygote, the starting point for the development of a new organism. By fertilizing the egg, the spermatozoon activates metabolic processes (respiration, protein and nucleic acid synthesis) and transfers genetic information to the egg. Several fundamental processes of cell biology, such as cell recognition, cell fusion, exocytosis and initiation of mitotic activity, are involved in the process of fertilization. We propose to examine these different phenomena in the fertilization of the sea urchin egg. The collection of sea urchin gametes is easy and fertilization can be obtained in the laboratory with an efficiency approaching 100%. Important progress in the analysis of fertilization in the echinoderms has been achieved at the ultrastructural and biochemical levels. The union of the gametes involves a sequence of events occurring at different stages in the fusion process, either in the spermatozoon or in the egg. These are: formation of the acrosomal process, fusion of this process with the egg plasma membrane, exocytosis of cortical granules and finally fusion of the 2 pronuclei.

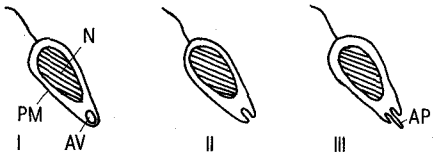

Fig. 1. Schematic representation of the acrosomal reaction. I, unreacted spermatozoa; II, opening of the acrosomal vesicle after fusion of the vesicle membrane with the sperm plasma membrane; III, elongation of the acrosomal process. N, Nucleus; AV, acrosomal vesicle; PM, sperm plasma membrane; AP, acrosomal process.

Formation of the acrosomal process

Spermatozoa liberated in sea water do not fertilize eggs immediately. First, they have to undergo the acrosome reaction (figure 1). The acrosome is a membrane-bounded vesicle located anterior to the nucleus in the spermatozoon. In a first step, fusion takes place between the sperm plasma membrane and the acrosomal membrane. The acrosomal vesicle then opens to the outside and its content scatters rapidly. In a second step, the acrosomal membrane everts and extends, forming the acrosomal process. It is the tip of this process which fuses with the plasma membrane of the egg. Through the fusion zone, egg cytoplasm progressively engulfs the nucleus, mitochondria and tail of the spermatozoon.

What is the nature of the inducer of the acrosomal reaction? The pioneering researches of Dan[1,2] showed that egg jelly solution induces acrosomal reaction. This jelly is a material coating eggs. It is formed of acidic mucopolysaccharides rich in sulfate groups[3]. The acrosome reaction depends on the pH of the medium and on the presence of calcium ions. Elevated pH acts as a trigger of the reaction. Low pH inhibits it. The acrosome reaction does not occur in calcium-free sea water. Lanthanum, an antagonist of calcium, inhibits the reaction. A 23187, a special ionophore which transports calcium ions through membranes, induces the acrosome reaction. The acrosomal triggers appear to act by increasing the permeability of the sperm to calcium ions[4].

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Removing the jelly coat from the eggs, by dissolving it in acidified sea water, does not impair fertilization. In eggs devoid of a jelly coat, the rate of fertilization is increased relative to normal eggs. The jelly coat would exert a protecting effect against polyspermy[5]. This observation raises the problem of the nature of the physiological trigger of the acrosome reaction when the egg is devoid of its jelly coating.

Surface interaction of the gametes

It is the tip of the acrosomal process which establishes the first contact with the most external investment of the egg, the vitelline layer. Some material from the former content of the acrosomal vesicle remains adherent to the exterior of the membrane of the acrosomal process. This material forms a morphological bond with the vitelline layer[6]. The vitelline layer covers the external surface of the egg and is in direct contact with the plasma membrane. Establishment of contact between the gametes and their subsequent fusion depends upon certain properties of the surfaces of the 2 gametes. It has been suggested that the interaction between the spermatozoon and the egg is mediated by the presence of sperm-binding sites at the surface of the egg. Experimental investigations have attempted to identify these sperm receptors. Reduction of fertilizability of eggs after treatment with the proteolytic enzyme, trypsin, suggests that the sperm receptors are proteins[7]. In contrast, the fertilizing capacity of the sperm does not appear to be reduced after trypsin treatment[8]. The use of lectins has proved very useful for studying the interaction between egg and sperm. These substances bind specifically to the terminal residues of polysaccharides and glycoproteins on the surface of cells. Several lectins specific for different saccharides are known. Lectins inhibit fertilization. The inhibition depends both upon the lectin used and upon the species of the sea urchin[9,10]. Concanavalin A is an important lectin specific toward α-D-mannopyranosides, α-D-glucopyranosides and α-N-acetyl-D-glucosamidines[11]. In one species of sea urchin, where the fertilization is inhibited by concanavalin A, the affinity of the lectin appears higher for the vitelline layer than for the plasma membrane[12]. Using concanavalin A, Schmell et al.[13] give indications that the sperm receptor at the surface of the eggs is a glycoprotein. In contrast, Aketa[9] suggests that a species-specific polysaccharide component is located on the apex of the sea urchin sperm head and that it constitutes the counterpart of the sperm-binding protein of the vitelline membrane. It is interesting to recall here that a material resembling an acidic mucopolysaccharide forms a bond between the tip of the acrosomal process and the vitelline layer[6].

The effects of modification of the egg surface by enzymatic or chemical treatment have been examined. The fertilizing capacity of eggs is altered by treatment with proteolytic enzymes such as trypsin and pronase[10,14,15], but not by α and β glucosidase, α and β amylase, dextranase, collagenase and hyaluronidase[10]. An oxidizing agent, sodium periodate, improves fertilizability[16]. Fertilization is obtained with an efficacy equal to normal fertilization in the presence of nonpermeable sulfhydryl blocking agents or nonpermeable thiols. Blocking of reactive amino groups accessible at the surface of eggs, by monocarboxylic or monosulfonic agents, or by pyridoxylation or succinylation does not inhibit fertilization. Some polysulfonated agents, such as Suramin and Evans blue, inhibit fertilization at very low concentrations. These agents react both with sperm and with eggs. The acrosomal reaction is inhibited by about 50% by Evans blue. Fertilization is reversibly inhibited by 100%. The inhibitory effect could result from the masking of sperm receptor sites and/or the stabilization of the cell surface by cross-linking of the inhibitors with protein component of cell surface[10,17].

The fusion between gametes takes place at the level of the egg plasma membrane. The material located at the surface of the vitelline layer establishes the first contact between gametes. Its function appears to be to fix the sperm and to ensure the specificity of cell recognition between the 2 gametes. The fusion between the membranes of the acrosomal process and the egg may involve sperm lipolytic activity. Phospholipase activity has been reported in sea urchin sperm[18,19]. The activity of phospholipase A appears to be correlated with the initiation of sperm-egg fusion, suggesting that this enzyme has a role in the fusion process, see Conway and Metz[20]. These authors propose a scheme for fusion, derived from the model of Lucy[21]. This scheme involves the enzymatic formation of lysophosphatides, resulting in an unstable membrane structure capable for fusion with another membrane in a similar condition.

Normal development of the egg is observed only after fertilization, with eggs and sperm of the same species. But the sperm can penetrate the eggs of other species.

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Hybridization has been obtained in this way between different sea urchin species. Activation of the eggs and cleavage are observed but development stops at the blastula stage. Recently, Longo[22] obtained cross-fertilization between the eggs of the sea urchin Arbacia and the sperm of the mussel Mytilus. The eggs were treated with trypsin. This treatment decreases the thickness of the vitelline layer. A highly variable percentage of fertilizations can be observed. The nucleus of Mytilus sperm can differentiate into male pronuclei in the Arbacia egg. In mammals, sperm are also able to penetrate somatic cells[23].

Fusion between the gametes induces a series of events in the eggs. The sequence of these events and the relationship between them has been established. The first reactions of the eggs appear very early, during the first 2 minutes following fertilization. These events are: the cortical reaction with exocytosis of cortical granules and the ensuing elevation of the fertilization membrane (figure 2); bioelectric phenomena involving the egg plasma membrane and ionic changes such as calcium release and sodium influx. Some change of enzymatic activity is apparent in the increase of the rate of respiration and the activation of nicotinamide kinase. Five minutes after fertilization, one sees an increase in permeability to phosphate, nucleosides and amino acids. Protein synthesis increases. After the fusion of the 2 pronuclei, DNA synthesis begins, preceding the first division of the egg into 2 blastomeres.

The cortical reaction

The first visible step following the contact between the gametes is the elevation of the fertilization membrane. At the microscopal level, the exocytosis of the cortical granules which lie beneath the egg plasma membrane can be observed. These granules are manufactured during oogenesis and are randomly distributed through the cytoplasm. They appear to be derived from the Golgi apparatus. In the mature egg, the granules migrate towards the periphery and come to lie below the plasma membrane. Exocytosis of granules begins at the moment of membrane fusion between the gametes, and extends rapidly to all the granules[24]. Exocytosis of cortical granules has important consequences. First the membrane surface is augmented by new membrane resulting from the fusion of the cortical vesicle membrane with the plasma membrane[12,25]. Then the contents of the granules is set free from the egg; this includes enzymes such as proteases, glucanase, peroxidase and proteins. Proteases appear to play a functional role in the elevation of the fertilization membrane and in the prevention of polyspermy. Carroll and Epel[26] have found 2 distinct protease activities. One protease appears to be involved in the breaking of bonds between the vitelline layer and the plasma membrane. The effect of the other would be to alter the sperm-binding sites located on the vitelline layer. The protecting effect exerted by proteases against polyspermy is indicated by the polyspermic effect of various proteolytic inhibitors[27]. Glucanase can play a role in the formation of the hyaline layer[28]. The hyaline layer is a protein layer that envelops the eggs and holds the blastomeres together. Peroxidase activity of catalase has been observed in cortical granules[29]. According to Foerder et al.[30], a peroxidase is released at fertilization into the sea water. The enzyme would intervene in the formation of cross-links during the stabilization of the fertilization membrane. The protein content of the cortical granules contributes to the formation of the fertilization membrane[31] and to the hyaline layer[32]. A surface material, probably a glycoprotein, is released at fertilization. Johnson and Epel[33] have suggested that this material is responsible for the metabolic block of the egg at the end of oogenesis. Exocytosis of cortical granules can be induced by ionophores A 23187 and X 537 A[34–36]. The effect is independent of the presence of calcium ions in the sea water. The ionophores appear to act by releasing calcium stored intracellularly. As well as inducing the cortical reaction, the ionophore initiates bioelectrical changes comparable to those observed in normal fertilization. Activation of respiration and protein and DNA synthesis are also observed with the ionophores. But cleavage is limited. α and β naphthols are also

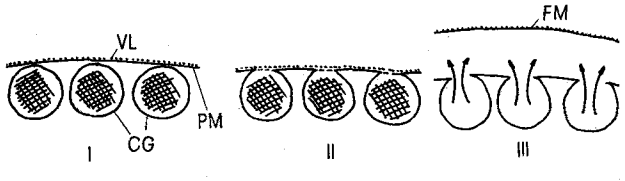

Fig. 2. Schematic representation of the cortical reaction. I, Surface of the unfertilized egg; II, fusion between the cortical granule membrane and the egg plasma membrane; III, breakdown of the cortical granules, extrusion of their content and elevation of the fertilization membrane. VL, Vitelline layer; PM, egg plasma membrane; CG, cortical granules; FM, fertilization membrane.

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very efficient inducers of exocytosis of cortical granules. These chemicals provoke the elevation of the fertilization membrane. Cleavage is not observed. The effect of naphthols is not dependent on the presence of calcium in the medium. Modifying the naphthol molecule by introducing hydrophylic groups suppresses exocytosis activity. The lipophylic character of the molecule appears necessary for the activity[37]. The ionophores A 23187 and X 537 A are also able to induce exocytosis in various cell systems and to promote cell fusion. Calcium ions play a fundamental role in these phenomena[38].

Fertilization induces membrane potential alterations in sea urchin eggs. Recent technical improvements in the measurement of transmembrane potential and resistance have permitted analysis of the sequence of bioelectric changes occurring during activation of eggs[39,40]. Three phases were observed in the membrane potential changes which accompany fertilization. Phase I is a depolarization resulting from fertilization; this phase is followed by phase II with a slow repolarization and a phase III with a rather rapid hyperpolarization. Sequential changes in ionic permeability appear to be associated with these alterations in membrane potential. Phase I corresponds to an increase in sodium permeability. Phase III is associated with an increase in potassium conductance.

The electrical depolarization of the egg plasma membrane that accompanies the entrance of the spermatozoon may be critical in preventing polyspermy. Changing membrane potential experimentally by passing current through microelectrodes demonstrates that the state of polarization of the egg membrane controls the entrance of the spermatozoon into the egg[41].

Recent work by Johnson et al.[42] has revealed the role of sodium ions in the activation of the sea urchin egg. Activation does not occur in sodium-free sea water, obtained by replacing sodium chloride with choline chloride. A very low concentration of sodium ions (40 mM) is sufficient for activation. In the presence of amiloride, an inhibitor of passive sodium transport across membranes, no activation is observed. Using radioactive sodium, these authors observed an influx of sodium ions at fertilization. This sodium influx is accompanied by an efflux of ions H^+. These ions H^+ account for the transient acid production that occurs after fertilization in sea urchin eggs. This efflux of ions H^+ results in an increase in intracellular pH. The authors suggest that the low intracellular pH in unfertilized eggs is responsible for the inhibition of metabolism. The rise in intracellular pH caused by efflux of H^+ ions would be responsible for the activation of metabolism. The efflux of H^+ ions can also be induced by treating the eggs with ammonia, and other organic amines such as nicotine and procaine, in the absence of extracellular sodium ions. These agents also activate the metabolism.

One consequence of fertilization is the activation of respiration. Part of the increase in oxygen consumption appears not to be mediated by the enzymatic mitochondrial system. It proved insensitive to the inhibitory action of cyanide. Perry and Epel[43] have suggested the existence of a respiratory activity mediated by the pigment echinochrome and activated by calcium ions.

The possibility of dissociating the series of events accompanying fertilization constitutes an important approach in the analysis of the stimulation of development. First, development can be obtained without sperm by using various chemical or physical methods. There are many methods of inducing parthenogenetic development. These methods vary greatly in their efficiency. Their mode of action is unknown. The fact that parthenogenetic development can be obtained indicates that the egg is an unstable system which can be activated by a variety of stimuli.

Elevation of the fertilization membrane and exocytosis of cortical granules can be suppressed by application of high hydrostatic pressure a few seconds after fertilization[44,45]. By treatment of eggs with chemicals such as acetone, butyric or lactic acids, it is possible to obtain development of eggs without a cortical reaction. Peculiarities such as defects in adhesivity of the blastomeres may result from the absence of hyaline layer. Reciprocally, the induction of exocytosis and elevation of the fertilization membrane by chemical agents, such as ionophores and naphthols, is not followed by development of eggs.

Important results have recently been obtained by Mazia[46] showing the initiation of DNA synthesis in sea urchin eggs exposed to ammonia-sea water (pH 9–9.1). These eggs incorporate labelled thymidine at a rate lower than normal fertilized eggs. The chromosomes condense and split but do not separate and regress into an interphase nucleus. The cycles repeat and a large number of chromosomes is formed. These eggs do not form a mitotic apparatus and they do not divide. Some phenomena accompanying normal fertilization, such as the cortical reaction, sodium influx and elevation of respiratory rate, are not observed in eggs treated with ammonia[47]. In contrast, fertilization acid is released in these eggs[42]. The development of

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potassium conductance is observed in ammonia-treated eggs, but appears independent of the increase in protein synthesis. Suppression of potassium conductance does not affect protein synthesis.

By changing the concentration of ammonia, it is possible to dissociate protein synthesis and chromosome condensation[47]. Ammonia, and also other chemical treatment which induce chromosome condensation and replication in unfertilized eggs, appear to remove a peripheral component of the plasma membrane. Material is also released upon fertilization of sea urchin eggs. This component may be involved in the repression of metabolism in the unfertilized egg[48]. When the released material is put back in the water with the partially activated eggs, the rate of protein synthesis is lowered to the level of the inactivated egg[33].

Fertilization by sperm is followed by the initiation of mitosis. Male and female pronuclei migrate and fuse at the approximate centre of the egg. Swelling of the pronuclei precedes fusion. A first aster forms then vanishes and the centrioles divide, taking up polar positions with respect to the nucleus. The nucleus enlarges and the nuclear membrane dissolves. The mitotic apparatus forms, along with centrioles, asters and spindle. The chromosomes condense, split and arrange themselves in relation to the spindle fibres. Mitosis proceeds.

In normal fertilization, the centriole is introduced by the spermatozoon. In artificial, parthenogenetic activation, when successful and followed by mitosis, asters appear in which the centrioles develop. The mitotic apparatus forms as an organized system of microtubules centred around the centrioles. Microtubules result from the polymerization of tubulin subunits. A pool of tubulin subunits produced during oogenesis is present in the unfertilized eggs. Synthesis of tubulin begins at fertilization and continues throughout cleavage. Tubulin would also be available from the dissolution of the mitotic apparatus after mitosis is completed[49]. In eggs activated by ionophores, breakdown of the nuclear membrane takes place. Formation of the cleavage furrow is generally unsuccessful. Some 2 cell aspects can be obtained but no further cleavage activity is observed[34].

In eggs activated by ammonia, no mitotic apparatus is formed. These eggs can be fertilized and the condensation of the paternal chromosomes together with the formation of a mitotic apparatus are observed. This observation suggests that chromosome condensation and mitotic apparatus formation are induced by the same factor[46].

Conclusion

The present state of research on sea urchin eggs fertilization has revealed a complex series of events. The use of activating agents has provided important evidence relative to the relationship between these events. None of these activating agents has allowed normal development of the eggs to be obtained. Further studies are needed in order to identify the additional factors responsible for the efficacy of sperm in the fertilization and for creating successful parthenogenetic conditions. Aspects of cell biology expressed in fertilization of sea urchin eggs are also observed in other cellular systems.

Complex polysaccharides are present in the surface membrane and cell surface coat of various cells. They can function in the cell recognition[50]. Calcium-induced exocytosis appears to be a general mechanism for the release of cellular secretory products. Ionophores promote exocytosis in many cell systems[51]. Recently Vacquier[52] proposed the use of isolated cortical granules of sea urchin eggs as model for the analysis of exocytosis. The state of cell surface appears to control the metabolism and the initial events of mitogenesis[53]. The potential level of the cell membranes appears to be linked to the mitotic activity[54]. Recently induction of mitosis in mature neurons was realized by depolarization with a variety of agents[55].

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SPECIALIA

N_a-demethyl-purpeline and N_a-demethyl-dihydropurpeline, new alkaloids from *Rauwolfia cumminsii* Stapf

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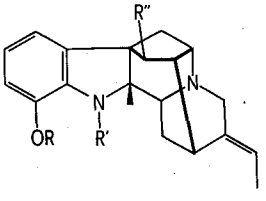
Summary. N_a-demethyl-purpeline and N_a-demethyl-dihydropurpeline, new indole alkaloids, were isolated from the stem bark of Rauwolfia cumminsii Stapf. Dihydroindole alkaloids have not previously been reported as occurring in this species.

Previous work concerning the alkaloids of Rauwolfia cumminsii Stapf showed the presence of the 18-hydroxy-yohimbine esters reserpine[1] and rescinnamine and the anhydronium base serpentine in the roots and traces of reserpine and rescinnamine in the stem[2]. Most of the Rauwolfia species so far investigated yield dihydroindole (indoline) bases of the ajmaline type and we have reinvestigated Ghanaian R. cumminsii roots and stems specifically to locate such alkaloids. None were detected in the roots but 2 new alkaloids were isolated from the stems. The first compound (**I**) occurred as yellow crystals, $[\alpha]_D = 0$ and yielded UV spectral maxima (MeOH) at 229, 254 and 290 nm (log ε 4.50, 3.92 and 3.45) and gave a violet colour when sprayed with 5% ferric chloride and 35% perchloric acid reagent on silica gel layers. This suggested a methoxy substituted dihydroindole alkaloid. IR measurements using KBr discs revealed bands at 2950 s, 1735 s, 1595 s, 1470 m, 1230 m, 820 m, 760 m, 740 m cm^{-1} agreeing with an indoline structure with a keto group attached to a 5-membered ring as in purpeline (**II**). The 60 MH_Z-NMR spectrum in $(CD_3)_2$ SO showed τ 1.68 (1H, s, indole NH proton), 3.30–3.45 (3H, aromatic protons, monosubstituted aromatic ring), 6.20 (3H, s, OCH_3 protons), 6.30 (1H, d, C-2H proton), 7.5 (2H, s) and 8.4 (1H, m). The mass spectrum produced peaks at m/e 322 (100%) (M^+, accurate mass 322.1682, calculated for $C_{20}H_{22}N_2O_2$), 293 (45%), 211 (10%), 199(30%), 198 (20%), 174 (10%), 173 (20%), 160 (60%), 108 (24%), 98 (20%). From this data is was concluded that the structure (**I**) of the compound resembled purpeline (**II**) and showed decrease of the molecular ion peak by 14 mass units. As the fragment ions embodying the indole part of the molecule demonstrated similar decreases and there was no shift of the UV spectral peaks in strong alkali, it was probable that the compound was N_a-demethyl-purpeline. The presence of MS peaks at m/e 199 and 198, corresponding to m/e 213 and 212 respectively in purpeline, and the absence of a peak at m/e 166 confirmed the β-orientation of the C-2 hydrogen[3]. From biogenetic considerations and the co-occurrence of mitoridine (19, 20-didehydro-12-hydroxy-ajmalan-17-one) (**IV**) it was concluded that the compound must be N_a-demethylpurpeline (norpurpeline or 1-demethyl-19, 20-didehydro-12-methoxy-ajmalan-17-one).

The second compound (**III**), a yellow amorphous powder, was found to be a dihydro derivative of N_a-demethyl-purpeline (M^+ 324.1186, calculated for $C_{20}H_{24}N_2O_2$). Reduction of N_a-demethylpurpeline with sodium borohydride in the cold yielded the dihydro derivative. Such reduction could only yield a C-17α hydroxyl group[4] and the compound was therefore the N_a-demethyl derivative of reflexine[5] (1-demethyl-19, 20-didehydro-12-methoxy-ajmalan-17-ol). The identity of mitoridine (**IV**) was confirmed by comparison with published data (m.p., $[\alpha]_D$, UV, IR, MS)[4].

The present communication is the 1st report of the isolation of dihydroindole alkaloids in R. cumminsii. The occurence of the N_a-demethyl alkaloids of the ajmaline series, e.g. norajmaline[6], norseredamine[7] and norpurpeline, may prove to be necessary in the production of the large group of ajmaline-derived alkaloids now known to occur in plants of the genus Rauwolfia.



I R = CH_3, R′ = H, R″ = O
II R = CH_3, R′ = CH_3, R″ = O
III R = CH_3, R′ = H, R″ = α-OH
IV R = H, R′ = CH_3, R″ = O

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