Early development of the horseshoe crab, Limulus polyphemus, L.: 1) ultrastructure of the cortical reaction and amino acid incorporation during egg activation, and 2) ultrastructure and protein synthesis of the extra-embryonic shell

Gary Anthony Bannon
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EARLY DEVELOPMENT OF THE HORSESHOE CRAB, LIMULUS POLYPHEMUS, L.: (1) ULTRASTRUCTURE OF THE CORTICAL REACTION AND AMINO ACID INCORPORATION DURING EGG ACTIVATION, AND (2) ULTRASTRUCTURE AND PROTEIN SYNTHESIS OF THE EXTRABRANCHIAL SHELL

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Early development of the horseshoe crab, *Limulus polyphemus*, L.: 1) ultrastructure of the cortical reaction and amino acid incorporation during egg activation, and 2) ultrastructure and protein synthesis of the extra-embryonic shell

by

Gary Anthony Bannon

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of

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### SECTION 4. ULTRASTRUCTURAL CHARACTERISTICS OF THE NON-EXPANDED AND EXPANDED EXTRA-EMBRYONIC SHELL OF THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS* L.

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GENERAL INTRODUCTION

Early embryonic development is a complicated process involving many structural and metabolic changes. In general, the changes which take place following sperm-egg attachment are grouped into the broad category of egg activation. The research reported in this dissertation utilized the horseshoe crab, *Limulus polyphemus* L., to examine some aspects of egg activation. Egg activation in *Limulus* embryos encompasses those events from sperm-egg attachment to the beginning of cleavage (approximately 24 hr of development at room temperature, 21°C).

One of the more conspicuous structural changes which takes place during egg activation is the cortical reaction. The cortical reaction has been studied in numerous organisms and has been implicated as a major effector of a block to polyspermy, as contributing some of the major structural components to the fertilization membrane in sea urchins and as contributing various enzymes utilized in polyspermic prevention and hardening of the fertilization membrane (See Schuel, 1978 for review). Since the egg cortical reaction is an important event in egg activation this event was studied in *Limulus*. Section 1 deals with elucidating the mechanisms of the egg cortical reaction of this primitive arthropod.

One of the major metabolic changes which takes place in egg activation is the derepression of protein synthesis. In sea urchins, protein synthesis is derepressed approximately 5-9 min after fertilization (Epel, 1967) and is dependent on an alkalization of the embryonic internal pH (pH$_e$; Shen and Steinhardt, 1978; Grainger, et al., 1979).
In Section 2 the time course of derepression of protein synthesis in *Limulus* is examined with respect to varying the embryonic pH. In depth studies on egg activation in other organisms have involved following the synthesis of a prominent protein or set of proteins which are important to the development of the future embryo. An excellent example of a set of proteins which have been extensively studied during egg activation (and later in development) are the sea urchin histone proteins. This set of proteins are amenable to this type of research because they are synthesized in sufficient quantities and are easily isolated from the rest of the cell. Section 3 examines the synthesis of *Limulus* extra-embryonic shell (EES) proteins during egg activation and later embryonic development. Finally, in Section 4, the ultrastructural aspects of the EES is examined in its nonexpanded and expanded forms.
EXPLANATION OF DISSERTATION FORMAT

The sections of this dissertation are modified manuscripts that have been submitted to scientific journals (Section 1 - published in Developmental Biology; Section 2 - submitted to Nature; Section 3 - submitted to Developmental Biology; Section 4 - published in Biological Bulletin). Each section has been modified to conform to the style required by the Iowa State University Thesis Office. Each section has its own introduction, materials and methods, results, discussion, and reference sections. Following the sections is a summary-discussion of the entire dissertation.
SECTION 1. VESICLE INVOLVEMENT IN THE EGG CORTICAL REACTION OF THE
HORSESHOE CRAB, LIMULUS POLYPHEMUS L.
INTRODUCTION

Egg activation, with particular emphasis on the cortical reaction, has been studied extensively by many investigators (Nicosia et al., 1977; Paul and Gould-Somero, 1976; Fallon and Austin, 1967). Sea urchin egg activation has received the majority of attention (cf. Epel, 1978) because of species availability and the ease with which a large number of gametes are procured. However, their cortical reaction is initiated quite rapidly and has a very short duration (approximately 60 sec), thus the examination of the chemical events is difficult. Recently egg activation has been examined in the horseshoe crab, Limulus polyphemus, and has been demonstrated to be an excellent system for the study of the cortical reaction (Brown and Clapper, 1980). As with sea urchins, this species is particularly satisfactory for fertilization studies since adults are readily available and easily maintained and viable gametes can be routinely obtained for use in the laboratory. As a greater advantage, female gametes are large (averaging 1.9 mm diameter) and thus are easy to observe for morphological changes and to manipulate for experimental procedures. Most importantly, light and scanning electron microscopic examination (Brown and Clapper, 1980) demonstrates a long duration (approximately 60 min) for the Limulus cortical reaction. Thus, examination of the morphological and chemical changes of this reaction should be considerably easier to perform in this species than in species with rapid cortical reactions.

Previous studies on Limulus fertilization have been limited to sperm-egg attachment (Brown, 1976; Mowbray and Brown, 1974; Brown and
Knouse, 1973; Cooper and Brown, 1972; Mowbray et al., 1970; Shoger and Brown, 1970) due to the relative impermeability of the egg envelope to standard fixation and embedding techniques. However, with the application of trialdehyde fixation techniques (Kalt and Tandler, 1971) the ultrastructure (TEM) of the cortical reaction has now been examined. The results presented in this study indicate that cortical vesicles of various sizes play an intimate role in the cortical reaction of inseminated Limulus eggs.
MATERIALS AND METHODS

Source of animals, gamete collection and insemination were accomplished according to the methods described by Brown and Clapper (1980).

Procurement of Events of Egg Cortical Reaction

Inseminated eggs were examined with a dissecting microscope and at the following intervals: 3, 6, 9, 12, 15, 20, 25, 30, 40, 50, and 60 min, an aliquot was removed and prepared for electron microscopy. During the fixation process the inseminated eggs were examined several times to ensure that fixation artifacts were not occurring on the surface. Uninseminated eggs were collected and treated in a similar manner.

Electron Microscopy

Each aliquot (uninseminated and inseminated) was transferred to a heptane solution saturated with 25% glutaraldehyde (Zalokar; 1971) for 1 min, fixed in a trialdehyde solution (glutaraldehyde, 0.33 M; formaldehyde, 0.66 M; acrolein, 0.178 M; DMSO, 0.320 M; cacodylate buffer, 0.012 M; pH, 7.0; Kalt and Tandler, 1971) for 3 hr, postfixed in 1% osmium tetroxide, and dehydrated (all fixation procedures were carried out at 4°C). Infiltration of the material was accomplished by cutting each egg in half with an industrial razor blade and embedding in Spurrs resin (Standard Medium A; Polysciences Data Sheet No. 127). All tissue was sectioned on a LKB Ultratome, stained with aqueous uranyl acetate and lead citrate, and examined on a Hitachi HU-11 E-1 electron microscope.
RESULTS

With *Limulus* fertilization, the staging of the cortical reaction is dependent upon the occurrence of morphological events and not on time after insemination. This is of utmost importance since the rate of the cortical reaction can vary between egg batches obtained from different females. For the results reported in this paper, the cortical reaction was completed in 60 min at 20°C. The following events represent major changes which are readily observable: Event I, uninseminated egg - smooth surface; Event II, inseminated egg - appearance and growth of pits; Event III, inseminated egg - coalescing of pits; Event IV, uninseminated egg - appearance of smooth surface (Fig. 5 a-d).

Event I, Uninseminated Egg - Smooth Surface

In an uninseminated egg four regions (Figs. 1a and 5a) are well defined in cross section. The first region is the egg envelope and is composed of an outer basement lamina (5 um) and a dense vitelline envelope (40 um) (Shoger and Brown, 1970; Dumont and Anderson, 1967). The second region is the small perivitelline space which contains numerous microvilli originating from the egg plasmalemma and closely apposing the vitelline envelope. Surrounding the microvilli is an electron dense granular matrix. The third region is the egg cortex which contains numerous membrane bound vesicles, glycogen rosettes, mitochondria and Golgi complexes. The cortex is separated into an upper and a lower layer. The upper layer, approximately one third of the cortex next to the perivitelline space, contains small vesicles (0.5 um)
Figure 1. Cross section of unfertilized and fertilized Limulus eggs

(a) Cross section through the cortex of an uninseminated Limulus egg. Note that the cortex of the egg can be divided into two distinct layers with the upper layer containing small vesicles (0.5 \(\text{um}\)) (SV) closely apposed to the plasmalemma. This layer is characterized by a general lack of other organelles. The lower layer contains large vesicles (4 \(\text{um}\)) (LV) with heterogeneous contents. This layer contains numerous cellular organelles. G, Golgi complex; GL, glycogen rosettes; L, lipid droplets; M, mitochondria; MV, microvilli; PS, perivitelline space; VE, vitelline envelope; Y, yolk. X 7600. (b) Cross section through the cortex of a Limulus egg 3 min after insemination. The initial response of the egg to insemination is the fusion of the small vesicles with the plasmalemma. Numerous vesicles are in the process of fusing with the plasmalemma (*) and as a result the plasmalemma assumes a more jagged appearance (arrows) than in the uninseminated egg. LV, large vesicles; VE, vitelline envelope. X 11,500
with homogeneous, electron translucent contents which are closely apposed to the plasmalemma. This layer is sparsely populated with mitochondria and other organelles. The lower layer of the cortex contains large vesicles (4.0 um) with variable contents. Mitochondria and glycogen rosettes are heavily concentrated in this layer. The fourth region is comprised primarily of yolk bodies, lipid droplets and glycogen rosettes. Rarely are other organelles observed in this area.

Event II, Inseminated Egg - Pit Formation and Growth

Several structural changes occur in the egg cortex within 3 min after insemination. More prominently, the plasmalemma has become more jagged appearing as compared to the plasmalemma of the unfertilized egg (compare Fig. 1a with Fig. 1b). This jagged appearance is caused by fusion of the small vesicles with the overlying plasmalemma (Fig. 1b). Just prior to and concomitant with fusion, the vesicular membrane adjacent to the plasmalemma becomes quite fuzzy on the upper hemispheric surface. The lower vesicular membrane (side away from the perivitelline space) remains sharply defined. All other regions of the egg appear unchanged from their uninseminated state.

By 6 min after insemination, most of the small vesicles have fused with the plasmalemma and are no longer evident in the cortex (Fig. 2a). The large vesicles are now in closer proximity to the plasmalemma and are larger than vesicles from the same area of the cortex 3 min after insemination. These enlarged vesicles are probably due to the fusion of one or more vesicles in the cortex. Mitochondria and glycogen rosettes are now throughout the cortex. The perivitelline space has
Figure 2. Cross section of fertilized *Limulus* eggs

(a) Cross section through the cortex of a *Limulus* egg 6 min after insemination. The cortex has lost many of the small vesicles through fusion with the plasmalemma. As a result the perivitelline space (PS) has expanded. The enlarged vesicles (EV), a result of an increase in size of the previous large vesicles may be due to fusion with one another (arrows). M, mitochondria; VE, vitelline envelope. X 7600. (b) Cross section through the cortex of a *Limulus* egg 9 min after insemination. An enlarged vesicle is observed fusing with the plasmalemma (arrows). As a result of this fusion the pit (P) is increased in size. Note that the fusing vesicle lacks microvilli. MV, microvilli; PS, perivitelline space; VE, vitelline envelope. X 7600
expanded when compared with the perivitelline space of the uninseminated egg (Fig. 1a).

At 9 min after insemination, the enlarged vesicles of the cortex begin fusing with the plasmalemma and form "pits" (Fig. 2b). These pits become progressively larger due to the fusion of additional vesicles. As fusion occurs, the vesicular membrane becomes incorporated into the plasmalemma. At this time, the vesicular membrane is smooth, and lacks microvilli (Fig. 2b).

By 12 min after insemination, the pits have continued to increase in size (Fig. 3a) and can be observed on the egg surface with a dissecting microscope. The growth of the pits is due to the fusion of numerous cortical vesicles with the plasma membrane. However, vesicles are still evident within the cortex. The vesicular membrane lining the basin of this pit now contains microvillar projections and represents the plasmalemma.

Event III, Inseminated Egg - Coalescing of Pits

At 20 min after insemination, the pits have grown to such an extent that their boundaries are difficult to discern. The result of pit growth is the coalescence of these structures to form ridges on the egg surface (Fig. 3b). At this time, the cortex has few cortical vesicles remaining. Numerous mitochondria and glycogen rosettes are still evident throughout this region (Fig. 3b).
Figure 3. Cross section of fertilized Limulus eggs

(a) Cross section through the cortex of a Limulus egg 12 min after insemination. This micrograph illustrates the result of numerous cortical vesicles fusing with the plasmalemma. Note that the cortical vesicles, once totally incorporated into the plasmalemma, develop microvillar projections (MV). P, pit; VE, vitelline envelope. X 5000. (b) Cross section through the cortex of Limulus egg 20 min after insemination. As pits increase their size, due to a succession of cortical vesicles fusing with the egg plasmalemma, they begin to coalesce. As a result of coalescence ridges form on the egg surface (arrows). EV, enlarged vesicle; VE, vitelline envelope. X 5000
Event IV, Inseminated Egg - Appearance of Smooth Surface

Sixty min after insemination the plasmalemma again becomes smooth as a consequence of pit coalescence. However, many changes have taken place in the cortex since the smooth stage in the uninseminated egg (compare Fig. 1a with Fig. 4). This region, which contained two morphologically distinct layers in the uninseminated egg now appears as a homogeneous layer. Sixty min after insemination the cortex is practically devoid of cortical vesicles (Fig. 4). The perivitelline space is definitely enlarged when compared with the perivitelline space of the uninseminated egg. The microvilli of the 60 min post-insemination egg have increased their size when compared with the microvilli of the uninseminated egg.
Figure 4. Cross section through the cortex of a Limulus egg 60 min after insemination. The cortical region of the egg has changed drastically: cortical vesicles are no longer evident in this region and the plasmalemma is again smooth. Note that the perivitelline space (PS) is expanded and microvilli (MV) are longer and wider. X 5000
Figure 5. Diagrammatic representation of the egg cortical reaction in Limulus. (a) Uninseminated egg. Four regions are present: Region 1, outer envelope; Region 2, perivitelline space; Region 3, cortex; Region 4, lipid-yolk center. (b) Inseminated egg, 1-8 min after mixing with spermatozoa. Small vesicles (SV), which were originally apposed to the plasmalemma, are now in the process of fusing, giving the egg surface a jagged appearance. (c) Inseminated egg 9 min after mixing with spermatozoa. Large vesicles (LV) and enlarged vesicles (EV) (formed by the fusion of one or more large vesicles) are observed fusing with the plasmalemma forming pits (P) on the egg surface. (d) Inseminated egg approximately 60 min to continued vesicle fusion. Note that the cortex is now lacking vesicles, the perivitelline space is expanded, and the microvilli are longer and wider. L, lipid droplets; M, mitochondria; VE, vitelline envelope; Y, yolk
DISCUSSION

The cortical reaction in *Limulus* is a dramatic event involving fusion to the plasmalemma of numerous vesicles and the exocytosis of their contents into the perivitelline space (Fig. 5 a-d). Although this event is comparable to the cortical reaction of most other organisms (rat and hamster - Szollosi, 1967; mouse - Nicosia et al., 1977; sea urchin - Anderson, 1968 and Longo, 1978) a major difference in *Limulus* is the development of large "pits" on the surface of inseminated eggs. These pits are the result of continued vesicle fusion with the overlying plasmalemma for a period of 30-60 min after egg activation. Although similar pit-like structures have been observed in the cortical reaction of other species, they are not as large and do not persist as long as those observed in the inseminated eggs of *Limulus*.

Fallon and Austin (1967) and Pasteels (1966) have described in the marine worm, *Nereis*, a cortical reaction which is very similar to that described for *Limulus*. These authors described the cortical region in *Nereis* as packed with various sized alveolar spaces which fused with the overlying plasmalemma at fertilization. Through the process of exocytosis these alveolar spaces dumped their contents into the perivitelline space. This material (believed to be the precursor to the jelly substance found on the exterior of the *Nereis* egg 15-18 min after fertilization) diffuses from the perivitelline space to the exterior through pores in the chorion. Inseminated eggs of *Limulus* exhibit a similar cortical reaction with enlarged vesicles being comparable to alveolar spaces of the *Nereis* egg. As with *Nereis* a sequential fusion
occurs until all vesicles are gone and the surface again becomes smooth. Exocytosis, exhibited by both organisms, does not serve the same function. *Limulus* possesses a dense, non-porous vitelline envelope which would block the passage of any precursor macromolecule from the embryo to the external environment. *Limulus* does, however, possess an extra-embryonic shell which becomes evident after the larvae hatches out of the initial egg investments at approximately 20 days after fertilization. Thus, exocytosis may be a mechanism which provides precursors for this extra-embryonic shell.

Egg activation has been thoroughly studied in the sea urchin (cf. Epel, 1978). The initial activation of the inseminated sea urchin egg involves a cortical granule fusion with the plasmalemma and the resulting exocytosis of their electron dense contents into the perivitelline space. The contents contribute to the formation of an activation calyx which may act as a block to polyspermy (Anderson, 1963). In *Limulus* the cortical vesicle contents which appear as a fine meshwork of fibers are morphologically different from sea urchin cortical granules. An activation calyx does not exist in the fertilized *Limulus* egg. Jaffe et al. (1978) reported that cortical granule fusion in sea urchins exhibit a latent period of approximately 30-45 sec with cortical granule exocytosis being completed approximately 60 sec after insemination (Rothschild and Swann, 1949). A latent period of cortical vesicle fusion in *Limulus* was not measured; however, cortical vesicle exocytosis was not completed until 60 min after insemination.
The cortical reaction described above occurs over the entire egg surface upon activation. Among all sections examined and with data from scanning electron micrographs (Brown and Clapper, 1980) no evidence exists to support a polarized response of cortical granules as observed in the mouse egg (Nicosia et al., 1977). The release of some cortical material prior to sperm contact with the vitellus, as described by Nicosia et al. (1977) has not been demonstrated in Limulus fertilization. The fact that sequential fusion of cortical vesicles occurs may indicate functional differences for vesicle contents. For example, small vesicles may be involved in polyspermy prevention while larger vesicles may be involved in embryonic shell formation. Since sperm penetration sites and time of penetration are presently unknown, these possibilities are purely speculative.

Microvilli are found over the entire egg surface before and after insemination. The only time the plasmalemma lacks microvilli is immediately after vesicle fusion. However, as soon as the vesicle membrane has been incorporated into the plasmalemma, microvilli are again formed.

Elongation of microvilli and their appearance and disappearance in various areas of inseminated eggs has recently received considerable emphasis in several species. In the sea urchins, Strongylocentrotus droebachiensis and S. purpuratus (Schroeder, 1978, 1979), microvilli are present before insemination and become elongated a few minutes after insemination. A second burst of elongation follows after the fertilization membrane forms. The diameter of these microvilli do not undergo any significant changes. In Xenopus laevis, microvilli are quite
obvious before and immediately after insemination, but are greatly reduced in number before the first cleavage (Monroy and Baccetti, 1975). Also, a considerable difference exists in the number of microvilli found on the animal pole and the vegetal pole. In teleosts, no structural change in the microvilli are reported; however, new microvilli appear in the membrane of the cortical alveloi after fusing with the plasma membrane (Iwamatsu and Keino, 1978). A similar lengthening of microvilli occurs during the cortical reaction in Limulus and could be due to vesicle fusion with the plasma membrane. As vesicles fuse with the plasmalemma the inner limiting membrane becomes incorporated into the egg plasmalemma and thus increases its surface area. Since this increase occurs in a relatively inflexible space, the vitelline envelope, the only way to incorporate the new membrane into the egg plasma membrane is by increasing the number and/or size of microvilli. The length and width of microvilli definitely increase during egg activation.
REFERENCES


SECTION 2. LOSS OF pH SENSITIVITY OF AMINO ACID INCORPORATION DURING EARLY DEVELOPMENT OF THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS* L.
INTRODUCTION

Structural and metabolic parameters of an unfertilized egg are altered as a result of sperm penetration. Collectively these changes are known as egg activation and are required for the normal development of the embryo. Although egg activation has been studied in many different organisms, most investigations have involved sea urchins, for which numerous structural and metabolic changes have been documented (cf. Epel, 1978). In sea urchins an increase in amino acid incorporation is observed 5-9 min after fertilization (Epel, 1967) and is accompanied by changes in internal pH ($pH_i$; Shen and Steinhardt, 1978). Grainger et al. (1979) have determined that one of the more important aspects of egg activation in sea urchins is an alkalization of the egg cytoplasm which appears to be linked to an increase in amino acid incorporation.

Two important questions may be asked. First, is cytoplasmic alkalization a general phenomenon of egg activation? Second, are changes in amino acid incorporation generally concomitant with changes in intracellular pH? If $pH_i$ and amino acid incorporation prove to be causally linked, then mechanisms by which $pH_i$ are changed could become implicated as control mechanisms which regulate the metabolic activity of a cell and ultimately its state of differentiation.

In the present study, we have measured $pH_i$ of the Limulus embryo from fertilization through 14 hr of development by the $^{14}$C-DMO (5,5-dimethyl-2,4-oxazolidinedione) method (Boron and Roos, 1976) and investigated amino acid incorporation over the same time period. Our
results indicate that the intracellular pH remains constant, at an alkaline level; while amino acid incorporation increases dramatically 5-50 fold. Although changes of internal pH and amino acid incorporation were not correlated during normal early development, we found that the initiation of accelerated amino acid incorporation may be blocked by acidifying the embryos with exposure to the weak penetrating acid, sodium acetate. However, cytoplasmic acidification after the increase of amino acid incorporation was found to have little effect. Thus, our data suggest a loss of pH sensitivity of amino acid incorporation during early development.
MATERIALS AND METHODS

Source of Animals

Specimens of *Limulus polyphemus* L. were obtained from Florida Marine Biological Specimen Co., Inc., Panama City, Florida, maintained at 15°C in 150 gal Instant Ocean Aquaria (Jungle Salts artificial sea water (ASW) Jungle Laboratories Corp., Comfort, Texas) and exposed to a light cycle of 14-10 LD.

Handling of Gametes and Embryos

Gametes were collected by brief electrical stimulation (1.5 v, 0.5-1.0 mA, AC) of the gonoducts proximal to the genital pores. Semen was diluted with millipore filtered ASW (MPFASW; 5 mM Hepes, pH 7.85) to produce a 10% sperm suspension (10⁹ spermatozoa/ml). Approximately 100 eggs were spawned and immediately transferred with wooden applicators to a Petri dish containing 25 ml of MPFASW. Two drops of the sperm suspension were added, and the dish swirled to ensure adequate mixing of gametes. All embryos were cultured at room temperature (21-22°C) and the percent of fertilization was better than 95% in all cultures. Embryos exposed to 10 mM NaAcetate MPFASW (pH 6.85) were transferred from MPFASW (pH 7.85) with extensive washing.

Amino Acid Incorporation

Amino acid incorporation was measured at 2 hr intervals for the first 16 hr of development. Radioactive labelling was accomplished by selecting embryos at each time point and placing them in MPFASW (pH 7.85) or 10 mM NaAcetate MPFASW (pH 6.85), which contained 1 uCi/ml
$^3$H-lysine or $^3$H-leucine, for 15 min. The labelled embryos were washed extensively, homogenized in 10% trichloroacetic acid (TCA) with 6 strokes of a Dounce homogenizer and allowed to extract for 1 hr on ice. The TCA homogenate was centrifuged in an Eppendorf microfuge (12,800 x g) for 10 min, the pellet was redissolved in 0.3 ml Protosol (New England Nuclear) and incorporated labelled amino acids were counted in Omnifluor (New England Nuclear) with a Beckman LS 230 LSC. An incorporation rate for unfertilized eggs was not obtained due to spontaneous activation of eggs approximately 10 min after spawning.

Measurement of Intracellular pH ($pH_1$)

Intracellular pH was measured for the first 14 hr of development. All $pH_1$ measurements were determined by utilizing DMO (5,5-dimethyl-2,4-oxazolidinedione) distribution according to the methods of Boron and Roos (1976). Distribution of $^{14}$C-DMO in Limulus embryos was accomplished by continuous incubation in either MPFASW (pH 7.85) of 10 mM NaAcetate MPFASW (pH 6.85) with 5 uM $[2-^{14}$C] DMO (56.5 Ci/mole, New England Nuclear) and 1 uCi/ml $[^3$H(G)] inulin. Embryos were added to radioactive media 30 min after fertilization in all experiments. At each time point embryos were selected and washed in MPFASW supplemented with $10^{-4}$ M unlabelled inulin prior to homogenization in 1 ml of 5% TCA. The embryos were homogenized by 6 strokes of a Dounce homogenizer and allowed to extract for 1 hr on ice. The TCA homogenate was centrifuged in an Eppendorf microfuge for 10 min and the supernatant was counted in Biofluor (New England Nuclear). Intracellular pH was calculated from
the DMO distribution by the following equation (Waddell and Butler, 1959):

\[ \text{pH}_i = \text{pK}_a + \log \left( \frac{[\text{DMO}^i]}{[\text{DMO}^o]} \left( 1 + 10^{\text{pH}_0 - \text{pK}_a} \right) - 1 \right) \]

where:  
\( \text{pH}_0 \) = external pH  
\( \text{pK}_a \) = pK of DMO (= 6.3)  
\( [\text{DMO}^o] \) = external concentration of DMO  
\( [\text{DMO}^i] \) = internal concentration of DMO

Utilization of DMO distribution to measure \( \text{pH}^i \) requires that an estimate of intracellular water be made for determining the portion of the egg volume accessible to DMO. Extracellular water volume must also be calculated to correct for extracellularly trapped DMO. For *Limulus* embryos, intracellular water was determined by blotting embryos dry, weighing them and then subjecting them to vacuum desiccation for 24 hr. At the end of this time, the eggs were reweighed and the loss in weight attributed to intracellular water which was estimated to be 1.25 ul/embryo. Extracellular trapping of DMO was calculated by using \(^3\text{H}\)-inulin as an extracellular space marker, which was constant during the 16 hr time period.

**Paper Chromatography**

Paper chromatography of labelled embryos was utilized to determine if \(^{14}\text{C}\)-DMO was metabolized in the cell. The procedures of Leguay (1977) were utilized with the following modifications: \(^{14}\text{C}\)-DMO labelled embryos (30 min-24 hr continuous label) were homogenized in 5% TCA
instead of ethanol. The homogenate was centrifuged in an Eppendorf microfuge (12,800 x g) for 1 min and the supernatant lyophylized to dryness. The lyophylized sample was brought up in ethanol and the insoluble salts pelleted. The supernatant was run on number 1 Whatmann filter paper in a solvent containing ammonia/butanol/water (5:84:14 v/v/v).
RESULTS

Amino Acid Incorporation in Developing Embryos

A typical incorporation curve of $^3$H-lysine into protein during the first 14 hr of Limulus development is shown in Fig. 6. In normal MPFASW (pH 7.85), the rate of incorporation of lysine increased approximately 7 fold from 8-14 hr following fertilization as compared to the rate of incorporation during the first 6 hr. An increase in incorporation rate was also observed when $^3$H-leucine was utilized (Fig. 6). The extent to which the rate of amino acid incorporation increased varied from 5-50 fold between different egg batches.

Intracellular pH of Developing Embryos

The time required for equilibration of $^{14}$C-DMO across Limulus embryos was approximately 60 min and no further uptake occurred after this time (Fig. 7). However, due to the relatively long incubation time (14 hr) utilized in the measurements of pH$_i$ in developing embryos a possibility existed that metabolism of the $^{14}$C-DMO occurred in the Limulus embryo. This was tested by employing paper chromatography (Fig. 8) of extracts of labelled embryos according to the methods of Leguay (1977). The results of these experiments have shown that $^{14}$C-DMO was not metabolized through 24 hr of incubation.

After equilibration had been reached (Figs. 7 and 9), the pH$_i$ of embryos incubated in MPFASW (pH 7.85) averaged 7.69 ± 0.02 (n = 17 mean ± SEM). No fluctuation of pH (alkalization or acidification) was observed before, during, or after the time of increased amino acid incorporation.
Figure 6. Amino acid incorporation during *Limulus* development. This graph illustrates the increase in rate of amino acid incorporation observed during the first 14 hr of development. Each point represents the counts (cpm) found in a TCA precipitable fraction of 5 embryos given a 15 min pulse of either $^3$H-lysine (•—•) or $^3$H-leucine (▲—▲). This graph represents one experiment which was repeated twice with similar results.
Figure 7. Equilibration curve for $^{14}$C-DMO in Limulus embryos. $^{14}$C-DMO was added to Limulus cultures 30 min after fertilization. The uptake of $^{14}$C into the TCA soluble fraction of embryos was monitored and plotted against time of exposure to the radioactive DMO. Equilibration was achieved approximately 60 min after addition of the DMO to the incubation media.
Figure 8. Ascending paper chromatography of $^{14}$C-DMO. Embryos were incubated in the presence of $^{14}$C-DMO for 24 hr. Samples were taken at 1 hr, 4 hr, 7 hr, and 24 hr and 25\% of embryo extracts were run on Whatmann filter paper (number 1) in a solvent containing butanol/ammonia/water (84:5:14 v/v/v). There was no metabolism of the DMO by Limulus embryos as evidenced by the lack of appearance of other radioactive spots on the chromatogram. Numbers along the bottom of this graph represent the number of fractions which the chromatogram was divided into for scintillation counting (each fraction represents 2 centimeters; $\uparrow$, origin; $\uparrow\uparrow$, solvent front).
Effects of Varying pH on Amino Acid Incorporation

The pH of the fertilized egg became acidic when incubated in 10 mM NaAcetate MPFASW (pH 6.85). During the time period examined, an average $pH_i$ of the developing embryo was $6.77 \pm 0.015$ ($n = 12$) (Fig. 9) and no increase in amino acid incorporation was observed (Fig. 10). However, incubation in 10 mM NaAcetate MPFASW (pH 6.85) was reversible when embryos were transferred to MPFASW (pH 7.85) as late as 9 hr after fertilization. The $pH_i$ of these embryos was shifted to a basic pH and a gradual increase in amino acid incorporation was observed.

Embryos were transferred from MPFASW (pH 7.85) to 10 mM NaAcetate MPFASW (pH 6.85) at different times after fertilization to determine if they were able to regulate against external pH ($pH_o$) changes. Embryos were transferred from MPFASW (pH 7.85) to 10 mM NaAcetate (pH 6.85) at 6 hr and 10 hr after fertilization. The pH of embryos transferred both at 6 hr and 10 hr became acidic (Fig. 9). However, surprising results were obtained when amino acid incorporation rates were determined for embryos treated in this manner. Instead of the expected drop in amino acid incorporation, those embryos transferred at 10 hr continued to exhibit an increased incorporation rate after an initial drop (Fig. 10). Embryos transferred to 10 mM NaAcetate MPFASW (pH 6.85) at 6 hr exhibited an incorporation rate which was less than control (Fig. 10).
Figure 9. pH$_i$ of Limulus embryos. Embryos incubated in MPFASW (pH 7.85; ●—●) had a pH$_i$ of 7.69 ± 0.02 (n = 17) after equilibration of 14C-DMO had been reached (90 min after fertilization). Embryos incubated in 10 mM NaAcetate MPFASW (pH 6.85; ○—○) had a pH$_i$ of 6.77 ± 0.015 (n = 12). Embryos shifted from MPFASW (pH 7.85) to 10 mM NaAcetate MPFASW (pH 6.85) at 6 (arrow 2; ▲—▲) and 10 hr (arrow 3; ■—■) after fertilization attained an acidic pH$_i$ shortly after transfer. Embryos shifted from 10 mM NaAcetate MPFASW (pH 6.85) to MPFASW (pH 7.85) at 10 hr (arrow 1; □—□) after fertilization attained a pH$_i$ of 7.6
Figure 10. Amino acid incorporation at different pH of Limulus embryos. The control (embryos incubated in MPFASW, pH 7.85) incorporation curve shows an increase at 8 hr after fertilization (a). The 10 mM NaAcetate MPFASW (pH 6.85) incorporation curve is greatly depressed with no increase observed at 8 hr after fertilization (d). Embryos were shifted from MPFASW (pH 7.85) to 10 mM NaAcetate MPFASW (pH 6.85) at 6 and 10 hr after fertilization. Embryos shifted at 6 hr (b) exhibited a depression of the incorporation curve with no increase observed at 8 hr while those embryos shifted at 10 hr (c) exhibited an incorporation curve which continued to increase after an initial drop. Embryos shifted from 10 mM NaAcetate MPFASW (pH 6.85) to MPFASW (pH 7.85) exhibited an incorporation curve with a gradual rate of increase (e).
DISCUSSION

Limulus embryos do not exhibit an increase in intracellular pH ($pH_i$) from 60 min-14 hr after fertilization. However, an increase in amino acid incorporation rate is observed at 8 hr. This is in direct contrast to sea urchin embryos which exhibit a rise in $pH_i$ (Shen and Steinhardt, 1978) immediately preceding an increase in protein synthetic rate (Epel, 1967). Grainger et al. (1979) have shown in sea urchins that the increased incorporation rate is dependent on cytoplasmic alkalization. In Xenopus laevis, where a rise in $pH_i$ occurs during egg activation (Webb and Nuccitelli, 1980), there is not a concomitant increase in amino acid incorporation (Woodland, 1974). In oocytes of the starfish, Pisaster ochraceus, no rise in $pH_i$ has been observed, however, there is a 5 fold increase in incorporation rate as a result of activation (Johnson and Epel, 1980). These results indicate that $pH_i$ and amino acid incorporation are not universally linked during egg activation.

Exposure of Limulus embryos to the weak acid, NaAcetate, within 6 hr after fertilization resulted in an acidification of the $pH_i$ and blocked the increase in amino acid incorporation. This result is in agreement with the finding of NaAcetate treated sea urchin embryos (Grainger et al., 1979) and starfish embryos (Johnson and Epel, 1980). However, Limulus differs from these organisms because after the rise in amino acid incorporation has occurred, a lowering of the $pH_i$ does not inhibit the rise in incorporation rate. These results indicate that protein synthesis is a pH sensitive process before the incorporation increases but becomes pH insensitive after the increase occurs.
The use of DMO as a pH$_i$ indicator involves precautionary difficulties which must be taken into account. The major difficulties being to ensure that the DMO is not bound by the embryo or metabolized by the embryo. If DMO binds within the embryo, the ratio of intracellular to extracellular DMO will change as binding sites become occupied with increasing DMO concentration. At 0.5 uM, 5 uM and 50 uM concentrations of DMO, the ratio was a constant, which suggested that DMO was not bound within the embryo. After equilibration of DMO with embryos, no further uptake of DMO was observed, which suggested that DMO was not being metabolized. As a further control, extracts of embryos which had been incubated with DMO for as long as 24 hr were chromatographed according to the methods of Leguay (1977). A single spot on the chromatogram was observed for all embryos. Thus, we concluded that DMO was neither bound or metabolized by *L. polyphemus* embryos.

NaAcetate is capable of uncoupling oxidative phosphorylation (McLaughlin and Dilgen, 1980) and has been implicated as causing disruption of the metabolism of the embryos (Johnson and Epel, 1980). Therefore, the use of NaAcetate to alter pH$_i$ may also alter the general metabolism of the embryo. If this were true, the inhibited amino acid incorporation rates observed as a result of NaAcetate treatment might be an artifact of the treatment and not the result of altered pH$_i$. Our results indicate that changes in incorporation rates are the result of altered pH$_i$ and not the general effect of NaAcetate-altered metabolism. The continued increase of amino acid incorporation after transfer of embryos from MPFASW (pH 7.85) to 10 mM NaAcetate MPFASW (pH 6.85) at
10 hr is good evidence against NaAcetate acting as an uncoupler of oxidative phosphorylation or of disrupting cell metabolism. As yet, the molecular mechanism underlying the change in pH sensitivity remains unclear.
REFERENCES


SECTION 3. SYNTHESIS OF EXTRA-EMBRYONIC SHELL PROTEINS IN EARLY EMBRYOS OF THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS* L.
INTRODUCTION

The development of an embryo from a single cell (the unfertilized egg) involves a myriad of gene products whose expression is carefully regulated. Some of the more prominent gene families which have been investigated and shown to be developmentally regulated are the sea urchin histone genes (cf. Kedes, 1979) and the tubulin genes (Raff and Kaumeyer, 1973; Raff et al., 1972; Raff et al. 1971). Both of these gene families produce recognizable gene products in relative abundance during specific portions of sea urchin embryonic development. From work done on the expression of these gene products during development, much has been learned about regulation of gene expression.

Embryos of the horseshoe crab, Limulus polyphemus L., possess an extra-embryonic shell (EES) which is a second protective sphere utilized when the egg envelope breaks 20 days after fertilization (Brown and Clapper, 1981). The EES is assembled between the egg envelope and embryo and is first recognizable 11 days after fertilization. The ultrastructure of the EES has been described for both the non-expanded (before the egg envelope cracks) and the expanded form (after cracking of the egg envelope; Bannon and Brown, 1980). Development of this specific, easily isolated structure relatively early in the development of this primitive arthropod leads to two interesting questions: (1) Are the proteins which comprise the EES synthesized after fertilization? (2) Are there specific periods of time when certain EES proteins are synthesized? The answer to these questions could lead to the development of this organism as an additional tool to study gene expression.
In the present study, we have determined that synthesis of EES components does occur after fertilization and that there are specific periods of time when certain EES proteins are synthesized. However, it is likely that the EES proteins are synthesized both before and after fertilization.
MATERIALS AND METHODS

Source of Animals

Specimens of Limulus polyphemus L. were obtained from Florida Marine Biological Specimen Co., Inc. Panama City, Florida. Animals were maintained at 15°C in 150 gal. Instant Ocean Aquaria (Jungle salts artificial sea water (ASW) Jungle Laboratories Corp., Comfort, Texas), and were exposed to a light cycle of 14-10 L-D.

Gamete Collection and Insemination

Gametes were collected by brief electrical stimulation (1.5 V, 0.5-1.0 mA, ac) of the gonoducts proximal to the genital pores. Semen was diluted with millipore filtered ASW (MPFASW) to produce a 10% sperm suspension (10^9 spermatozoa/ml). Approximately 100 eggs were spawned and immediately transferred with wooden applicators to a Petri dish containing 25 ml of MPFASW. Two drops of the sperm suspension were added, and the dish swirled to ensure adequate mixing of gametes. All cultures were maintained at room temperature (21-22°C).

Labelling of Embryos

Embryos were labelled in MPFASW supplemented with an appropriate radioactive precursor (³H-leucine (1 uCi/ml; 134.2 Ci/mMole) or ³⁵S-methionine (3.5 uCi/ml; 1103 Ci/mMole) New England Nuclear). Embryos were labelled during a 21 hr period sometime after fertilization. Labelling of unfertilized eggs was not possible due to spontaneous activation of eggs approximately 10 min after spawning. After a
labelling period, embryos were placed in unlabelled MPFASW and allowed
to develop to stage 14 (11 days after fertilization at 21°C).

Evidence that pulse-chase conditions were adequate to prevent
continued incorporation of labelled amino acids and reutilization
during the chase is as follows: Embryos cease to incorporate labelled
amino acids 3 hr after its withdrawal from the external media, as
evidenced by the flattening of the TCA precipitable curve (Fig. 11).
Our results indicate that some proteins made during each of the
labelling periods were broken down during the chase, as evidenced by
the lower number of counts in the TCA precipitable fraction at 11
days. However, reutilization of labelled amino acids was judged unlikely
to have occurred because of the observation that Limulus embryos
possess extremely large amino acid pools (pools measured by the methods
of Rubin and Goldstein, 1970, as modified by Goustin and Wilt, 1981;
preliminary results indicate that the leucine and lysine pools are
greater than 550 picomoles per embryo). The contribution of labelled
amino acids, which result from protein breakdown, to the amino acid
pools would be so small that reutilization of radioactive amino acids
would be unlikely to have an effect on the experiments performed.

Isolation of the Extra-Embryonic Shell

The extra-embryonic shell (EES) was isolated from stage 14 embryos
(see Brown and Clapper, 1981 for staging of embryos) by removing the egg
envelope with watchmakkers forceps. The EES was ripped open, the embryo
pushed out, and the EES turned inside out. The EES was washed exten-
sively in MPFASW and distilled water to remove any embryonic debris.
Figure 11. This graph represents the continuous incorporation of $^3$H-leucine (●—●) and $^{35}$S-methionine (▲—▲) into the TCA insoluble fraction of homogenized Limulus embryos. Embryos were cultured in MPFASW supplemented with the appropriate radioactive precursor for the first 24 hr after fertilization and then chased in unlabelled MPFASW for the following 24 hr. As can be seen from the graph, TCA insoluble counts level off within 3 hr of withdrawal of labelled amino acids from the media.
Isolation of the Extra-Embryonic Shell Proteins

The isolated EES were placed in protein isolation buffer (modified from Neville, 1975; 0.1 N NaOH, 1% SDS, 0.5 M Tris pH 6.8, 5 M urea, $10^{-4}$ M phenylmethylsulfonylfluoride) for 1 hr on ice. The shells were separated from the supernatant (containing the EES proteins) by centrifugation. These proteins were either run immediately on SDS polyacrylamide gels or stored at $-70^\circ$C until needed.

Gel Electrophoresis and Fluorography

All samples were run on 5-20% SDS polyacrylamide gradient gels according to the methods of Laemmli (1970). Gel fluorography was accomplished according to the methods of Bonner and Laskey (1974).
RESULTS

Protein Components of Stage 14 EES

There are 8 µg of extractable protein present in each extra-embryonic shell (EES). These proteins represent 1.5% of the total protein of the embryo (approximately 500 µg of protein as determined by the Lowrey protein determination method, 1951). The extractable protein components of an 11 day EES are shown in Fig. 12. The EES protein range in molecular weight from slightly less than 14,000 to greater than 250,000. Of the approximately 17 bands observed, 5 appear particularly prominent. These 5 bands migrate with apparent molecular weights of 117,000; 90,000; 28,000; 16,000; and 14,300 as judged by their co-migration with molecular weight standards.

Post-Fertilization Synthesis of EES Components

The EES is first recognizable, between the egg envelope and embryo, 11 days after fertilization (stage 14 embryo; room temperature, 21°C). Embryos were labelled for different 24 hr periods from 1-10 days after addition of a sperm suspension to determine if any EES components were synthesized after fertilization. Embryos were then chased in unlabelled MPFASW to the 11th day, the EES isolated and placed in scintillation vials to be counted. The results from this experiment are shown in Fig. 13.

Our results indicate that there was synthesis of EES components after fertilization. Synthesis of EES components occurred for each of the 10 twenty-four hr labelling periods. However, the synthesis of EES
Figure 12. EES protein pattern on a 5-20% SDS polyacrylamide gradient gel. Numbers on the right hand margin represent the migration pattern of molecular weight markers (BioRad molecular weight markers; 200K-myosin; 116.5K-B-galactosidase; 94K-phosphorylase B; 68K-bovine serum albumin; 43K-ovalbumin; 30K-carbonic anhydrase; 21K-soybean trypsin inhibitor; 14.3K-lysozyme). EES proteins are designated by letters on the left hand margin from high to low molecular weights.
Figure 13. Time course of EES synthesis. Different batches of embryos were labelled with $^{3}$H-leucine (1 uCi/ml; 134 Ci/mmmole New England Nuclear) for one of 10 different 24 hr periods after fertilization. After labelling, the embryos were chased in unlabelled MPFASW to day 11 and the EES isolated. Percentage of the total cpm incorporated, which was found associated with the EES, is plotted against the day of label.
components was not at a constant level throughout the periods examined. The highest percentage of total TCA precipitable counts found associated with the EES occurred when embryos were labelled during the first 24 hr after fertilization (Fig. 13). Ten percent of the $^3$H-leucine found in the TCA insoluble fraction of the embryo during the first 24 hr labelling period was found associated with the EES on the 11th day after fertilization. The second and third 24 hr labelling periods resulted in a continued decrease in the percentage of counts found associated with the EES (3.7% and 0.9%, respectively). While the remaining labelling periods ($4^{th}$-$10^{th}$) showed incorporation of counts into the EES, the percentage of total TCA insoluble counts never again rose above 2.0%.

Synthesis of EES Proteins

Determination of the time sequence of synthesis of EES proteins was accomplished by labelling different batches of embryos with $^{35}$S-methionine during a 24 hr period sometime after fertilization. Typical fluorograms of EES proteins synthesized during the first 10 days after fertilization are shown in Fig. 14. An SDS polyacrylamide gel of stained EES protein is included for comparison of radioactive and stained protein bands. The radioactive bands do not always line up with one another because they were not run on the same gel.

All of the EES proteins were not synthesized at detectable levels during each of the 10 day labelling periods. We have concentrated on delineating the time course of synthesis of 6 EES proteins. These 6 proteins can be placed into three groups according to their pattern of synthesis: (1) EES proteins synthesized at detectable levels throughout
Figure 14. $^{35}$S-methionine labelled EES proteins. Embryos were labelled for a 21 hr period sometime after fertilization. After labelling, the embryos were allowed to develop to stage 14 (11 days after fertilization at 21°C) and the EES proteins isolated and run on 5-20% SDS polyacrylamide gradient gels. (A) stained pattern of EES proteins with proteins F,M,N,O,P, and Q labelled. Numbers represent the day after fertilization during which these embryos were labelled.
all 10 labelling periods (proteins F and G); (2) EES proteins which are
not detectably synthesized after fertilization (proteins P and Q); EES
proteins which are synthesized early (days 1 and 2) with no detectable
synthesis for the last 8 days (proteins M and N).
The extra-embryonic shell (EES) of Limulus is a specific structure produced by the embryo early in development. The EES represents a second protective envelope, utilized by the embryo, as an expanded area in which to continue development. The EES first appears as a defined structure between the egg envelope and embryo 11 days after fertilization (Bannon and Brown, 1980). Even though the EES is not recognizable until 11 days after fertilization, some of the protein components are being synthesized as much as 10 days before. Not all of the stainable EES proteins are synthesized after fertilization. The occurrence of proteins which are in sufficient quantity to be visualized by stain, but not detectably synthesized after fertilization, indicates that they were made in the developing oocyte or unfertilized egg.

In sea urchins, the synthesis of histone proteins during embryonic development has been extensively investigated (cf. Kedes, 1979). Sub-types of the 5 common histones (H1-H2A-H2B-H3-H4) have been shown to be differentially synthesized during specific portions of sea urchin development (Newrock et al., 1977). The expression of these specific histone subtypes has been shown to be the result of transcriptional or post-transcriptional control of histone mRNA and not post-translational modification of histone proteins (Newrock et al., 1978). Another group of proteins which have been shown to be developmentally regulated in the sea urchin embryo are the microtubule proteins. Soluble pools of microtubule proteins exist in sea urchin eggs and embryos (Raff, et al., 1971) which is used in assembling microtubule containing organelles
(Hynes et al., 1972). Synthesis of microtubule proteins after fertilization has been shown to occur as the result of translation of maternal mRNA which is stored in the unfertilized egg (Raff et al., 1972). Thus, the sea urchin embryo contains a microtubule protein pool acquired during oogenesis, with synthesis of new microtubule proteins to replenish this pool being under the control of a post-transcriptional process (i.e., the unmasking of maternal mRNA coding for microtubule proteins at fertilization).

In *Limulus* embryos, the molecular mechanisms controlling EES protein synthesis are unclear. However, assuming that *Limulus* embryos possess protein synthetic rates similar to frog embryos (i.e., a low synthetic rate of 20-40 ng of protein/hr for meiotic *Rana* oocytes and a high of 200 ng/hr for *Xenopus* blastula stage embryos; Davidson, 1976) calculations can be made to determine if it was possible to make all 8 ug of EES proteins after fertilization. Utilizing the low synthetic rate of meiotic *Rana* oocytes, only 0.170 ug of EES proteins could be synthesized in the 10 days following fertilization \[\text{(30 ng/hr x 24 hr x 10% for the first day) + (720 ng x 9 x 1.5% for the last 9 days) = 0.170 ug of EES proteins}.\] Utilizing the high synthetic rate of a *Xenopus* blastula embryo, only 1.13 ug of EES proteins could be synthesized \[\text{(200 ng/hr x 24 hr x 10% for the first day) + (4800 ng x 9 x 1.5% for the last 9 days) = 1.13 ug}.\] Utilization of the protein synthetic rates of other developing embryos indicates that even a smaller quantity of EES proteins could be synthesized (Davidson, 1976). This suggests that the *Limulus* egg either contains a store of EES proteins which were synthesized
during oogenesis or EES proteins are being synthesized in the unfertilized egg. If the majority of EES proteins are synthesized during oogenesis or in the unfertilized egg, these proteins must be transported in some manner from the embryo to the perivitelline space. A possible mechanism for transport of EES proteins is the cortical reaction. The demonstration of EES protein transport via cortical vesicle fusion or by any other mechanism remains to be determined.
REFERENCES


SECTION 4. ULTRASTRUCTURAL CHARACTERISTICS OF THE NON-EXPANDED AND
EXPANDED EXTRA-EMBRYONIC SHELL OF THE HORSESHOE CRAB, *LIMULUS
POLYPHEMUS* L.
INTRODUCTION

The envelopes of developing embryos are important for providing protection from mechanical injury, for maintaining a constant environmental media, and for allowing the passage of respiratory gases (Wigglesworth, 1946). These envelopes, depending on their origin, have been classified as primary, secondary, or tertiary (Quattropani and Anderson, 1969; Ludwig, 1874). Such structures produced by the oocyte are primary envelopes, those produced by ovarian follicle cells are secondary envelopes, and those produced by an extra-ovarian source (e.g., the developing embryo) are tertiary envelopes (Quattropani and Anderson, 1969). The formation, structure and function of primary and secondary envelopes have been described for a number of invertebrates (Regier et al., 1980; Mazur et al., 1980; Turner and Mahowald, 1976; Barbier and Chauvin, 1974; Salkeld, 1973; Quattropani and Anderson, 1969; Cheung, 1966). The tertiary envelope, however, has been less thoroughly examined probably because many organisms do not form a true envelope of this origin (i.e., the fertilization membrane of sea urchins can be described as one of primary and tertiary origins because it forms from a combination of the oocyte-produced vitelline layer and materials excreted by the embryo during the cortical reaction; Chandler and Heuser, 1980; Boyan, 1970a, b).

In the American horseshoe crab, Limulus polyphemus, all three types of envelopes are found. Eggs possess an egg envelope consisting of an inner vitelline envelope of primary origin and an outer basement lamina of secondary origin. The formation and structure of these layers have
been previously described (Dumont and Anderson, 1967; Shoger and Brown, 1970). However, in *Limulus* and the closely related Japanese horseshoe crab, *Tachypleus tridentatus*, the formation and structure of the extra-embryonic shell (a true tertiary envelope) has been only superficially examined (Kingsley, 1892; Sekiguchi, 1970).

The extra-embryonic shell (EES) appears in the periembryonic space during early development of the *Limulus* embryo and is completed prior to stage 17 (Brown and Clapper, 1980). During stage 20 (approximately 21 days after insemination at 20-21°C), the egg envelope cracks off and the EES slowly expands to approximately twice its previous diameter. In this expanded sphere, the larva is active and several days after the cracking of the egg envelope moults into a trilobite larva (stage 21). Eventually, the trilobite larva will hatch from the EES and become a free swimming form. Similar observations have also been recorded by Sekiguchi (1973) and Sugita and Sekiguchi (1979) on *Tachypleus tridentatus*.

This report describes the organization of the extra-embryonic shell (EES), both before and after expansion. Possible mechanisms by which the EES expands are also discussed.
MATERIALS AND METHODS

Source of Animals

Specimens of *Limulus polyphemus* L. (obtained from the Florida Marine Biological Specimen Co., Inc., Panama City, Florida) were maintained at 15°C in Instant Ocean Aquaria in artificial sea water (ASW) (Aquarium Systems, Inc., Eastlake, Ohio) on a 14-10 LD photoperiod.

Gamete Collection and Insemination

Gametes were collected by brief electrical stimulation (1-1.5 V, 0.5-1.0 mA, ac) of the gonoducts proximal to the gonopores (Brown and Clapper, 1980). Semen was diluted with 100% ASW (960 m Osmoles) to produce a 10% sperm suspension (10^9 spermatozoa/ml). Eggs (40-50) were placed in a plastic Petri dish containing 25 ml of ASW. Two drops of the sperm suspension were added and the mixture was gently swirled. All cultures were maintained at room temperature (20-21°C).

Procurement of Embryos Before and After Expansion of EES

Specimens representing different steps of expansion of the EES were collected for electron microscopical preparation (1) before, (2) during, and (3) after the cracking of the egg envelope and subsequent expansion of the EES. The first step was obtained when embryos were in stage 19 (approximately 19 days after fertilization). The second step was obtained after stage 20 appeared and the cracking of the egg envelope was in process. Specimens for the third step were usually collected several days after the cracking had occurred. The expansion process was
measured daily until hatching and was recorded photographically using a Wild M-5 dissecting microscope.

**Electron Microscopy**

Properly staged specimens were fixed in a 2.5% glutaraldehyde solution (cacodylate buffer; 4°C) for 12-24 hr, post-fixed in 2% osmium tetroxide and stored in dd H$_2$O for one or more days. For critical point drying, utilizing liquid carbon dioxide, fixed specimens were dehydrated in ethanol and cleared into amylacetate. Some dried specimens were fractured with a sharp razor blade. For freeze-drying, fixed specimens were snap-frozen, one at a time, in liquid nitrogen, placed on precooled brass blocks, and evacuated 5 x 10$^{-5}$ Torr) overnight. All specimens were mounted on stubs with silver paint, carbon and gold coated, and examined on a Cambridge Mark 2A "Stereoscan" scanning electron microscope.

Similarly staged specimens, to be examined by transmission electron microscopy, were fixed in a trialdehyde solution (Kalt and Tandler, 1971; acrolein, 0.178 M; DMSO, 0.32 M; formaldehyde, 0.66 M; glutaraldehyde, 0.33 M; pH 7.0) for 3 hr, post-fixed in 1% osmium tetroxide and dehydrated (all fixation procedures were carried out at 4°C). Infiltration of the sample was effected by puncturing the embryo with a sharp probe prior to embedding in spurrs resin (Standard Medium A; Polysciences Data Sheet No. 127). All tissue was sectioned on a LKB Ultrotome, stained with aqueous uranyl acetate and lead citrate and examined on a Hitachi HU-11E-1 electron microscope.
RESULTS

General Observations

Approximately 14 days elapse from the first step of the expansion process (Figs. 15 a-c) to when the larva hatches out (19th to 33rd day after fertilization). The diameter of the enclosing envelopes during this process increases from an initial 3.8 mm to a final 6.4 mm when hatching occurs. This increase in diameter of the EES is very slow during expansion, even immediately after the cracking off of the egg envelope (Fig. 15b). The EES forms in the periembryonic space between the egg envelope and embryo proper and appears to be morphologically complete by stage 14 (limbbud stage, 11 days after fertilization). The very obvious polygonal structures can be easily observed after the egg envelope cracks (Fig. 15d).

Extra-Embryonic Shell Before Expansion

The EES before expansion is evident in a section through a stage 19 embryo (Fig. 16a). Also apparent are the egg envelope and the developing embryo. The egg envelope consists of two morphologically distinct layers, a 5 \( \mu \)m thick basement lamina and a 40 \( \mu \)m thick vitelline envelope (Shoger and Brown, 1970; Dumont and Anderson, 1967). The EES is approximately 33.16 \( \mu \)m thick and consists of swirls of non-cellular materials with the outer third containing regularly spaced indentations. Closely opposed to the inside surface of the EES is the chitinous exoskeleton of the embryo.
Figure 15. Expansion of the extra-embryonic shell: (a) The embryo is enclosed by the extra-embryonic shell and the egg envelope. X 7. (b) The embryo has moulted into stage 20 and the egg envelope is cracking away. The extra-embryonic shell is exposed and is slowly expanding. Full expansion takes several days. X 7. (c) The fully expanded extra-embryonic shell allows an increased space in which the stage 20 embryo can be active and undergo the final embryonic moult into the trilobite larva (stage 21). X 7. (d) Scanning electron micrograph (SEM) of the cracking of the egg envelope (EN). The extra-embryonic shell (EES) is characterized by the presence of numerous packed polygonal structures which gives the surface a rough, irregular appearance. X 40
Figure 16. Electron microscopy of non-expanded shell: (a) SEM of fractured stage 19 embryo and surrounding coats. The EES develops between the egg envelope (EN) and the chitinous exoskeleton (*) of the embryo (EM) in the periembryonic space. Indentations in the EES are clearly visible (arrows). X 1,750. (b) TEM of non-expanded EES showing large indentations and fibrous bundles (FB). X 2,700. (c) Longitudinal section of an indentation observed in the non-expanded shell. Dense bodies are found associated with the indentations. X 12,000. (d) Cross section of the non-expanded EES detailing the three layers which comprise this structure. Layer 1 (1) is 0.02 \( \mu \text{m} \) thick, is electron translucent and possesses numerous hair-like projections. Layer 2 (2) is 0.14 \( \mu \text{m} \) thick and electron dense. Layer 3 (3) is 33.0 \( \mu \text{m} \) thick and is intermediate in its electron density between layer 1 and layer 2. X 35,000. (e) Fibrous bundles of layer 3 found in non-expanded EES. X 22,000
The EES is composed of three distinct layers (Figs. 16b and 16d). The outermost layer (layer 1) is approximately 0.02 μm thick, electron translucent (Fig. 16d) and is the source of numerous hair-like projections. The middle layer (layer 2) is approximately 0.14 μm thick and electron dense. The innermost layer (layer 3) comprises the majority of mass of the EES and is approximately 33 μm thick and intermediate in electron density between layer 1 and layer 2. Fibrous bundles are observed throughout layer 3 (Figs. 16b and 16e). Associated with the indentations observed in the EES in Fig. 16a and 16b are electron dense bodies (Fig. 16c).

Extra-Embryonic Shell After Expansion

After expansion, the exterior surface of the EES consists of numerous variable-shaped polygonal structures separated by a large interpolygonal region which has a very irregular surface (Fig. 17a). As the EES expanded, this region was formed by the elevation and flattening of the previously described indentations between the polygonal structures.

On the surface, the interpolygonal region appears to be composed of many ridges or "fibers" radiating from each polygonal structure which interdigitates with "fibers" from other polygonal structures (Fig. 17a). This arrangement can also be observed on an unfixed expanded EES with light microscopy. Between these "fibers", hair-like projections give the appearance of cross-linking (Fig. 17b). Cross sections reveal the surface to be very irregular and these "fibers" to be ridges on the outer surface of the EES (Fig. 17c). The thickness of the fully expanded shell (measured at widest portion) is reduced to 12 μm, a 64% reduction from
Figure 17. Electron microscopy of expanded shell: (a) SEM of exterior surface of expanded EES showing polygonal structures (PS) and the large interpolygomal region which is characterized with interdigitating "fibers." X 1,750. (b) SEM of interpolygomal region. This region is very irregular and numerous hair-like projections can be seen originating from the "fibers." X 13,600. (c) TEM of expanded shell. The large indentations, fibrous bundles, and electron dense bodies are no longer present. X 4,950. (d) Cross-section of expanded EES showing that the basic structure of the shell does not change after expansion (1, Layer 1; 2, Layer 2; 3, Layer 3). (See Fig. 17d for comparison). The irregular surface topography represent the "fibers" observed in 18a. X 32,000.
the non-expanded shell thickness. The three layers which comprise the EES retain their original characteristics (i.e., as seen in the non-expanded form) with the exception of the absence of fibrous bundles and electron dense bodies in layer 3 (Figs. 17c and 17d).
DISCUSSION

For xiphosurid embryology, the use of the term extra-embryonic shell (EES) is recommended since this structure is formed outside the embryo, is composed of non-cellular material and is formed by embryonic secretions which may occur over several developmental stages. Other terms have been used: blastoderm cuticle (Roonwal, 1944), blastodermhaut (Kingsley, 1892), deutovum (Iwanoff, 1933), inner egg membrane (Sekiguchi, 1970), and egg membrane (French, 1979) but are inappropriate because of the above characteristics.

However, the EES of Limulus has some similarities with a typical insect cuticle. An insect cuticle is constructed asymmetrically with the outermost layer representing the first layer produced by the epidermis and subsequent layers distinguished by their different electron densities as observed with electron microscopy (cf. Neville, 1975). The insect cuticle may be composed of numerous layers (Caveny, 1971). The EES is also asymmetrical but consists of only three layers which could correspond, at least spatially, to epicuticle, exocuticle, and endocuticle of the insect cuticle. The EES differs from a typical insect cuticle in its relative simplicity and the separation from any cellular layer by a periembryonic space.

The expansion of the EES involves utilization of preformed surface area which is stored in large indentations observed in the non-expanded shell (Fig. 16b). However, the increase in size of the totally expanded shell requires more surface area than found in these indentations. For example, before expansion, the thickness of the EES from the bottom of
the indentations to the inside surface of the EES is 22 μm. But, when the expanded shell reaches maximum size, its width is only 12 μm at the widest point indicating that the non-cellular components of the EES must be rearranged (stretched) to compensate for the decreased thickness of the expanded shell. As measured in this study and others (Sekiguchi, 1970; Roomwal, 1944), the EES continues a slow expansion until hatching of the larva.

The mechanisms for the expansion phenomenon has been considered by various investigators (Kingsley, 1892; Iwanoff, 1933; Roomwal, 1944; Sugita and Sekiguchi, 1979). Several theories have been presented. Basically, the favorite mechanism has involved osmotic effectors produced by the embryo to allow an increase in fluid content in the periemphryonic space. However, little evidence has been provided for support. Recently, using Tachypleus, Sugita and Sekiguchi (1979) have examined the complement of proteins present in the periemphryonic ("perivitelline") space and described four classes of proteins, H, B-1, B-2, and "rest". Changes in concentration of these proteins were followed during development. The "rest" proteins increased dramatically prior to expansion of the EES indicating the possible involvement of these proteins in this process.

According to Yamamichi and Sekiguchi (1974) and Sugita and Sekiguchi (1979), the EES is formed from material secreted into the periemphryonic space by blastoderm cells. The time table for secretion of these components into the perivitelline space and the mechanisms of transport are not known. However, the possibility exists that some of the components reach the perivitelline space via the egg cortical reaction (Bannon and
Brown, 1980). The question now arises as to when these components are synthesized. Are they synthesized during oogenesis and stored in the early embryo until needed or are they synthesized by the embryo during development? These questions are currently being addressed in our laboratory.
REFERENCES


SUMMARY-DISCUSSION

This dissertation research investigated some aspects of egg activation in the horseshoe crab, *Limulus polyphemus* L. Structural and metabolic changes were studied, with particular emphasis on: (1) the egg cortical reaction, (2) intracellular pH (pH$_i$) and amino acid incorporation and (3) the synthesis of EES proteins during egg activation and later in development.

The egg cortical reaction in *Limulus* is an easily observed phenomenon which occurs on the fertilized egg surface, resulting in the formation of large indentations of "pits" (Brown and Clapper, 1980). The formation of pits was investigated and found to result from fusion of cortical vesicles with the egg plasma membrane. The cortical vesicles in the unfertilized egg could be divided into two groups according to their size and location in the cortex. The smallest vesicles (0.5 μm) contained electron translucent contents and were closely apposed to the plasmalemma. The largest vesicles (4.0 μm) contained electron dense material which was variable in its morphology. Shortly after sperm-egg fusion the small vesicles began to fuse with the egg plasma membrane, followed by fusion of large vesicles which dump their contents into the perivitelline space and form large pits on the egg surface. There was some ultrastructural evidence presented indicating that fusion between two or more of the large vesicles to form an enlarged vesicle was possible. The enlarged vesicle eventually fused with the egg plasma membrane. The fusion of vesicles with the egg plasma membrane continued until none were remaining in the cortical region of the fertilized egg.
One of the possible functions for cortical vesicle fusion might be the transport of macromolecular components to the perivitelline space for utilization in the assembly of the extra-embryonic shell later in development.

In Section 2, amino acid incorporation was studied and changes in this metabolic parameter as a result of egg activation were documented. An increase in rate of amino acid incorporation was observed approximately 6-8 hr following fertilization in embryos cultured in MPFASW (pH 7.85). Intracellular pH was determined for these embryos and a concomitant alkalization of $pH_i$ and amino acid incorporation did not occur. The $pH_i$ of embryos cultured in MPFASW (pH 7.85) was 7.69 from 90 min-14 hr after fertilization. It was not possible to determine if there was an alkalization of egg cytoplasm at fertilization because of the long equilibration time required for the pH indicator utilized (DMO; 5,5-dimethyl-2,4-oxazolidinedione). By artificially lowering the $pH_i$, before the increase in amino acid incorporation occurs, it was possible to suppress the increase in incorporation rate. However, the suppression of amino acid incorporation was reversible, as evidenced by an increased incorporation rate shortly after re-alkalization of $pH_i$. Acidification of embryos (pH 6.77) at 10 hr after fertilization (after the increase in incorporation rate had occurred) did not result in a suppressed incorporation rate. These data suggest that protein synthesis in the Limulus embryo is pH sensitive before the increase in amino acid incorporation, but becomes pH insensitive after the increase in incorporation has occurred.
The synthesis of a specific set of proteins, the extra-embryonic shell proteins, was studied during egg activation and later in development. The EES is a structure first recognizable on the 11th day after fertilization. The EES is assembled, in the periembryonic space, between the egg envelope and embryo and is utilized as a second protective shell after sloughing of the egg envelope. The EES consisted of approximately 8 μg of extractable protein which produced at least 17 protein bands on one dimensional SDS 5-20% polyacrylamide gradient gels ranging in molecular weight from slightly less than 14,000 to greater than 250,000. This group of proteins was not accumulated at a constant rate throughout the 10 day period examined. The first 24 hr after fertilization (egg activation) exhibited the highest percentage (10%) of total TCA precipitable counts associated with the EES. The remaining 9 days after fertilization exhibited some accumulation of EES proteins, but at a greatly reduced rate (average for the 9 days was 1.5%). Of the 17 protein bands observed, 15 were synthesized at some time after fertilization. The fact that two EES proteins were not synthesized during this time period indicates that some synthesis of these proteins took place during oogenesis or in the unfertilized egg. Assuming that Xenopus and Rana protein synthetic rates (Davidson, 1976) are similar to Limulus embryos, it was discovered that a maximum of 1.13 μg of EES proteins could be synthesized during the 10 days following fertilization. This strengthens the assumption that some of the EES protein were synthesized before fertilization.
Finally, the ultrastructural aspects of the non-expanded and expanded EES were examined. The EES consisted of 3 layers which were differentiated according to their varying morphology. The non-expanded EES, before sloughing of the egg envelope, exhibited large indentation in the outer third of the shell. These indentations represented a store of surface area, utilized as the EES began to swell and the egg envelope was sloughed. The expanded EES lacked these indentations, but measurements made indicated that the width of the EES was less than expected. This indicated that the EES was expanding as a result of utilization of preformed surface area stored in the indentations and by stretching.

The results reported in this dissertation indicate that Limulus embryos may be an excellent system in which questions pertaining to egg activation may be answered. Some of the attributes of Limulus embryos are: (1) availability of a large number of gametes year round, (2) ease of culturing embryos in the laboratory, (3) an easily observed cortical reaction of long duration (for studying function of cortical vesicle fusion), (4) a definite turn on of amino acid incorporation which is affected by intracellular pH (for studying the role of pH in development), (5) a group of proteins whose synthesis appears to be developmentally regulated (for the study of gene regulation in early development). These attributes are impressive, however, before they can be fully realized two inherent difficulties must be confronted. The first difficulty is artificial activation of unfertilized eggs. Without being able to measure parameters of unfertilized eggs it will be difficult to study egg activation any further than it has already been pursued.
Second, the nuclear events, from the unfertilized eggs through blastulation, should be documented. Both of these problems are approachable, and when solved, will make the Limulus embryo amenable to further study.
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