# CYTOCHEMICAL STUDIES OF NUCLEOSIDE PHOSPHATASE ACTIVITY IN THE EGGS OF *STRONGYLOCENTROTUS PURPURATUS*

by

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Blow, blow thou winter wind
Thou art not so unkind
As man's ingratitude.
Thy tooth is not so keen
Because thou art not seen
Although thy breath be rude.

Freeze, freeze thou bitter sky
Thou dost not bite so nigh
As benefits forgot.
Thou, thou the waters warp
Thy sting is not so sharp
As friend remembered not.

(William Shakespeare)

To those who, because of their nature, have extended a hand out of kindness I owe much. These debts I can repay only by remembering that a man's accomplishments reflect not only his abilities but the magnanimity of his friends as well.

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# INTRODUCTION

## A. Historical aspects.

The history of the study of sea urchins, as detailed in Harvey's The American Arbacia and Other Sea Urchins (22), parallels that of the development of modern science. Not unexpectedly, the account began with Aristotle (384-322 B.C.), in the Historia Animalium, De Partibus Animalium, and De Generatione Animalium, in which appeared the earliest accounts of sea urchin natural history, anatomy, and embryology. These works were partially copied and added to by Pliny (23-79 A.D.) in his Natural History, where he also commented upon their non-scientific uses as food, medicine, and artifacts. Throughout the Middle Ages virtually nothing was added to any scientific knowledge and sea urchin studies were no exception, although various authors did hand down their impressions of the first two original works. These studies also shared in the Renaissance, however, with Rondelet's description of sea urchin anatomy, which was the first study not to be derived from either Aristotle or Pliny. Shortly thereafter, the systematic scientific approach to sea urchins was taken beginning with Linnaeus and culminating, in this early period, with the works of Louis Agassiz. This climax also represented the beginning of another epoch in which the classic embryology of sea urchins was examined thoroughly by workers like Th. Mortensen, H. L. Clark and Johannes Muller (22). Indeed, the history of sea urchin studies parallels not only that of science in general but the development of embryology in particular.

## B. Modern approaches.

Just as the sea urchin studies shared in the growth of the scientific method during and after the Renaissance, they also shared in the

full application of this approach in the late 19th and 20th centuries. Thus, the two outstanding reviews of the classic studies of sea urchins by Harvey and Hyman (22, 24) were published in 1955 and 1956 and covered a period of 23 centuries in the growth of this body of knowledge. In contrast, the next review appeared only 10 years later in 1966 (7) and represented a massive increase in knowledge in this area during a period of about 20 years.

The work in the modern period has been directed toward many aspects of sea urchins, but it was the work on the eggs which has a particular bearing to this study. In general, the eggs were examined by two disciplines, biochemistry and anatomy. The biochemical approach was used to examine enzymes (22), proteins (19), oxidative phosphorylation (10), respiration (63), high energy phosphate metabolism (38), and many other components and phenomena of the sea urchin egg (7, 22).

The anatomical examinations, on the other hand, generally delved into the cytology, the embryology, and the cytochemistry of the eggs. The cytological studies were primarily an extension of the early light microscopy work (22) utilizing the electron microscope (1, 3, 4, 5). The embryologic studies were continuations of the earlier works with the added resolution of electron microscopy and with new emphasis upon the chemical basis of development (7, 22).

C. Cytochemical analysis.

The cytochemical studies utilized both the biochemical approach to the constituents of the egg and the anatomical examination of its morphology and development. Some understanding was thereby gained of structural-functional relationships by examining such aspects as reduction gradients (23), various chemical constituents (7, 22), and enzy-

matic activity (7, 22) in the sea urchin egg.

These last studies, on enzymes, represented a serious attempt to dissect the functional anatomy of the egg in an effort to understand the earliest basis of development, and as yet have been pursued very little. Almost all of these reports have dealt with phosphatases which reflects an interest in the central role of these enzymes and the phosphates in cellular functions. Thus, the distribution of acid phosphatase has been studied in the egg (13, 14, 47, 48) along with a few cytochemical (13, 14, 36) and several biochemical studies (12, 34, 37, 43, 55) of ATPase. This latter enzyme is of particular interest, since the substrate upon which it acts is virually the hub of high energy phosphate metabolism, along with a few other nucleoside triphosphates, and is thus one of the key agents in almost all cellular functions (31, 59, 60). Furthermore, in sea urchin eggs ATP has been observed to increase greatly following fertilization (8, 9, 30) and the enzyme which acts upon it, ATPase, has been implicated in the activation of development following gamete fusion (57).

Since ATPase is probably concerned with the developmental functions of the sea urchin egg, it was decided to study its cytochemical demonstration in the mature egg. The previous work on ATPase in sea urchin eggs was quite useful in formulating an incubation medium and an experimental approach, but at the same time the results of these studies were quite perplexing in their contradictions. For example, in the biochemical studies, Conners and Scheer (12) described $Ca^{++}$ activated and $Mg^{++}$ inhibited activity, whereas Miki (36) determined exactly the opposite and described the inhibition of the system by $K^{+}$ as well. Ohnishi (43) found an ATPase activity in the cortex which was $Na^{+}$ and $K^{+}$ dependent, $Mg^{++}$

activated and Ca++ inhibited, and also inhibited by ouabain, monoiodoacetic acid, azide, cyanide and dinitrophenol. Furthermore, Conners and Scheer (12) found the optimal pH to be 8.5 as did Ohnishi (43), whereas Miki (36, 37) and Ricotta (55) found two pH maxima at 6.2 and 8.2 and 6.4 and 8.2, respectively.

The histochemical studies, on the other hand, were less confusing but were also far less convincing in their demonstration of ATPase. Both Miki (36) and Dalcq (14) demonstrated separate localizations for acid phosphatase and ATPase, but in both cases the latter activity was assumed to be ATPase on the basis of the substrate used. In addition, Dalcq (13, 14) found activity with CTP, UTP, GTP, ITP, ADP, AMP, and CMP, but admitted no difference in localization of activity with these substrates. In none of the histochemical studies were activators or inhibitors used and the long incubation times (16 hours) used by Dalcq (13, 14) cast considerable doubt upon the validity of his findings.

Accordingly, it appeared relevant to study the egg cytochemically, in order to preserve the structural-functional relationships, and to examine not only the localization of enzymatic activity but some characteristics of the system as well. These characterization studies examined both the substrate specificity and the response to activators and inhibitors and, along with the studies on the reactivation phenomenon, revealed considerable information regarding activity demonstrable with ATP and the other substrates.

# MATERIALS AND METHODS

## A. General techniques.

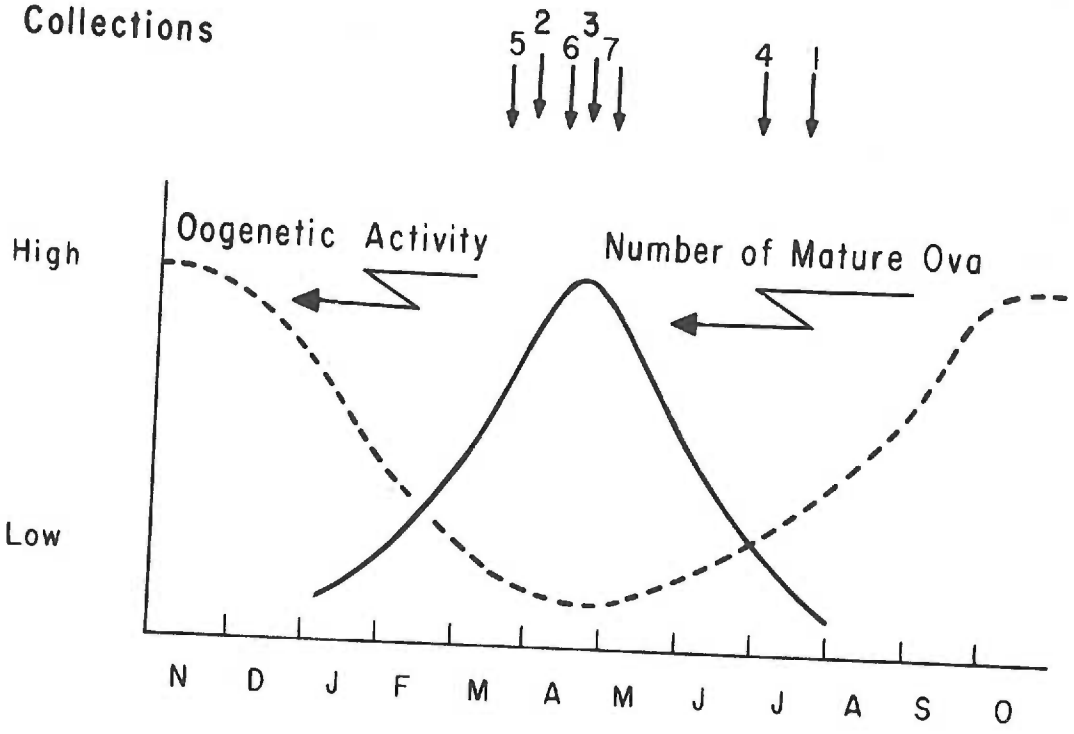
1. Collection and care of animals and collection of eggs. The ova used for experimentation were those of the common purple sea urchin, Strongylocentrotus purpuratus (Stimpson), and were collected at the Oregon coast. Collections were coincident with the annual reproductive cycle beginning between January and March and ending in May or June (16), (Figure 1). The animals were transported, immersed in buckets of sea water which were surrounded by ice, to Portland. In the laboratory they were stored in sea water aquaria at 10-15° C. Usually the animals were sacrificed within one to three weeks. Food was provided in the form of eel grass (Zostera) collected from the urchin beds.

Upon removal from the aquaria, the outer surface of the animals was rinsed thoroughly with distilled water to remove debris and any contaminating spermatozoa. For the collection of eggs, the dissection method was chosen over that of forced shedding (22, 61). After the eggs were shed from the collected gonads, a sample from each collection dish was tested for ability to be fertilized with similarly collected male gametes. Eggs which could not be fertilized were discarded. The ova were cleaned by rinsing in sea water, and were then concentrated by centrifugation at 2,000 xg for 3-5 minutes in a Lourdes AX centrifuge.

2. Density-gradient centrifugation and staining. The only pre-fixation step was density-gradient centrifugation. The density-gradient technique was an adaptation of Harvey's method (22) which was applied to produce both stratified and stretched eggs. Stratified eggs were produced by centrifugation for 10 minutes at 10,300 xg in a Lourdes AX centrifuge. The gradient was produced with sea water layered above

Figure 1.

Composite of collection times, oogenetic activity, and number of mature ova in the gonads. Note the optimum collecting range extends from early March to late May. It was during this period that large quantities of eggs could be consistently collected.




| Collection | Date | Locale | Collector |
|---|---|---|---|
| 1 | 12 July, 1966 | Depoe Bay | WAS |
| 2 | 29 March, 1967 | Depoe Bay | WAS |
| 3 | 17 April, 1967 | Boiler Bay | WAS |
| 4 | 31 June, 1967 | Boiler Bay | WAS |
| 5 | 19 March, 1968 | Yaquina Head | JB |
| 6 | 14 April, 1968 | Boiler Bay | WAS |
| 7 | 21 April, 1968 | Boiler Bay | WAS |

0.85 M sucrose in distilled water.

Stretched eggs were prepared similarly, except for a triple density-gradient of 0.85 M sucrose in distilled water, a one-half dilution of 0.85 M sucrose in distilled water with sea water, and sea water. The eggs were centrifuged for 10 minutes at 21,600 xg in a Lourdes AX centrifuge. Following centrifugation concentration was accomplished as outlined above.

After fixation the density-gradient centrifuged eggs were stained with the azure II-methylene blue stain of Richardson (54) and then mounted on slides as whole mounts.

3. Whole mount technique. Whole mounts were made by the method of Roulet (50) by adding drops of egg suspension to 3 drops of warm glycerol-gelatin (Sigma GG-1), adding the coverslip, and allowing to harden for 12 hours before examining.

4. Fixation and electron microscopy techniques. Whole mounts were fixed with an adaptation of the glutaraldehyde method of Sabatini, Bensch, and Barrnett (58). The final aldehyde concentration was 2.5% in a Veronal-acetate buffer at pH 7.4. All components were dissolved in a 50% dilution of sea water with distilled water, and the eggs were fixed for one hour at 4° C. After fixation, the eggs were rinsed and stored in the Veronal-acetate buffer at pH 7.4 (prepared in distilled water) at 4° C. before use.

Eggs used for electron microscopy were fixed in 1% $OsO_4$ in sea water buffered with Veronal-acetate at pH 7.4 for 2 hours at 4° C. (5) or in the glutaraldehyde fixative as used for the whole mounts. In the latter case, the eggs were post-fixed with $OsO_4$. The eggs were then embedded in Epon (32) and sectioned and stained according to standard

methods (51).

B. Cytochemical techniques.

1. Incubation medium and experimental procedure. The basic incubation medium was a variation of the original Wachstein-Meisel (50) technique:

| | | Final concentration |
|---|---|---|
| Substrate: 125 mg.% concentration | 20 ml. | 0.00083 M |
| Buffer: Michaelis, pH 6.2 | 20 ml. | |
| 2.0% $Pb(NO_3)_2$ | 3 ml. | 0.0036 M |
| 0.005 M $MgSO_4$ | 5 ml. | 0.0005 M |
| Distilled water | 2 ml. | |
| Total volume | 50 ml. | |

The basic experimental procedure was the following:

1. Rinse out storage buffer with three 5 minute changes of distilled water.
2. Treat controls with three 5 minute changes of 95% ethanol.
3. Rinse with one 15 minute change of incubation buffer before incubation.
4. Incubate for 1, 2, and 3 hours at room temperature.
5. Stop incubation by three 5 minute changes of distilled water.
6. Rinse with one 5 minute change of 2.0% acetic acid.
7. Rinse with one 5 minute change of distilled water.
8. Develop with one 5 minute change of 1.0% $(NH_4)_2S$.
9. Rinse with one 5 minute change of distilled water.
10. Mount by permanent mount.

Nonspecific activity, which was not due to the 1.0% ammonium sulfide treatment but to the retention of lead salts, was removed with a 2.0% acetic acid rinse, as in the original Gomori technique (18), provided the treatment preceded the development with 1.0% ammonium sulphide. Control eggs were incubated in the medium with substrate after treatment with 95% ethanol or incubated without substrate. Alkaline phosphatase (AlPase) and acid phosphatase (AcPase) activity was evaluated with the same basic procedure. The pH was changed to 5.0 and 9.0, and both $\alpha$- and $\beta$-glycerophosphate at 125 mg.% were used as substrates.

2. Method of recording results. The final reaction product (FRP)

was PbS, a crystal which appeared brown to black depending upon its density in the egg. The results were recorded in two ways:

1. Positive (+) if final reaction product present and negative (-) if not.
2. Intensity of reaction product graded from 1+ to 4+.

The grading method was utilized according to the following guide-lines:

| Grade of reaction | Criteria |
|---|---|
| - | No evidence of final reaction product; eggs same as unincubated eggs. |
| 1+ | Final reaction product usually golden-brown to brown; coloration only slightly, but perceptably, different from unincubated eggs; localized to medulla. |
| 2+ | Final reaction product brown to dark brown; coloration obviously different from unincubated and 1+ eggs; no reaction product in cortex; concentration of FRP in medulla. |
| 3+ | Reaction product not entirely limited to medulla; cortex usually pale golden-brown and slightly darker than unincubated egg; medulla brown or dark-brown and generally with areas of black coloration suggesting, but not definitive of, granule formation. |
| 4+ | Same as 3+ but with definitive black granule formation. |

3. Localization studies. The studies on the localization of enzymatic activity were an integral part of all of the experiments. Thus, whenever there was a positive reaction, its localization was recorded either photographically or described in the experimental record. In mature eggs the FRP was produced with the standard incubation medium and technique and its localization was evaluated at varying times before and after 4 days post-fixation. Those eggs incubated after 4 days post-

fixation were reactivated with KCl, $KH_2PO_4$, NaCl, $MgSO_4$, $MgCl_2$, $CaCl_2$, or sea water, and some experiments utilized various activators and inhibitors.

Other localization studies on mature eggs were done after sucrose density-gradient centrifugation. Layered eggs were incubated only in the period before 4 days post-fixation, whereas stretched eggs were also studied at 9 days post-fixation. The reactivating agents used were sea water, $KH_2PO_4$, $MgSO_4$, $CaSO_4$, and $NaH_2PO_4$.

Immature eggs and atypical reaction forms were studied only in the period before 4 days post-fixation. The reactivating agents used were sea water, $KH_2PO_4$, $MgSO_4$, $CaSO_4$, and $NaH_2PO_4$. At the same time L-cysteine, 2,4-dinitrophenol, sodium fluoride, sodium azide and ouabain were included in the experimental system (see Section 7).

4. Period of reactivity and reactivation. The activity of the preparations in the immediate post-fixation period was studied by allowing the eggs to remain in storage buffer after fixation for 1, 2, 3, and 4 days. Following this lag, they were assayed by the standard method outlined above with di-sodium-ATP as substrate. Subsequently, the time of loss of activity in days post-fixation, or $R_t$ (reactivation time), was established.

Re-establishing activity in the eggs, or reactivation, was first produced by treatment with sea water. In these experiments the incubation medium remained the same, but the experimental procedure was changed to the following:

1. Rinse storage buffer out with three 5 minute changes of sea water.
2. Store overnight in sea water at 4° C.
3. Rinse out sea water with three 5 minute changes of distilled water.
4. The remainder of the procedure remained the same as above.

A number of studies were aimed at evaluating several components of sea water as reactivating agents. The experiments were done at 4, 9, 22, 30, 40, 62, and 67 days post-fixation. The reactivation method was the same as above, but the different compounds employed were used as follows:

| Reactivating agent | Concentration |
|---|---|
| KCl | 0.01 M |
| $KH_2PO_4$ | 0.01 M |
| NaCl | 0.5 M |
| $NaH_2PO_4$ | 0.5 M |
| $MgSO_4$ | 0.04 M |
| $MgCl_2$ | 0.04 M |
| $CaCl_2$ | 0.01 M |
| $CaSO_4$ | 0.01 M |

5. Substrate specificity. Substrate specificity was studied by varying the substrate used in the incubation medium. In all cases, the substrates were prepared as 125 mg.% stock solutions. In cases where reactions were done post-$R_t$, sea water was used as the reactivating agent. Reactions with ATP as substrate were run concurrently as part of each experiment in order to assure comparability. In all other respects the standard medium and procedure were employed. All substrates were evaluated in the pre-$R_t$ period, whereas in the post-$R_t$ period only those nucleosides which were reactive in the pre-$R_t$ period and the non-nucleoside substrates were assayed. The organization of this series of experiments was as follows:

| Days post-fixation | Substrates evaluated |
|---|---|
| 2 | ATP, UMP, CDP, CMP, GDP, GMP, IDP, IMP, TTP |
| 3 | ATP, ADP, AMP, TDP, TMP, CTP, GTP, ITP, G1P, G6P, ThPOP, NaPOP, $\alpha$GP, $\beta$GP |

| Days post-fixation | Substrates evaluated |
|---|---|
| 20 | ATP, ADP, TTP, TDP, ITP, UTP, CTP, UDP |
| 33 | ATP, αGP, βGP, G1P, G6P, NaPOP, ThPOP |
| 36 | ATP, ADP, TTP, TDP, ITP, UTP |

ATP adenosine-5′-triphosphoric acid, di-sodium salt.
ADP adenosine-5′-diphosphoric acid, sodium salt.
AMP adenosine-5′-monophosphoric acid, sodium salt.
CTP cytidine-5′-triphosphoric acid, sodium salt.
CDP cytidine-5′-diphosphoric acid, sodium salt.
CMP cytidine-5′-monophosphoric acid, di-sodium salt.
GTP guanosine-5′-triphosphoric acid, sodium salt.
GDP guanosine-5′-diphosphoric acid, sodium salt.
GMP guanosine-5′-monophosphoric acid, sodium salt.
G1P alpha-D-glucose-1-phosphoric acid, di-sodium salt.
G6P D-glucose-6-phosphoric acid, di-sodium salt.
αGP DL-alpha-glycerophosphoric acid, di-sodium salt (95-55% alpha isomer).
βGP DL-beta-glycerophosphoric acid, di-sodium salt (0.1% maximum DL-alpha isomer impurity).
ITP inosine-5′-triphosphoric acid, sodium salt.
IDP inosine-5′-diphosphoric acid, sodium salt.
IMP inosine-5′-monophosphoric acid, sodium salt.
NaPOP tetra-sodium pyrophosphate.
ThPOP thiamine pyrophosphate (or aneurine pyrophosphate, or cocarboxylase), chloride salt.
TTP thymidine-5′-triphosphoric acid, sodium salt.
TDP thymidine-5′-diphosphoric acid, sodium salt.
TMP thymidine-5′-monophosphoric acid, sodium salt.
UTP uridine-5′-triphosphoric acid, sodium salt.
UDP uridine-5′-diphosphoric acid, sodium salt.
UMP uridine-5′-monophosphoric acid, sodium salt.

6. Activators and inhibitors. Analysis of activating and inhibiting agents involved only a minor change in the incubation medium and none in the basic experimental procedure. The incubation medium was changed so that instead of 2 ml. of distilled water, 2 ml. of an activator or inhibitor were added. Incubations without any agents, or with the standard incubation medium, were included to verify activity of the preparations towards ATP and thus allow comparisons to be made with

other studies. Eggs which were incubated post-$R_t$ were reactivated with sea water. A complete list of activators and inhibitors, the concentrations in which they were prepared, their final concentration in the incubation medium, and the authors from whose work they were adapted follows:

| Agent evaluated | Prepared as | Final concentration | Author |
|---|---|---|---|
| L-cysteine (L-cys) | 0.00625 M<br>756 mg.% | 0.00025 M | Colowick and Kaplan (11) |
| 2,4-dinitrophenol (DNP | 0.0015 M<br>27.5 mg.% | 0.00006 M | Meyers and Slater (35) |
| iodoacetamide (IAA) | 0.125 M<br>2.3 gm.% | 0.05 M | Colowick and Kaplan (11) |
| sodium azide ($NaN_3$) | 0.025 M<br>162 mg.% | 0.001 M | Novikoff, et al. (41) |
| ouabain | 0.00025 M<br>18.3 mg.% | 0.00001 M | Katz and Epstein (27) |
| glutathione (GSH) | 0.0025 M<br>77 mg.% | 0.0001 M | Colowick and Kaplan (11) |
| p-chloromercuri-benzoic acid (pCMB) | 0.01 M<br>178.5 mg.% | 0.0004 M | Colowick and Kaplan (11) |
| mersalyl acid (MA) | 0.0004 M<br>20.2 mg.% | 0.000016 M | Padykula and Herman (44) |
| sodium-L(+)-tartrate (NaT) | 0.02 M<br>300 mg.% | 0.0008 M | Colowick and Kaplan (11) |
| zinc chloride ($ZnCl_2$) | 0.01 M<br>13.6 mg.% | 0.0004 M | Colowick and Kaplan (11) |
| sodium fluoride (NaF) | 0.01 M<br>41.9 mg.% | 0.0004 M | Colowick and Kaplan (11) |

In the first analysis L-cys, DNP, GSH, pCMB, IAA, MA, NaT, $ZnCl_2$, and ouabain were evaluated. Further examination of activators and inhibitors was also conducted in which DNP, L-cys, $NaN_3$, NaF, and IAA

were evaluated at 4, 5, 22, and 40 days post-fixation, with sea water as a reactivating agent for those incubations in the post-$R_t$ period.

7. Influence of reactivating agents on activator and inhibitor patterns. In these studies, the influence of reactivating agents on activator and inhibitor patterns in post-$R_t$ eggs was evaluated. The reactivating agents used were sea water, 0.01 M $KH_2PO_4$, 0.04 M $MgSO_4$, 0.01 M $CaSO_4$, and 0.5 M $NaH_2PO_4$. Documentation of a loss of activity in post-$R_t$ eggs was done by including an incubation which used distilled water instead of a reactivating agent. The activators and inhibitors, at final concentrations as above, were DNP, L-cys, NaF, $NaN_3$, and ouabain. The experiments were done at 14, 15, and 16 days post-fixation, respectively, as follows:

| Days post-fixation | Reactivating agents |
|---|---|
| 14 | none, sea water |
| 15 | $KH_2PO_4$, $MgSO_4$ |
| 16 | $CaSO_4$, $NaH_2PO_4$ |

# RESULTS

## A. Morphology of the sea urchin egg.

1. Whole mounted immature eggs. Immature forms were identified by their large pre-pronuclear nuclei (germinal vesicles). These eggs were commonly of a smaller diameter ranging from 61.5-67.5 μ. The non-nuclear ooplasm could be divided, although with less facility than in mature eggs, into cortical and medullary cytoplasm (corticoplasm and medulloplasm). The interface between these two regions, the cortico-medullary junction, was a poorly defined zone of gradual transition. The interface between the medulloplasm and the germinal vesicle, on the other hand, was a distinct boundary. The germinal vesicle was a round, 35.0 μ body which was eccentrically located within the medullo-plasm. The nucleoplasm was homogeneous throughout except for the 6.0-8.0 μ nucleolus (Figures 2 and 3 - see Appendix for Tables and Figures).

2. Whole mounted mature eggs. In contrast to the germinal vesicle stages, the mature eggs were larger, being about 70.0 μ in diameter with remarkably little variation. These eggs were uniformly spherical with a pronuclear configuration of their nuclei. The ooplasm was divisible into nuclear and non-nuclear portions only with considerable difficulty when using standard, or non-phase, optics. However, the delineation between cortico- and medulloplasm was clearer than in the immature eggs. The pronucleus most commonly was found within the deepest medulloplasm or at the boundary of the outer and inner medulloplasm, but never within the corticoplasm and was homogeneously agranular and anucleolate. Around neither immature nor mature eggs could jelly coats be identified (see Figures 2 and 3).

3. Whole mounted density-gradient centrifuged eggs. Only mature

eggs, as judged by size and nuclear morphology, were submitted to density-gradient centrifugation. By varying the conditions of centrifugation the eggs were either layered or stretched. The layered eggs retained their same general shape while the stretched eggs were elongated by the process.

Layered eggs were generally spherical except for some irregularity at the most centripetal aspect. The ooplasm was still divisible into nuclear and non-nuclear portions, although the same difficulty in localizing the nucleus as in the other mature eggs was found. Similarly, the non-nuclear portion of ooplasm was easily divided into corticoplasm and medulloplasm. The medulloplasm was greatly changed by the centrifugation process. The effect was one of layering the components of the medulloplasm within the boundaries formed by the oolemma and the corticoplasm. At the most centripetal pole the lipid hood (layer no. I) extended into the region of the corticoplasm and deformed the outer boundary of the egg by its presence. At the interface between the lipid hood and first granular layer (layer no. II) the nucleus was found. It was unchanged in size, shape, or consistency and appeared as a 7.0-10.0 $\mu$ spherical body which lay mostly within the first granular layer.

The first granular layer was distinctly separate from the corticoplasm, nucleus, and lipid hood which bounded it laterally and centripetally. The hyaline layer was a region of more or less clear cytoplasm interposed between the first and second granular layers. This layer had indistinct boundaries at either end but was clearly discontinuous from the cortical layer. The second granular layer (layer no. IV) shared an indistinct boundary with the hyaline layer, but laterally the corticoplasm and centrifugally the third granular layer formed definite bound-

aries. The most centrifugal layer, except for the centrifugal cap of corticoplasm, was the third granular layer (layer no. V). This layer was distinctly bounded by the second granular layer centripetally and the corticoplasm laterally and centrifugally.

By submitting the eggs to a stronger gravitational field, the ability to resolve the separate components of the medulloplasm was enhanced and the eggs were stretched in the region of layer no. III, the hyaline layer. Depending upon the particular egg, the stretching was moderate to great and produced both oblate and dumbbell-shaped cells. Occasionally, cells were fragmented into separated centripetal and centrifugal portions. When the elongated or stretched eggs were fixed immediately after centrifugation, they did not resume their spherical shapes.

It was remarkable, in particular, that no other discernable layers were produced by this treatment. The lipid hood bulged more conspicuously from the centripetal pole and the nucleus still lay partially within this layer. The first and second granular layers were more distincly separated from the hyaline layer, and the hyaline layer itself was variously elongated and appeared more clear and glassy. The third granular layer was located in the same position and was still composed entirely of the larger granules (Figures 4, 5, 6, and 7).

Further differentiation of the stratified medulloplasm was produced with the azure II-methylene blue stain. The effect upon the layered egg was not distinctive because the layered medulloplasm stained evenly throughout and usually all evidence of layering was obscured. The stretched centrifuged egg, however, differed in its staining reaction with the azure II-methylene blue stain by the differential staining

properties of the various strata in the medulloplasm. In both types of eggs the corticoplasm, lipid hood and nucleus were not stained.

In the stretched eggs the most intense staining occurred in the two poles of the egg with the greatest basophilia being in the centripetal pole. This pole, which contained primarily the first granular layer (layer no. II), was divided into two almost equal portions with the line of division normal to the gravitational axis, or the long axis of the cell. The most centripetal half was stained intensely while the centrifugal half was stained little, if at all. Furthermore, the interface between the two differently staining halves was sharp, whereas based upon strictly morphological criteria there was no such differentiation of the first granular layer possible. The hyaline layer (layer no. III) was not stained.

The centrifugal pole was evenly stained throughout but not with the same intensity as that observed in the centripetal half of the first granular layer. The staining was light and produced a slightly blue-green coloration among the yellowish granules. There was no differential staining between the second and third granular layers and the clear interface between the two was obscured (Figure 8). Detailed descriptions of the light microscopy of sea urchin eggs and oogenesis can be found elsewhere (7, 22).

4. Electron-microscopic morphology. The eggs were commonly irregular in general outline and the outer cell surface was folded outwards to form microvilli which were, for the most part, evenly distributed over the surface of the egg. Over the tips, and, to some extent, between the microvilli, there was a filamentous material which was not present on each egg but was uniformly distributed when present, and

which was probably the remnants of the jelly coat.

The oolemma, at lower magnification, appeared as an electron-dense layer of 300-400 Å thickness, but at higher magnification this outer boundary could be resolved into three 100 Å components; the fertilization membrane, a clear space, and the plasmalemma proper. Congruent with the light-microscopic findings, the ooplasm was divisible into cortical and medullary components. The most remarkable feature of the corticoplasm was the presence of the cortical granules. These varied in size from 2.0-4.0 μ and lay immediately subjacent to the oolemma. Within the 100 Å limiting membrane the substance of the cortical granules was composed of lamellar and amorphous components of variable extent and configuration. Among the other organelles found within the corticoplasm were ribosomal particles and 0.2-0.8 μ vesicles. The ribosomes were often arrayed in masses interposed between the cortical granules and in part between the cortical granules and the inner layer of the oolemma. These ribosomal masses were not of uniform density and had irregularly disposed, within a dense matrix, clumps of 50-100 granules.

The corticomedullary junction was indistinct, but there was a definite difference between the two components of the ooplasm. Whereas the cortex was distinguished by the presence of the cortical granules, the medulloplasm was marked by the presence of the yolk granules. The yolk granules varied from small spherical masses somewhat less than 1.0 μ in diameter to large elliptical bodies measuring 2.0 x 4.0 μ. Evenly distributed throughout the medulloplasm between the yolk granules were large numbers of mitochondria which were round to ellipsoidal bodies from 0.5-1.0 μ in maximum length and about 0.5 μ in width. Lipid droplets were also scattered throughout the medulloplasm, but were by no

means as common as the yolk granules or the mitochondria. These droplets were spherical, about 1.0 μ in diameter, usually highly electron dense, and without a limiting membrane. Groups of mitochondria often partially or completely surrounded single lipid droplets forming mitochondrial-lipid droplet complexes or rosettes.

Another major component of the medulloplasm was the endoplasmic reticulum which appeared for the most part as 0.2-2.0 μ empty vesicles bounded by granular or agranular membranes.

Besides the vesicular endoplasmic reticulum there was also a lamellar component. Part of the lamellar system was uniformly granular and, when present in lengths greater than 0.5 μ, almost invariably in the form of annulate lamellae. These annulate lamellae, while found separately throughout the cytoplasm, were more commonly associated with the heavy bodies (2, 5). The heavy bodies appeared as 0.8-1.0 μ circular masses of densely packed ribosomes around which the annulate lamellae were distributed, usually in sets of three, each up to 1.0-1.5 μ in length. The lamellae were arranged triangularly with the corners separated and thus forming an incomplete boundary. One of the limbs of the triangle was often missing or ectopically placed alongside one of the other annulate lamellae.

The agranular components of the lamellar membrane system were more commonly encountered within the medulloplasm than the granular. These structures consisted of stacked double membrane systems, with little intermembranous space, embedded in a dense but homogeneous matrix in the manner of Golgi complexes.

The only irregularly disposed component of the medulloplasm was the anucleolate nucleus. It appeared as highly irregularly shaped,

double membrane bounded body with its maximum dimension ranging from 5.0-15.0 μ. The folding of the limiting nuclear membrane was often such as to form complicated circular and linear "intra-nuclear" masses of membranes.

Glutaraldehyde fixed eggs varied from osmium fixed eggs only in the homogeneous nature of the ooplasmic matrix and the better preservation of membranes and membrane bounded organelles (Figures 9-13). More extensive descriptions of the ultrastructure with osmium fixation are presented by Afzelius (1, 2, 3, 4, 5), while Anderson (6), Jubinville (26), and Verhey (62) discuss the findings with aldehyde fixation.

B. Localization studies.

1. Controls, specificity, and AcPase activity. For the localization studies the standard incubation medium and procedure, with ATP as substrate, were used. In both the localization and the cytochemical studies controls, both denatured and without substrate, were consistently negative and there was never any nuclear localization of FRP (Table 1 and Figures 14 and 15). Deposits of PbS were never encountered with the use of specific enzymatic inhibitors such as L-cys and NaF. The reactions were usually first seen after one hour of incubation and changed only in intensity and not localization as the incubation was allowed to continue.

In eggs examined for AlPase and AcPase activity, FRP was seen only at pH 5.0 with beta-glycerophosphate as a substrate at 1, 2, and 3 hours and appeared as a homogeneously distributed, intense dark brown to black stain. A control reaction with ATP as substrate at pH 6.2 was used to show activity of the preparations and FRP was present in these eggs as a yellow-brown stain in the medullary portion of the egg at 2 and 3 hours.

The other two controls were both negative, under all conditions, at 3 hours (Table 2).

2. The frequency of the reactions. The cytochemical studies, with the exception of the studies on the influence of reactivating agents on FRP morphology, were neither designed nor conducted procedurally in a manner allowing a quantitative analysis of the frequency of the reactions. However, certain qualitative impressions were obtained in this regard and deserve to be mentioned.

In the case of mature and layered eggs, a reaction that worked did so in virtually all of the eggs with very few remaining unreactive. This was true with different buffers, pH, substrates, activators, inhibitors, and reactivating agents. However, in stretched eggs, approximately 80% exhibited FRP in the pre-$R_t$ period and approximately 50% were reactive in the post-$R_t$ period, no matter which of the reactivating agents were used.

In the case of the immature eggs, microcytes, and atypical reactions the situation was somewhat more complex. Of the germinal vesicle stages and microcytes, which could be definitely identified, about 3/4 appeared to be reactive. But among the mature forms, some appeared reactive in the standard manner, some atypically, and some not at all, and it was not possible to tell which group the unreacted eggs belonged to. However, as an approximation, about half of the mature eggs appeared reactive in the standard manner, a quarter atypically, and a quarter not at all.

3. Activity in mature eggs. In eggs incubated in the standard manner, with ATP as substrate, the FRP was localized exclusively intracellularly, almost always within the medulloplasm, and usually no closer than 5.0-8.0 $\mu$ to the oolemma. The FRP appeared as either discrete

0.5-2.0 μ black granules, as a relatively homogeneously distributed agranular yellow-brown or brown-black stain, or as a combination of the two. Most frequently the reaction occurred as a combination or merely the amorphous type, whereas the solely granular variety was relatively uncommon.

There was also an occasional egg in which there was some localization of FRP within the corticoplasm in either the granular or the amorphous forms. The granular type of corticoplasmic reaction, which was uncommon, appeared only in association with the granular-amorphous and never with a solely amorphous medulloplasmic reaction. These granules measured 3.0-5.0 μ, were dissociated from the medullary mass of FRP, lay immediately subjacent to or, more commonly, attached to the inner surface of the oolemma, and occurred singly in the egg. The amorphous corticoplasmic reaction was quite common, appeared in almost all eggs with an amorphous medulloplasmic reaction, and never in association with a reaction with granules present. The amorphous cortical reaction was usually weaker by one or two grades than the medullary one. Corticoplasmic reactions of either kind never occurred with a solely granular reaction (Figures 16-21).

4. Activity in density-gradient centrifuged mature eggs. The localization of FRP was also studied in density-gradient centrifuged eggs. Layered eggs were incubated during the pre-$R_t$ period only, whereas stretched eggs were evaluated both pre- and post-$R_t$ with a variety of reactivating agents. The controls, both denatured and without substrate, were uniformly non-reactive with the layered and stretched eggs alike and there was no nuclear localization of FRP.

In the layered eggs the medulloplasm was distinctly separated into

regions, as discussed in the morphological section above, but the localization of FRP within the medulloplasm of these eggs was not differential. The reaction was mostly amorphous, was usually 2-3+ in intensity, and there were granules occasionally present. On the other hand, solely granular rections were never seen. The corticoplasm and the lipid hood, whether it lay within the cortex or medulla, were uniformly devoid of FRP.

With increased centrifugal force, the layering of the medulloplasm was more distinct and the eggs were stretched at the hyaline layer and there was a resultant localization of FRP within the two poles of the stretched egg. The corticoplasm, the lipid hood, and the hyaline layer were non-reactive.

In the pre-$R_t$ stretched eggs, the reaction, in the centripetal pole, was present in the first granular layer. Furthermore, it was restricted within this layer to the centrifugal half and there was a sharp boundary between this and the unreactive centripetal half. The FRP always occurred as a very intense amorphous reaction.

The reaction in the centrifugal pole, in this same type of egg, was also always amorphous and of similar intensity. This reaction was present only in the second granular layer, and there was a distinct boundary between it and the unreactive third granular layer.

These localizations were the same in the post-$R_t$ stretched eggs after treatment with the reactivating agents, $KH_2PO_4$, $MgSO_4$, $CaSO_4$ or $NaH_2PO_4$. When sea water was used as a reactivating agent, however, the reaction was limited to the centrifugal pole and was of slightly lesser intensity than in the pre-$R_t$ eggs. Also, localization was not as clear since the boundary with the unreactive third granular layer

was less distinct and some FRP appeared within the corticoplasm adjacent to the second granular layer (Table 3 and Figures 22-27).

5. Activity in immature eggs and atypical reactions. In the mature eggs incubated with ATP, the localization of FRP was similar in all the studies undertaken. The reaction appeared almost wholly within the medulloplasm and could be granular, amorphous, or both in form. In the experiments on the influence of reactivating agents on activator and inhibitor patterns different FRP localizations appeared. With the $CaSO_4$ reactivation, activity was present in the germinal vesicle stages and microcytes and as atypical reactions (band and dot forms). With the $NaH_2PO_4$ reactivation only the dot forms of activity could be observed. Along with these atypical reactions and reactive immature forms, there were also standard reactions in apparently mature eggs. For all egg stages both types of controls were negative and there was no nuclear localization of activity.

In immature eggs the FRP was all cytoplasmically located with no deposit on their outer surfaces or in the germinal vesicles. FRP was present in both an amorphous and granular form with the granules being either 0.5-1.0 $\mu$ or 5.0 $\mu$ in size. There were usually no more than 3-4 of the 5.0 $\mu$ granules per egg, and these appeared to be attached to the inner surface of the oolemma. Although these granules were commonly round, they were often oblong, being up to 10.0-15.0 $\mu$ in length and 5.0 $\mu$ wide. On the inner surface of these granules, next to the corticomedullary junction, there were large masses of 0.5-1.0 $\mu$ granules which appeared to be fused with the larger mass of FRP.

The smaller (0.5-1.0 $\mu$) granules were also scattered throughout the cytoplasm, but not within the germinal vesicle, and thus were

found in both the cortex and the medulla. The cortically located granules were less common and were frequently immediately adjacent to the inner surface of the oolemma. The great majority of these small granules were located in the medulloplasm throughout the large amorphous mass of FRP, but they were never in association with the membrane of the germinal vesicle.

Almost all of the extra-nuclear portion of the medulloplasm was occupied by the amorphous FRP. This mass was eccentrically located, crescentic in shape, and was molded about half way around the large nucleus. The reaction was absent in the corticoplasm.

The band form of the atypical reaction was so named because of the shape of the amorphous FRP. It occurred in eggs which, when judged by size and nuclear configuration, were apparently mature. This band reaction occurred within the medulloplasm as a crescentic mass of FRP which extended its arms roughly half-way around the cell along the cortico-medullary junction, and which was slightly less intense than that observed in the germinal vesicle stages. Between these two arms, lying within non-reacted medulloplasm, lay the small 5.0-10.0 μ pronucleus. Since the preparations were whole mounts, these crescentic masses were often seen in views other than direct side view. When they were seen "end on" they appeared as an eccentrically located circular mass of amorphous FRP within the medulloplasm.

In the band reactions there was never any amorphous FRP localized in the corticoplasm, but there were granular forms of FRP of both sizes present. The smaller granules (0.5-1.0 μ) were not as common as in the germinal vesicle stages and were more often encountered over the region of the amorphous FRP. The large granules were encountered in the same

frequency, morphology, and distribution as in the germinal vesicle stages.

The other atypical reaction was the dot form which also occurred in apparently mature eggs. In this reaction type the FRP was granular and appeared both below the oolemma and within the medulloplasm. The medulloplasmic FRP occurred as a dense, concentrated mass of the 0.5-1.0 μ granules, most of which were within a 5.0-10.0 μ radius, in an eccentric position. There were also occasional small granules distributed throughout both components of the cytoplasm, but they were encountered with decreasing frequency as the distance from the central mass increased.

The other component of the granular FRP present in the dot forms was the 5.0 μ granules located immediately subjacent to the oolemma and attached to its inner surface. These were generally of a size and configuration similar to those found in the germinal vesicle stages and the band reactions, but were not as frequently encountered. These subooleммal granules were also frequently smaller, in which case they did not have the 0.5-1.0 μ granules associated with their inner aspects.

Along with these atypical FRP localizations, there were also reactive microcytes which were identified by their smaller size and lack of nuclei. The FRP in these small cells was diffusely spread throughout the cytoplasm without any apparent corticomedullary differentiation and was amorphous. The intensity of the reaction was never greater than 2+ and there were rarely a few small granules (0.5-1.0 μ) present, but never any large granules (Tables 9 and 10 and Figures 28-33).

C. Characterization studies.

1. Period of reactivity and reactivation. Since fixed and stored eggs had not been used previously, the viability of the system over a

period of time had to be evaluated. In the initial assay, eggs were incubated in the early post-fixation period at 1, 2, 3, and 4 days post-fixation. The reaction was first clearly detectable at 2 hours at 1 day post-fixation; thereafter, it steadily increased in intensity up to 3 days post-fixation, and then became less intense and appeared later at 4 days post-fixation. Incubations done at 29 days post-fixation failed to demonstrate any activity at all.

This loss of activity was then studied to determine exactly when the event occurred. The results again indicated an increase in intensity of reaction after the first day, a waning at 4 days, and a total absence of activity thereafter.

In another group of similarly prepared eggs there was still a strong reaction present at 4 days post-fixation, but this was subsequently absent at 5 and 9 days post-fixation. This indicated that the time of loss of activity, designated $R_t$, occurred between 4 and 5 days post-fixation. Therefore, the pre-5 day period was designated the pre-$R_t$ period and the period after 5 days the post-$R_t$ period (Table 4). By rinsing the eggs in sea water until all storage buffer was removed, storing in sea water overnight at 4° C. for 12-16 hours, and then incubating in the standard manner, the reaction could be reinstated to the same intensity as in the 3 day post-fixation eggs. Even with this treatment, however, the intensity began to wane at 34 days post-fixation until at 67 days post-fixation the reaction was absent.

After these studies, an attempt was made to identify the component or components of sea water responsible for the reactivation phenomenon. These experiments considered the effectiveness of selected reactivating agents from 4 to 67 days post-fixation. It was found that 0.5 M NaCl

prevented reaction in the pre-$R_t$ period and failed to reactivate the eggs in the post-$R_t$ period. Contrarily, 0.5 M $NaH_2PO_4$ would reactivate eggs in the post-$R_t$ period and would not prevent a reaction in the pre-$R_t$ period. Between the two potassium salts, 0.01 M KCl prevented a reaction in the pre-$R_t$ period and was associated with only a 1+ reaction in the post-$R_t$ period. On the other hand, 0.01 M $KH_2PO_4$ did not prevent a reaction in the pre-$R_t$ period and was the best reactivating agent up to as long as 67 days post-fixation. Of the two magnesium salts, both of which were good reactivating agents, 0.04 M $MgSO_4$ was more consistent in the results obtained than 0.04 M $MgCl_2$. The two calcium salts (0.01 M $CaSO_4$ and $CaCl_2$), likewise, were good reactivating agents, but there appeared to be little difference between the two. Using sea water as a reactivating agent never resulted in a reaction stronger than 3+, but it was consistent in its action. All agents used showed a waning of reactivating ability after 40 days post-fixation, but the 0.01 M $KH_2PO_4$ reactivations seemed to be affected the least (Table 5).

2. Substrate specificity. For these experiments the standard incubation medium and experimental procedure were used except that different substrates, all at 125.0 mg.% concentration, were substituted for ATP. With each set evaluated an incubation with ATP as substrate was included to assure that activity toward ATP was present and that results could therefore be compared. The reactivating agent for reactions carried out in the post-$R_t$ period was sea water.

In the pre-$R_t$ period, ATP, ADP, CTP, CMP, GTP, GMP, TTP, TDP, ITP, IMP, and UMP were all active substrates, whereas the others evaluated were not. UTP was not evaluated in the pre-$R_t$ period because it was

not available then, but it was active at 20 and 36 days post-fixation. Of the substrates demonstrating activity in the pre-$R_t$ period, CMP, GTP, GMP, IMP and UMP were not available for analysis in the post-$R_t$ period, but all others, except CTP, were active up to 36 days post-fixation. The activity with GTP, ADP and TDP was less than that observed with the other triphosphates while GMP, IMP and UMP produced an intense reaction when used as substrates.

Those substrates which did not show activity in the pre-$R_t$ period were CDP, GDP, IDP, AMP, TMP, whereas G1P, G6P, ThPOP, $\alpha$GP, and $\beta$GP did not show activity in either the pre- or post-$R_t$ period (Table 6).

3. Activators and inhibitors. The first experiment indicated activity without any agents in the medium, but none with the sulfhydryl containing agents L-cysteine (L-cys) and glutathione (GSH). Conversely, sulfhydryl antagonizing agents such as p-chloromercuribenzoic acid (pCMB) and mersalyl acid (MA) allowed activity, although it was reduced in intensity and time of appearance. Dinitrophenol (DNP) and iodoacetamide (IAA) appeared to stimulate the earlier appearance of reaction product, but did not seem to increase the intensity of the reaction. With sodium tartrate (NaT) and zinc chloride there was no reaction. Ouabain was also evaluated and did not inhibit the system (Table 7).

In the second analysis of the effect of activators and inhibitors, careful attention was paid to eggs in the pre- or post-$R_t$ periods. The agents evaluated were DNP, L-cys, sodium azide ($NaN_3$), sodium fluoride (NaF), and IAA. With the use of $NaN_3$, NaF, and IAA there was no reaction in the pre-$R_t$ period, while with L-cys and NaF there was none in either the pre- or the post-$R_t$ periods. The only agent with which there was a reaction in the pre-$R_t$ period was DNP and it was of the same intensity

as the standard reaction controls but appeared only at 3 hours. In the post-$R_t$ period the reaction with DNP appeared earlier and was of greater intensity than the standard incubation. However, at 40 days post-fixation this effect with DNP had waned so much that only a questionable reaction was produced while the reaction in the standard control was still strong.

Treatment with the other two agents, $NaN_3$ and IAA, similarly appeared to allow the reaction to occur in the post-$R_t$ period. With $NaN_3$ there was not much difference from the standard control, while with IAA there appeared to be a slight increase in the intensity of the reaction in the early post-$R_t$ period. The effect of both of these agents appeared to wane by 40 days post-fixation although there was still some reaction present in the eggs treated with IAA (Table 8).

4. Influence of reactivating agents on activator and inhibitor patterns. As with the other activator and inhibitor studies, there was no reaction seen with L-cys or NaF treatment, either with or without reactivation. Without reactivation, at 14 days post-fixation, strong reactions were seen with DNP and ouabain treatment, while a weak reaction was observed with $NaN_3$ treatment. With sea water reactivation, at 14 days post-fixation, this same reaction pattern persisted, although the intensity of the reaction was decreased with DNP and was increased with $NaN_3$ (Table 9). Reactivation with $KH_2PO_4$, at 15 days post-fixation, demonstrated a moderate reaction with DNP and $NaN_3$, while none was seen with ouabain. Conversely, with $MgSO_4$ reactivation at 15 days post-fixation, ouabain treated eggs were the only ones which reacted at all, and then only moderately. However, with $CaSO_4$ and $NaH_2PO_4$ reactivation at 16 days post-fixation, strong reaction patterns were seen with DNP,

$NaN_3$, and ouabain treatments (Table 10).

All of these above reactions occurred with the normal medullary localization and in eggs which were ostensibly mature according to size and nuclear configuration. However, in these experiments not all eggs were mature by morphological characteristics, some being germinal vesicle stages and some differing in size from both the mature and immature forms. In addition, there were also eggs which appeared to be mature, but which did not have the characteristic medullary reaction of the mature eggs.

The larger and smaller eggs were termed macrocytes when the average diameter was about 100 μ (range 90-110 μ) and microcytes when the average diameter was about 50 μ (range 45-65 μ) and nuclei could not be identified in either. The eggs presumed to be mature with atypical reaction types were classified as either band or dot forms. The reactions in immature and irregularly sized forms and the atypical reactions appeared only after 3 hour incubations.

The case of the macro- and microcytes is the most simple in that reactions were never seen in the macrocytes and in the microcytes only after $CaSO_4$ reactivation and $NaN_3$ treatment. Only about 3/4 of the microcytes appeared to be reactive and the reaction product appeared diffusely spread throughout the eggs without corticomedullary differentiation.

Of the two varieties of atypical reactions, whose appearance was described in the localization studies, the band type reaction appeared only after $CaSO_4$ reactivation and ouabain treatment. The dot reaction, however, appeared after both $CaSO_4$ and $NaH_2PO_4$ reactivation and with DNP, $NaN_3$ and ouabain treatment.

The reactions in the germinal vesicle stages, which were also described in the section on localization, were present only with $CaSO_4$ reactivation and after DNP, $NaN_3$ and ouabain treatment. Since the reactions in the immature forms and the atypical reactions differed from the medullary reactions in mature eggs, they were not graded as to intensity, but recorded as present (+) or absent (-) (Table 10).

# DISCUSSION

These studies revealed for the first time the localization of nucleoside phosphatase activity with ATP as substrate in the eggs of Strongylocentrotus purpuratus. Activity had previously been demonstrated both biochemically (36, 37, 43, 55) and cytochemically (13, 14, 36) in other species of sea urchins, but had only been shown biochemically in the S. purpuratus eggs (12).

The incubation times used were considerably shorter than those used by Dalcq (13, 14) and the same as those recorded by Miki (36). Final reaction product was never encountered in either substrate-free or denatured controls, was absent with the use of specific enzymatic inhibitors such as L-cys and NaF, and was never nuclear in localization. The reaction was usually visible after a 1 hour incubation and changed only in intensity and not localization as the incubation was allowed to proceed further. These findings agreed with generally accepted criteria of true enzymatic activity demonstrated histochemically, as discussed by Lillie (29) and Pearse (50).

One of the ways chosen to study some of the characteristics of the preparation was the analysis of the substrate specificity as suggested by Novikoff (39). Hydrolysis of substrate by non-specific acid phosphatase was excluded by lack of activity with either α- or β-glycerophosphate as substrate at pH 6.2. This lack of activity and the demonstration of activity with β-glycerophosphate at pH 5.0 agreed with Dalcq's (13, 14), Pasteels' (47, 48, 49), and Miki's (36) cytochemical, and Gustafson's (21) and Jackson's (25) biochemical description of AcPase activity in the sea urchin egg. Alkaline phosphatase was similarly excluded by the absence of activity at pH 9.0 which concurred

with Pfohl's (52) findings that this enzyme was present only in minute quantities before gastrulation in sea urchin eggs. Hexose phosphatase, organic and inorganic phosphatase activity was not present at pH 6.2 and had not been previously described in sea urchin eggs. These data, along with the absence of activity with AMP as substrate and the difference between the reactions with ADP and ATP indicated that the enzymatic hydrolysis was probably acting upon the terminal phosphate of ATP when it was used as substrate.

There was, however, activity with many other nucleoside tri-, di-, and monophosphates as had been reported previously by Dalcq (13, 14). The differences observed with GTP, CTP, ADP, TDP, and CMP and the intense reactions with GMP, IMP, and UMP as substrates made it impossible to distinguish clearly between the nucleoside tri-, di-, and monophosphatases as had been done with mammalian tissues (15, 17, 42). Consequently, the activity demonstrated cytochemically with ATP as substrate could not be termed ATPase as Dalcq (13, 14) and Miki (36) had done, but rather was more accurately described as a nucleoside phosphatase activity. In spite of these objections, it was possible to describe the characteristics and the localization of activity in the system with ATP as substrate.

Additional characterization of the preparation was done by determining which agents served as activators and inhibitors of the enzyme activity. This approach has been used by Novikoff (39, 41), Maengwyn-Davies (33), and Padykula (44) in mammalian tissues and by Ohnishi (43) in sea urchin eggs with considerable success. The results obtained with the sulfhydryl containing agents, such as L-cysteine and glutathione, and with sulfhydryl antagonists, such as p-chloromercuribenzoic and

mersalyl acids, indicated that the activity was inhibited by the presence of sulfhydryl groups. These results along with the inhibition with $ZnCl_2$ indicated a similarity to AlPases (11, 29, 50), whereas the inhibition with tartaric acid indicated a similarity to certain AcPases (11), although the results in the substrate specificity studies indicated that the activity was not due to such phosphatases acting either directly or indirectly. In addition, dinitrophenol, iodoacetamide, and ouabain stimulated the earlier appearance of FRP, which was the opposite of the results with these agents reported by Ohnishi for another sea urchin ATPase (43). The results with the selected agents used to describe the response of the system over a period of time suggested the existence of more than one enzyme acting upon ATP in the pre- and post-$R_t$ periods.

In the study with activators and inhibitors which suggested the presence of multiple enzymatic activities, sea water had been used to reinstate activity in the post-$R_t$ period. The period of reactivity and reactivation studies had also suggested the presence of more than one enzymatic activity by the variation in response to the cations. Since the reactivation had been produced by sea water, which is a poly-electrolyte solution, it was necessary to re-evaluate the effects of activators and inhibitors when reactivation was induced by specific cations.

The intent was to activate each enzyme separately, if indeed there were more than one, and identify them by their responses to the activators and inhibitors. For this purpose NaF and L-cysteine were used again, since they had appeared to result in inactivation of all activity following sea water reactivation. Ouabain was substituted for iodoacetamide since the former appeared to have a specific relationship to

the Na-K-ATPase of sea urchin eggs (43) and vertebrate tissues (59), and since my results with this agent in the pre-$R_t$ period were at odds with those for sea urchin egg ATPase reported by Ohnishi (43). The experiments were all done in the post-$R_t$ period and an incubation without the reactivation step was included since in this series there clearly would be no FRP present without activation of the enzyme.

In the standard reaction the appearance of activity following treatment with dinitrophenol, $NaN_3$, and ouabain, when no reactivating agent was used, indicated that these agents were activating the enzymatic hydrolysis. Thus, the system was different from the Na-K-ATPase of sea urchin eggs reported previously (43) which was inhibited by these compounds. This ouabain stimulation appeared to be real and had been described in vertebrate systems (45, 46, 53).

In general, the response to the various agents was the same as seen previously when sea water, $CaSO_4$, and $NaH_2PO_4$ were used as reactivating agents, whereas with $KH_2PO_4$ and $MgSO_4$ the results were quite different. Hence, on the basis of the patterns of response to the activators and inhibitors, the enzymatic activity was divisible into K+-sensitive, Mg++-sensitive, and Ca++-Na+-sea water-sensitive types. The latter variety may have represented two separate types, Ca++- and Na+-sensitive, respectively; or a Ca-ATPase which was responsive to Na+, as had been reported in vertebrate tissue (56); or a Na-ATPase which was responsive to Ca++, which would have been in contradiction to the results obtained in sea urchin eggs (43), although it had been seen in vertebrate tissues (56).

Miki (36) found that her preparation was Mg++ stimulated and Ca++ and K+ inhibited. This was similar to Ohnishi's (43) cortical ATPase

which was Na+-K+ dependent and inhibited by ouabain in the manner of classical Na-K-ATPases of mammals (59). On the other hand, Conners and Scheer (12), working with S. purpuratus egg homogenates, described a Ca++ stimulated and Mg++ inhibited ATPase system. The difference between these results and those reported here probably reflect the preferential extraction or demonstration of one enzyme system in the biochemical preparations and the presence of all systems in the cytochemical method. This concept is further strengthened by Ricotta's finding (55) of Mg++ activation and inhibition of ATPase activity depending upon the fraction of the sea urchin egg assayed.

The impressions of the frequency of occurrence of the different reactions were subjective ones because, for the most part, the experiments were neither designed nor conducted for a statistical analysis. The standard reaction in both the mature uncentrifuged and layered eggs occurred in almost all of the eggs regardless of the buffer, pH, substrate, activators, or reactivating agents used. Thus, except late in the post-$R_t$ period, there was no problem in determining if a reaction had occurred. The fact that the frequency of standard reactions was less for stretched eggs, especially when evaluated post-$R_t$, probably indicated a result of harsher treatment of the eggs rather than a loss in reliability of the reaction system.

Of the atypical reactions, those in the germinal vesicle stages occurred only after 3 hour incubations, under special conditions, and only in about 3/4 of the eggs. This suggested that the incubation medium was suboptimal for these eggs, although still capable of producing reliable and reproducible results, and indicated a difference between the enzymatic activity in the less mature and more mature eggs. The

other atypical reactions, the band and dot types, occurred in about 1/4 of the mature type eggs present. Of the other 3/4 of this variety of egg, 1/2 appeared reacted in the standard manner, while the remainder were unreactive. This unreactive group may very well have belonged among the atypical types since the reaction system was probably also suboptimal for them.

The standard reaction of the mature egg was entirely intracellularly located and was diffusely spread throughout the medulloplasm without any activity demonstrable in the nucleus and little in the corticoplasm. It did not seem likely that this medulloplasmic localization merely represented the activity remaining after the acetic acid rinse since there was some corticoplasmic activity observed. This observation, along with the fact that the jelly coat was quite soluble in acids (22), indicated that the non-specific activity was located in the remnants of the jelly layer, and that it was these lead impregnated remnants that were being removed by the acid rinse rather than the lead itself. Consequently, it appeared that the observed localization of enzymatic activity was real, that the technique demonstrated a medullary nucleoside phosphatase, which had been observed in other species by Dalcq (13, 14) and Miki (36), and that there was little cortical activity present, in contrast to results found by Miki (37) and Ohnishi (43) in biochemical studies of other species of sea urchins.

The homogeneous distribution of the FRP throughout the medulloplasm was consistent with the homogeneous distribution of the organelles in this region of the egg as observed elsewhere (2, 3, 4, 5, 6, 41). Thus, in speculating with which organelles the enzymatic activity might be associated, it was apparent that it was not in the cortical

granules or in the nuclei and that it was associated with part or all of the medulloplasmic organelles, i.e., the yolk granules, the vesicles, the lipid droplets, the mitochondria, and the smooth and rough components of the endoplasmic reticulum. The nature of the FRP gave no definite indication as to the organelles with which the activity was associated.

Further resolution of the localization of activity in whole mounts was obtained with the use of density-gradient centrifuged eggs. This procedure had demonstrated that the components of the medulloplasm could be separated in vivo on the basis of their density, as revealed by light and electron microscopic studies (20, 22, 28, 47, 48, 49). The FRP in both the layered and the stretched eggs was again all intracellularly located within the medulloplasm, but in the layered egg there was an incomplete separation of the components and no differential localization of activity. In the stretched eggs, however, the activity was differentially localized within the strata of the medulloplasm and was of such an intensity as to suggest concentration of the enzyme in certain regions. There was no activity in the corticoplasm, lipid droplets, or the nucleus; nor in the centripetal half of the first granular layer in the centripetal pole (which in other species was high in AcPase activity and contained the Golgi complexes)(14, 47, 48, 49); nor in the hyaline zone, which probably contained atypical tubular endoplasmic reticulum, annulate lamellae, and heavy bodies (14, 47, 48, 49). The third granular layer in the centrifugal end of the centrifugal pole, which contained the mitochondria (14, 20, 22, 47, 48, 49), was also unreactive, although a mitochondrial ATPase with maximum activity at pH 6.2 had been demonstrated biochemically in the eggs of another sea urchin by Ricotta (55). Thus, the possibilities of localization of activity among the medullo-

plasmic organelles was considerably reduced by this study and it appeared that the activity was either associated with the yolk granules or the typical endoplasmic reticulum. Dalcq (13) found that such activity might very well be associated with the yolk granules, or their derivatives, the multivesicular bodies, although experience with mammalian tissues stressed the association of ATPases with the membranous systems of the cell, particularly the endoplasmic reticulum (40).

The atypical reactions allowed correlations to be made between the specific enzymatic activities identified and the localization of FRP in certain eggs. It was evident that the reactions in the germinal vesicle stages and those in the mature eggs that were typically reactive represented the types of localization of activity found at the extremes of the segment of oogenesis present. The atypical reactions occurring in eggs which appeared to be mature then may have represented intermediate stages in development.

Thus, a developmental sequence could be hypothesized in which the germinal vesicle stage came first and contained a $Ca^{++}$-sensitive enzyme which was activated by dinitrophenol, $NaN_3$, and ouabain. The next step would be the band reaction, which was essentially a germinal vesicle type of reaction without the presence of a germinal vesicle, and which was due to a ouabain activated $Ca^{++}$-sensitive enzyme. After this the dot type reaction would follow in which the enzymatic activity was sensitive to both $Ca^{++}$ and $Na^{+}$, responded in a standard pattern to the activators and inhibitors, and might have been due to 2 enzymes. Finally, in the mature egg, the activity was due to a $K^{+}$, a $Mg^{++}$, and a $Ca^{++}$-$Na^{+}$-sensitive component of which the latter might have represented 2 separate enzymes, especially in view of the separate $Ca^{++}$-system present

in the immature eggs (Table 11). The reacted microcytes and unreacted macrocytes presumably represented eggs wasted in the course of oogenesis. Regardless of the theory, however, there were definable changes occurring in nucleoside phosphatase activity during the portion of oogenesis examined, and there appeared to be separate nucleoside triphosphatase systems in immature and mature eggs, even though both types were inhibited by L-cysteine and NaF.

The data presented have dealt with only a small fraction of oogenesis and the characteristics of activity with one substrate. A more thorough analysis of the other substrates and the gametes from oogonium to mature ova could easily be done using whole mounts. In addition, a considerable amount of information probably could be obtained in regard to this system if it could be adapted so that sections of the eggs could be obtained for light microscopic evaluation. Such a technique could then also be applied to the evaluation of cytochemical changes during the course of oogenesis. A quantitative study of these changes could easily be undertaken using either whole mounts or sectioned eggs.

Once such preliminary cytochemical work had been accomplished, it would then be timely to examine the biochemical characteristics of the system. Such work would include the determination of Michaelis-Menton constants, pH and substrate concentration maxima, cofactors and their concentration maxima, and the effects of activators and inhibitors. These data could be determined from both whole egg homogenates and subcellular fractionations at various stages of oogenesis.

The areas for investigation discussed above would form a logical basis for detailed analysis of localization of enzymatic activity throughout the course of oogenesis with attention directed toward both

light and electron microscopic studies. Once having obtained more information regarding the system during oogenesis, it would then be appropriate to apply the same techniques to the analysis of development after fertilization.

## SUMMARY

The demonstration of nucleoside phosphatase activity was consistent and reproducible when the pH and the Mg++ concentration of the incubation medium were changed from those in the original Wachstein-Meisel medium to 6.2 and 0.0005 M, respectively. This activity was lost in the early post-fixation period, but was reinstated by treatment with sea water or solutions of its major ionic components. The characterization studies indicated that ATP and other nucleoside tri-, di-, and monophosphates were reactive and that the activity was most accurately described as a nucleoside phosphatase. This activity was inhibited by L-cysteine, glutathione, NaF, sodium tartrate, and $ZnCl_2$; probably not inhibited by p-chloromercuribenzoic and mersalyl acids; and stimulated by 2,4-dinitrophenol, $NaN_3$, iodoacetamide, and ouabain. Other studies indicated, on the basis of reaction patterns of eggs treated with activators and inhibitors and selected reactivating agents, that there were at least three separate enzymatic activities. Of these activities, the first two were sensitive to K+ and Mg++, respectively, while the third responded to both Na+ and Ca++ and may have represented two separate enzymes. These separate activities were associated with particular localizations and led to the hypothesis of changing enzymatic patterns and localizations during the course of oogenesis.

In the whole mounts of mature eggs, the activity in the medulloplasm suggested many possible organellar localizations. The results in the density-gradient centrifuged stretched mature eggs, however, indicated that this activity probably was not associated with the lipid droplets, nuclei, Golgi complexes, atypical endoplasmic reticulum, heavy bodies, annulate lamellae, or mitochondria, but rather with the yolk

granules or the typical endoplasmic reticulum.

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# APPENDIX

The appendix compares the incubation medium of the study with the original Wachstein-Meisel technique and contains the data presented in the Results in tabular and pictorial form. The tables are concerned with the characterization of the enzymatic activity while the figures demonstrate its localization.

## A. Incubation media.

1. Wachstein-Meisel:

| | | Final concentration |
|---|---|---|
| 125 mg.% di-sodium-ATP | 20.0 ml. | 0.00083 M |
| 0.2 M tris-HCl buffer at pH 7.2 | 20.0 ml. | 0.08 M |
| 2.0% $Pb(NO_3)_2$ | 3.0 ml. | 0.0036 M |
| 0.1 M $MgSO_4$ | 5.0 ml. | 0.01 M |
| Distilled water | 2.0 ml. | |
| Total volume | 50.0 ml. | |

2. Modified Wachstein-Meisel (medium of study):

| | | Final concentration |
|---|---|---|
| 125 mg.% di-sodium-ATP | 20.0 ml. | 0.00083 M |
| Buffer | 20.0 ml. | |
| 2.0% $Pb(NO_3)_2$ | 3.0 ml. | 0.0036 M |
| 0.005 M $MgSO_4$ | 5.0 ml. | 0.0005 M |
| Distilled water | 2.0 ml. | |
| Total volume | 50.0 ml. | |

B. Tables.

Table 1.

With the Michaelis buffered incubation medium at pH 6.2 with 0.0005 M $Mg^{++}$ eggs demonstrate activity with ATP as substrate. EtOH controls are those denatured by ethanol treatment and w/o controls are those incubated without substrate.

Table 2.

Activity with beta-glycerophosphate as a substrate is only present at pH 5.0. Activity is also demonstrated towards ATP, but only at pH 6.2. There is no activity with alpha-glycerophosphate as substrate.

Table 1.

Incubation in the standard reaction system.

| Substrate | Incubation time (hours) Experimental with substrate 1 | 2 | 3 | Controls EtOH 3 | w/o 3 |
|---|---|---|---|---|---|
| ATP | 1+ | 2+ | 4+ | - | - |

Table 2.

Alkaline and acid phosphatase activity.

| Incubation medium Substrate | pH | Incubation time (hours) Experimental with substrate 1 | 2 | 3 | Controls EtOH 3 | w/o 3 |
|---|---|---|---|---|---|---|
| ATP | | - | 2+ | 4+ | - | - |
| ∝GP | 6.2 | - | - | - | - | - |
| β GP | | - | - | - | - | - |
| ∝GP | | - | - | - | - | - |
| β GP | 9.0 | - | - | - | - | - |
| ∝GP | | - | - | - | - | - |
| β GP | 5.0 | 2+ | 4+ | 4+ | - | - |

Table 3.

Tabular presentation of data from experiments demonstrating activity in density-gradient centrifuged eggs. Notice that polar localization of FRP, in the pre-$R_t$ period, in layered eggs is not possible, but is present when the eggs are stretched. In the post-$R_t$ period FRP appears only in the centrifugal pole with sea water reactivation, while it is seen in both poles with the other reactivating agents. CPP: centripetal pole; CFP: centrifugal pole.

Table 3.

Activity in density-gradient centrifuged eggs.

| Days post-fixation | Substrate | Reactivating agent | Egg type | Incubation time (hours) Experimental with substrate 3 | Pole localization CPP | Pole localization CFP | Controls EtOH 3 | Controls w/o 3 |
|---|---|---|---|---|---|---|---|---|
| 3 | ATP | none | layered | 4+ | - | - | - | - |
| | | | stretched | 4+ | + | + | - | - |
| 9 | ATP | 0.01 M $KH_2PO_4$ | stretched | 3+ | + | + | - | - |
| | | 0.04 M $MgSO_4$ | | 2+ | + | + | - | - |
| | | 0.01 M $CaSO_4$ | | 3+ | + | + | - | - |
| | | 0.5 M $NaH_2PO_4$ | | 4+ | + | + | - | - |
| | | sea water | | 3+ | - | + | - | - |
| | | none | | - | - | - | - | - |

Table 4.

The asterisks indicate reactions done with a different batch of eggs. Although the duration of activity varies slightly between the two batches of eggs, it can be seen that after the 4th day post-fixation there is no demonstrable activity in either group of eggs. Consequently, $R_t$, or the time of loss of activity, occurred between 4 and 5 days post-fixation.

Table 4.

Determination of time of loss of activity.

| Days post-fixation | Substrate | Incubation time (hours) Experimental with substrate 1 | 2 | 3 | Controls EtOH 3 | w/o 3 |
|---|---|---|---|---|---|---|
| 1 | ATP | ± | 1+ | 3+ | - | - |
| 2 | | - | 2+ | 4+ | - | - |
| 3 | | - | 2+ | 4+ | - | - |
| 4 | | - | 2+ | 2+ | - | - |
| 4* | | - | 2+ | 4+ | - | - |
| 5* | | - | - | - | - | - |
| 6 | | - | - | - | - | - |
| 9 | | - | - | - | - | - |
| 9* | | - | - | - | - | - |
| 14 | | - | - | - | - | - |
| 28 | | - | - | - | - | - |
| 29 | | - | - | - | - | - |

Table 5.

Tabular presentation of a synopsis of data from the analysis of reactivating agents. The effect of the individual salts over the time period of the experiment can be seen. Of all the salts, $KH_2PO_4$, $MgSO_4$, and $CaSO_4$ appear to be the most efficacious reactivating agents. Blanks in the table indicate that the agents were not evaluated at that time.

Table 5.

Synopsis of data from analysis of reactivating agents.

| Reactivating agent | Reaction measured after 3 hour incubation. Days post-fixation: 4 | 9 | 22 | 30 | 40 | 62 | 67 |
|---|---|---|---|---|---|---|---|
| 0.5 M NaCl | - | - | - | - | - | - | - |
| 0.5 M $NaH_2PO_4$ | 4+ | 4+ | 4+ | | | | |
| 0.01 M KCl | - | 1+ | ± | 1+ | ± | 1+ | 1+ |
| 0.01 M $KH_2PO_4$ | 3+ | 3+ | 4+ | 4+ | 4+ | | 2-3+ |
| 0.04 M $MgSO_4$ | 4+ | 4+ | 4+ | 4+ | 4+ | 1+ | 2+ |
| 0.04 M $MgCl_2$ | 4+ | 2+ | 2+ | 4+ | 1+ | | - |
| 0.01 M $CaCl_2$ | 4+ | 2+ | 3+ | 4+ | 4+ | 1+ | 1+ |
| 0.01 M $CaSO_4$ | 4+ | 2+ | 4+ | 4+ | 4+ | | 1+ |
| sea water | 2+ | 2+ | 1+ | 3+ | 3+ | ± | ± |

Table 6.

Tabular presentation of a synopsis of the data from the substrate specificity studies. Blanks in the table represent times when the particular substrates were not evaluated. The asterisks (*) indicate that the substrates were not available.

Table 6.

Synopsis of data from substrate specificity studies.

| Substrate | Reaction at 3 hours Days post-fixation 2 | 3 | 16 | 20 | 36 | 40 |
|---|---|---|---|---|---|---|
| ATP | 4+ | 4+ | | 3+ | 2+ | 2+ |
| ADP | | 2+ | | 1+ | 1+ | |
| CTP | | 4+ | | - | | - |
| CMP | 2+ | * | * | * | * | * |
| GTP | | 1+ | * | * | * | * |
| GMP | 3+ | * | * | * | * | * |
| TTP | 4+ | | | 1+ | 2+ | 2+ |
| TDP | | 2+ | | 1+ | 1+ | |
| ITP | | 4+ | | 1+ | 1+ | |
| IMP | 4+ | * | * | * | * | * |
| UTP | * | * | * | 3+ | 3+ | * |
| UDP | * | * | 2+ | * | * | * |
| UMP | 4+ | * | * | * | * | * |
| CDP | - | | | | | |
| GDP | - | | | | | |
| IDP | - | | | | | |
| AMP | - | | | | | |
| TMP | - | | | | | |
| G1P | | - | | | - | |
| G6P | | - | | | - | |
| ThPOP | | - | | | - | |
| NaPOP | | - | | | - | |
| ∝GP | | - | | | - | |
| β GP | | - | | | - | |

Table 7.

Tabular presentation of data from the first analysis of activators and inhibitors. Sulfhydryl containing agents, such as L-cysteine (L-cys) and glutathione (GSH) appear to inhibit the reaction, while sulfhydryl antagonists, such as p-chloromercuribenzoic acid (pCMB) and mersalyl acid (MA), probably do not. Dinitrophenol (DNP) and iodoacetamide (IAA) stimulate a more intense earlier appearance of FRP, while there is no reaction with tartaric acid (TA) or zinc chloride treatment. Ouabain does not appear to be an inhibitor.

Table 7.

Analysis of activators and inhibitors-I.

| Days post-fixation | Substrate | Reactivating agent | A/I agent | Incubation time (hours) Experimental with substrate 1 | 2 | 3 | Controls EtOH 3 | w/o 3 |
|---|---|---|---|---|---|---|---|---|
| 40 | ATP | sea water | L-cys | - | - | - | - | - |
| | | | DNP | 2+ | 2+ | 2+ | - | - |
| | | | GSH | - | - | - | - | - |
| | | | pCMB | - | - | 1+ | - | - |
| | | | IAA | 2+ | 2+ | 2+ | - | - |
| | | | MA | ± | ± | 1+ | - | - |
| | | | NaT | - | - | - | - | - |
| | | | $ZnCl_2$ | - | - | - | - | - |
| | | | none | 1+ | 1+ | 2+ | - | - |
| 2 | ATP | none | ouabain | - | 2+ | 4+ | - | - |
| | | | none | 1+ | 2+ | 4+ | - | - |

Table 8.

Data from the second analysis of activators and inhibitors. Notice that the patterns of activity change throughout the course of the experiments.

Table 8.

Analysis of activators and inhibitors-II.

| Days post-fixation | Substrate | Reactivating agent | A/I agent | Incubation time (hours) Experimental with substrate 1 | 2 | 3 | Controls EtOH 3 | w/o 3 |
|---|---|---|---|---|---|---|---|---|
| 4 | ATP | none | DNP | - | - | 3+ | - | - |
| | | | L-cys | - | - | - | - | - |
| | | | $NaN_3$ | - | - | - | - | - |
| | | | NaF | - | - | - | - | - |
| | | | IAA | - | - | - | - | - |
| | | | none | - | 2+ | 3+ | - | - |
| 5 | ATP | sea water | DNP | 1+ | 2+ | 3+ | - | - |
| | | | L-cys | - | - | - | - | - |
| | | | $NaN_3$ | - | 1+ | 2+ | - | - |
| | | | NaF | - | - | - | - | - |
| | | | IAA | - | 2+ | 3+ | - | - |
| | | | none | - | - | 2+ | - | - |
| 22 | ATP | sea water | DNP | 1+ | 2+ | 2+ | - | - |
| | | | L-cys | - | - | - | - | - |
| | | | $NaN_3$ | - | 1+ | 2+ | - | - |
| | | | NaF | - | - | - | - | - |
| | | | IAA | - | 1+ | 1+ | - | - |
| | | | none | - | ± | 2+ | - | - |
| 40 | ATP | sea water | DNP | - | - | ± | - | - |
| | | | L-cys | - | - | - | - | - |
| | | | $NaN_3$ | - | - | - | - | - |
| | | | NaF | - | - | - | - | - |
| | | | IAA | - | - | 1+ | - | - |
| | | | none | - | - | 2+ | - | - |

Table 9.

Tabular presentation of data from the study on the influence of reactivating agents on activator and inhibitor patterns appears here and in Table 10. There are no reactions in immature eggs, atypical eggs, or atypical reactions, but the mature eggs are reacted. Notice that DNP, $NaN_3$, and ouabain stimulate activity in the post-$R_t$ period without a preceding reactivation incubation. ATP was the substrate for all reactions.

Table 9.

Influence of reactivating agents on activator and inhibitor patterns.

| Days post-fixation | Reactivating agent | A/I agent | Mature eggs 1 | 2 | 3 | Germ. yes. | Experimental with substrate: Atypical eggs Macro | Micro | Atypical rxns. Band | Dot | Controls EtOH 3 | w/o 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 14 | none | DNP | - | 2+ | 4+ | - | - | - | - | - | - | - |
| | | L-cys | - | - | - | - | - | - | - | - | - | - |
| | | $NaN_3$ | - | - | 1+ | - | - | - | - | - | - | - |
| | | NaF | - | - | - | - | - | - | - | - | - | - |
| | | ouabain | ± | 1+ | 3+ | - | - | - | - | - | - | - |
| | sea water | DNP | - | 1+ | 2+ | - | - | - | - | - | - | - |
| | | L-cys | - | - | - | - | - | - | - | - | - | - |
| | | $NaN_3$ | - | 1+ | 2+ | - | - | - | - | - | - | - |
| | | NaF | - | - | - | - | - | - | - | - | - | - |
| | | ouabain | ± | 2+ | 4+ | - | - | - | - | - | - | - |

Table 10.

Tabular presentation of data from studies on the effect of reactivating agents on activator and inhibitor patterns. Notice the reaction patterns in the mature eggs and the appearance of reactions in immature eggs, atypical eggs, and also atypical reactions after incubation for 3 hours with $CaSO_4$ and $NaH_2PO_4$ reactivation. ATP was the substrate for all reactions.

Table 10.

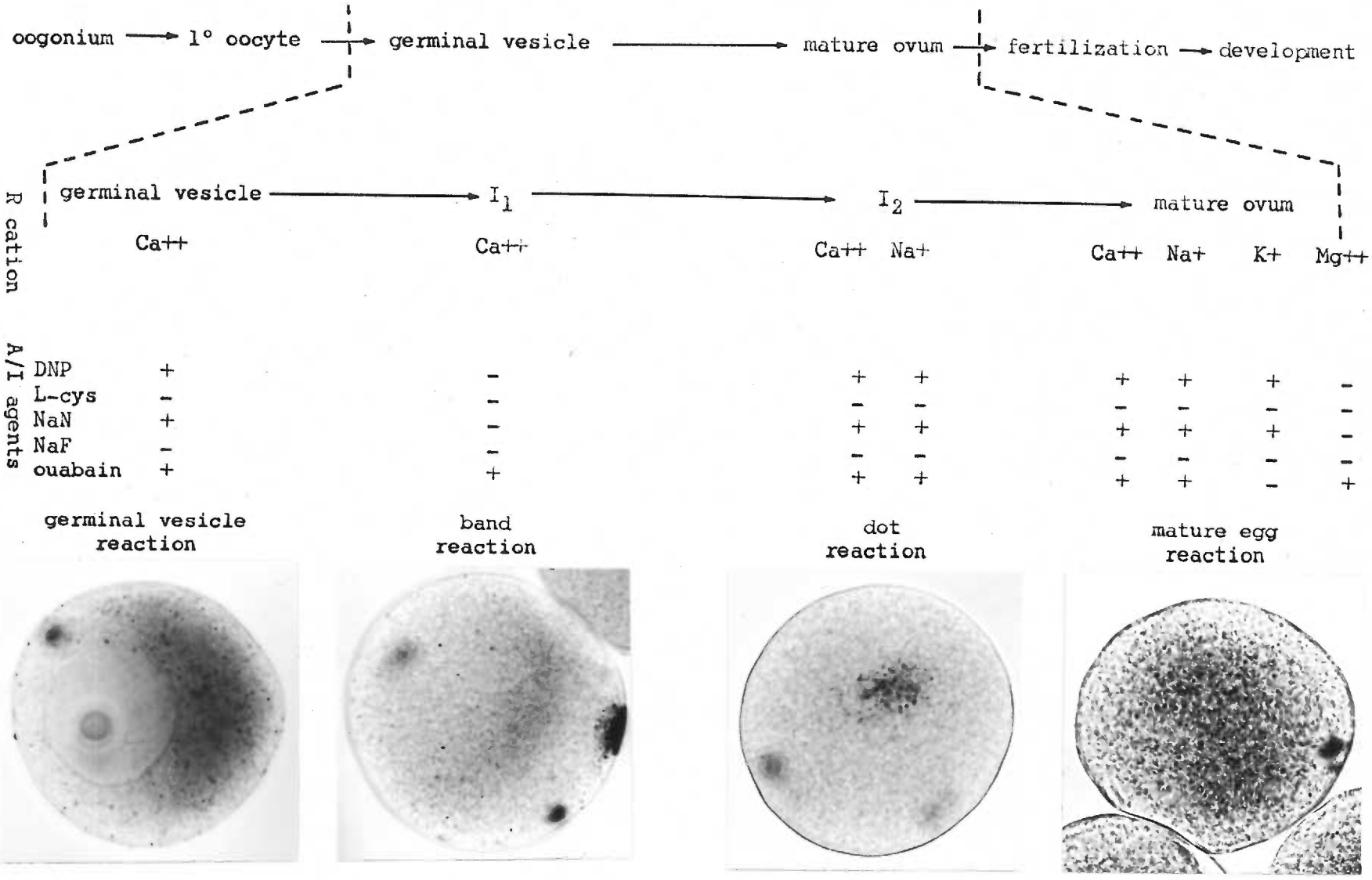
Influence of reactivating agents on activator and inhibitor patterns.

| Days post-fixation | Reactivating agent | A/I agent | Incubation time (hours) Experimental with substrate: Mature eggs 1 | 2 | 3 | Germ. ves. | Atypical eggs Macro | Micro | Atypical rxns. Band | Dot | Controls EtOH 3 | w/o 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 15 | 0.01 M $KH_2PO_4$ | DNP | - | 1+ | 2+ | - | - | - | - | - | - | - |
| | | L-cys | - | - | - | - | - | - | - | - | - | - |
| | | $NaN_3$ | - | - | 1+ | - | - | - | - | - | - | - |
| | | NaF | - | - | - | - | - | - | - | - | - | - |
| | | ouabain | - | - | - | - | - | - | - | - | - | - |
| | 0.04 M $MgSO_4$ | DNP | - | - | - | - | - | - | - | - | - | - |
| | | L-cys | - | - | - | - | - | - | - | - | - | - |
| | | $NaN_3$ | - | - | - | - | - | - | - | - | - | - |
| | | ouabain | - | - | 2+ | - | - | - | - | - | - | - |
| 16 | 0.01 M. $CaSO_4$ | DNP | - | - | 4+ | + | - | - | - | + | - | - |
| | | L-cys | - | - | - | - | - | - | - | - | - | - |
| | | $NaN_3$ | - | - | 4+ | + | - | + | - | + | - | - |
| | | NaF | - | - | - | - | - | - | - | - | - | - |
| | | ouabain | - | - | 4+ | + | - | - | + | + | - | - |
| | 0.5 M $NaH_2PO_4$ | DNP | - | - | 4+ | - | - | - | - | + | - | - |
| | | L-cys | - | - | - | - | - | - | - | - | - | - |
| | | $NaN_3$ | - | 2+ | 3+ | - | - | - | - | + | - | - |
| | | NaF | - | - | - | - | - | - | - | - | - | - |
| | | ouabain | - | 1+ | 4+ | - | - | - | - | + | - | - |

Table 11.

The sequence of development, as described classically, appears at the top of the table. When judged upon a morphological basis, the transition from the "germinal vesicle" oocyte to mature ovum appears to be a one stage process. When data from localization and characterization studies of nucleoside phosphatase activity are correlated, however, at least two intermediate steps ($I_1$ and $I_2$) can be hypothesized. R cations: reactivating cations; A/I agents: activating and inhibiting agents.

Table 11.
Developmental hypothesis.



## C. Figures.

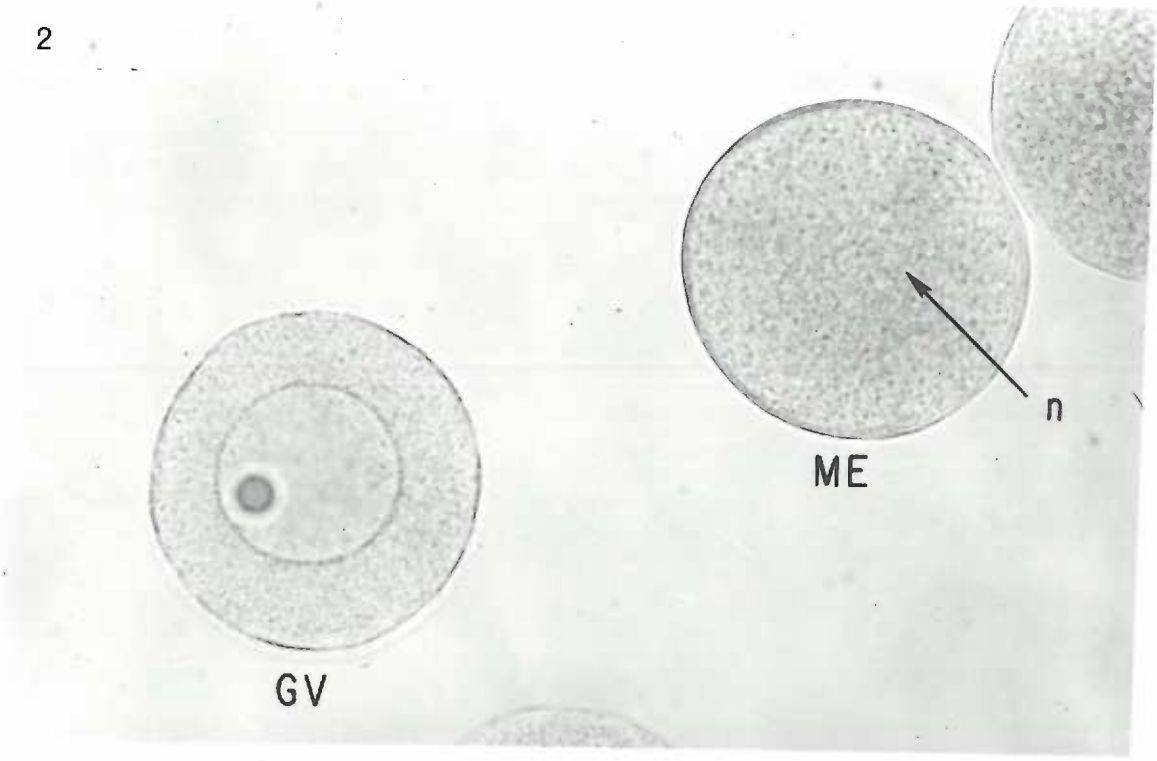
### Figure 2.

The distinction between the germinal vesicle stage and mature egg is clear. These two stages differ not only in their nuclear configuration but character of cell surface, nature of cortico- and medulloplasm, and size as well. The nucleus in the mature egg is seen only with difficulty, but could be definitely identified by varying the focal plane or with modified phase optics. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained. GV: germinal vesicle stage; ME: mature egg stage; n: nucleus.

### Figure 3.

Another germinal vesicle stage is present among a group of mature eggs. Notice that the nucleus of the immature stage is nucleolate. Also the difference between the various components of the cytoplasm can be clearly seen in the mature and immature eggs. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained. n: nucleus; no: nucleolus.

2

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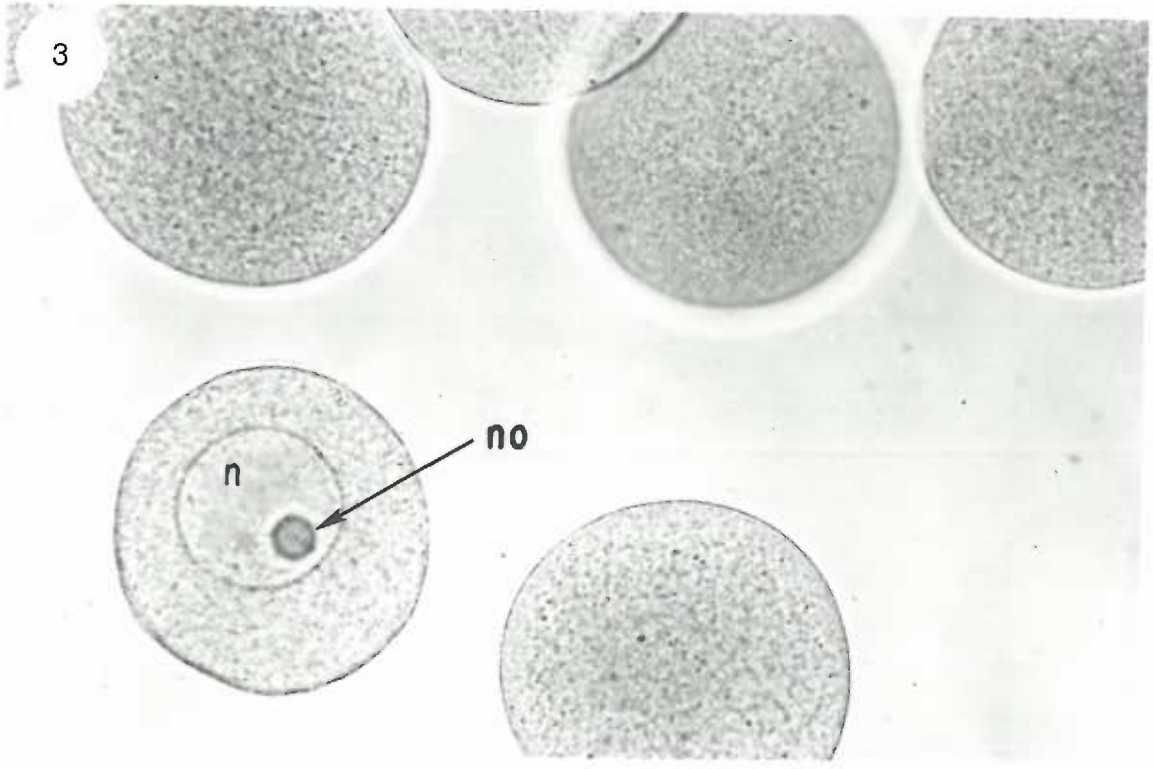


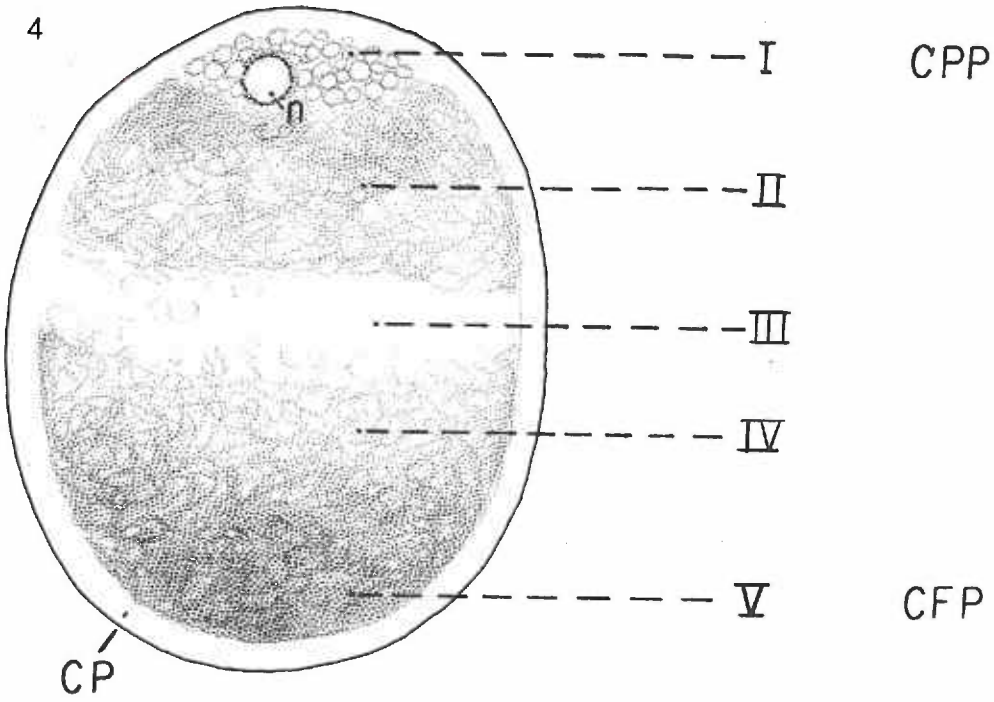
Figure 4.

Artist's conception of a density-gradient centrifuged layered egg. Notice that except for the region of the centripetal pole where there is disruption by the lipid hood the corticoplasm is intact. The other four layers of the layered medulloplasm are present, but their boundaries are not distinct. Mag. approx. 950x. n: nucleus; CP: corticoplasm; I: layer no. I, lipid hood; II: layer no. II, first granular layer; III: layer no. III, hyaline layer; IV: layer no. IV, second granular layer; V: layer no. V, third granular layer; CPP: centripetal pole; CFP: centrifugal pole.

Figure 5.

Artist's conception of a density-gradient centrifuged stretched egg. Again the corticoplasm, except in the region of the lipid hood, is intact. Stretching has occurred through the hyaline zone and the boundaries between the different layers are more distinct. Mag. approx. 550x. n: nucleus; CP: corticoplasm; I: layer no. I, lipid hood; II: layer no. II, first granular layer; III: layer no. III, hyaline layer; IV: layer no. IV, second granular layer; V: layer no. V, third granular layer; CPP: centripetal pole; CFP: centrifugal pole.

4

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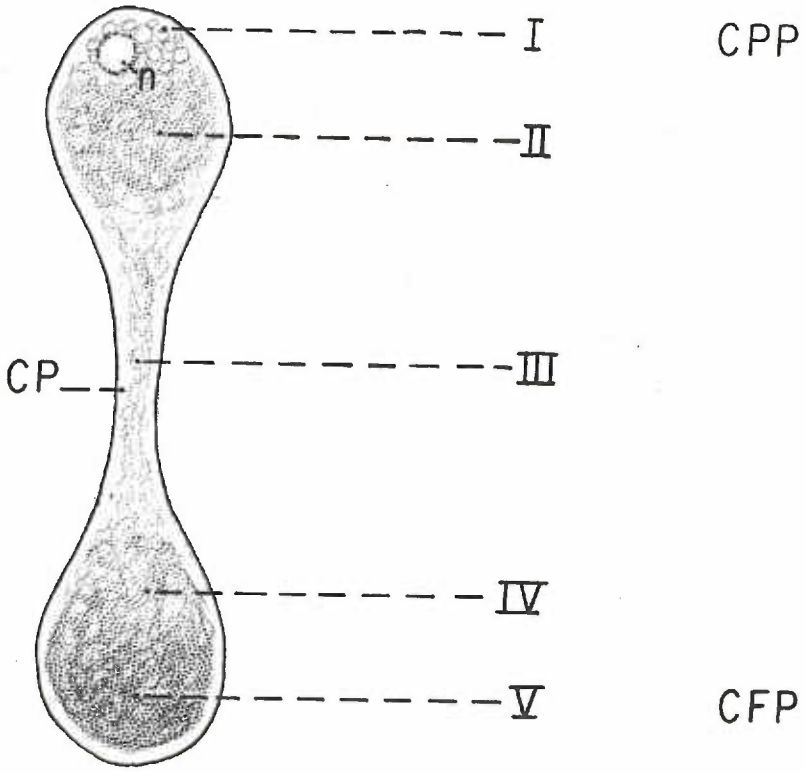


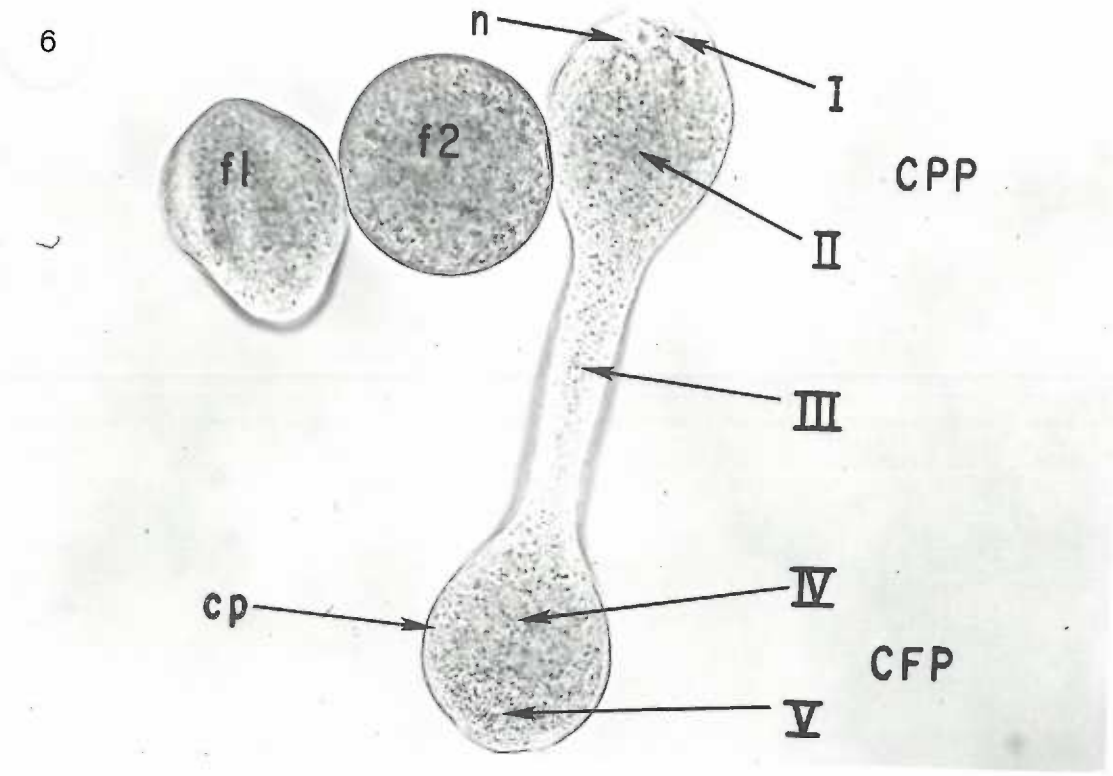
Figure 6.

A density-gradient centrifuged egg. All of the layers of the medulloplasm can be seen. Centripetal and centrifugal pole fragments of an egg which has been separated at the hyaline layer can be seen at $f_1$ and $f_2$, respectively. Mag. 450x; glutaraldehyde fixation; permanent whole mount; unstained. n: nucleus; cp: corticoplasm; f1: centripetal pole fragment; f2: centrifugal pole fragment; I: lipid hood; II: first granular layer; III: hyaline layer; IV: second granular layer; V: third granular layer; CPP: centripetal pole; CFP: centrifugal pole.

Figure 7.

A higher magnification view of the same egg as in Figure 6. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained. f1: centripetal pole fragment; f2: centrifugal pole fragment; CPP: centripetal pole; CFP: centrifugal pole.

6

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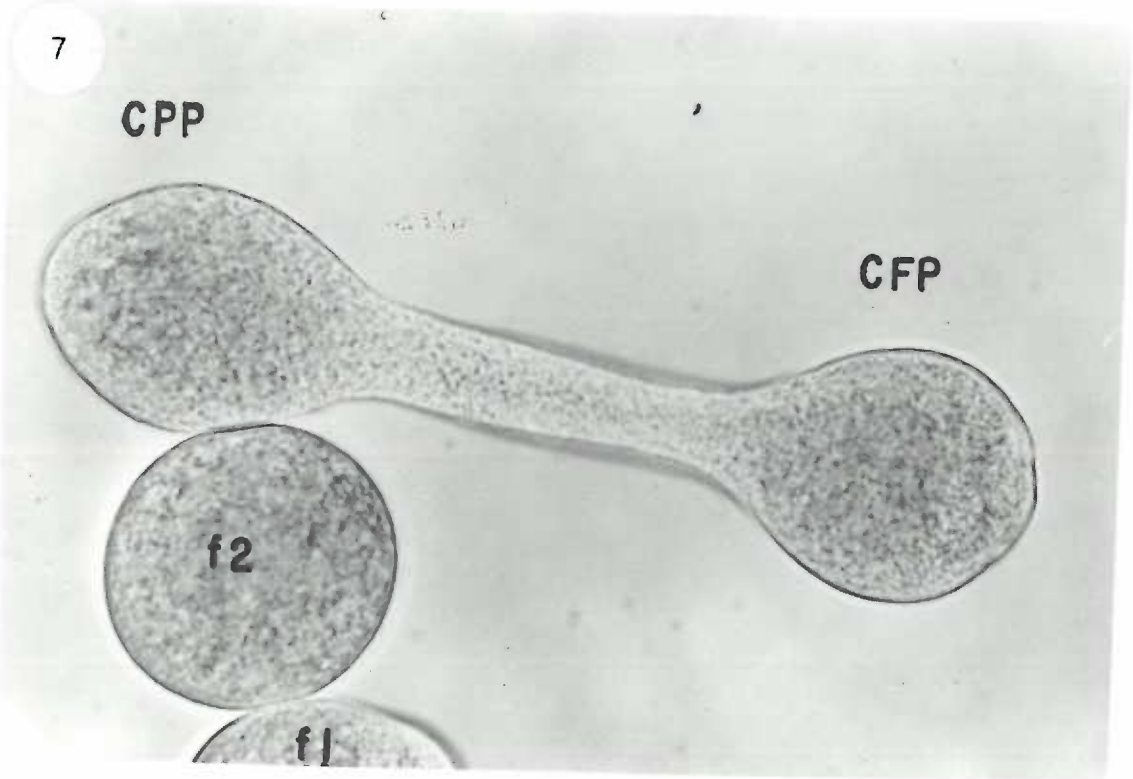


Figure 8.

A density-gradient centrifuged stretched egg which has been stained post-fixation in azure II-methylene blue. There is a faint staining of the corticoplasm and the hyaline layer and the separation of the centripetal pole into staining and non-staining halves is apparent. The centrifugal pole is stained lightly and the discrimination between the second and third granular layers is lost. The nuclear membrane appears to be stained when viewed in the region of the lipid hood. Mag. 550x; glutaraldehyde fixation; permanent whole mount; azure II-methylene blue stain. n: nucleus; cp: corticoplasm; I: lipid hood; IIa: basophilic portion of first granular layer; IIb: non-basophilic portion of first granular layer; III: hyaline layer; IV: second granular layer; V?: region of third granular layer.

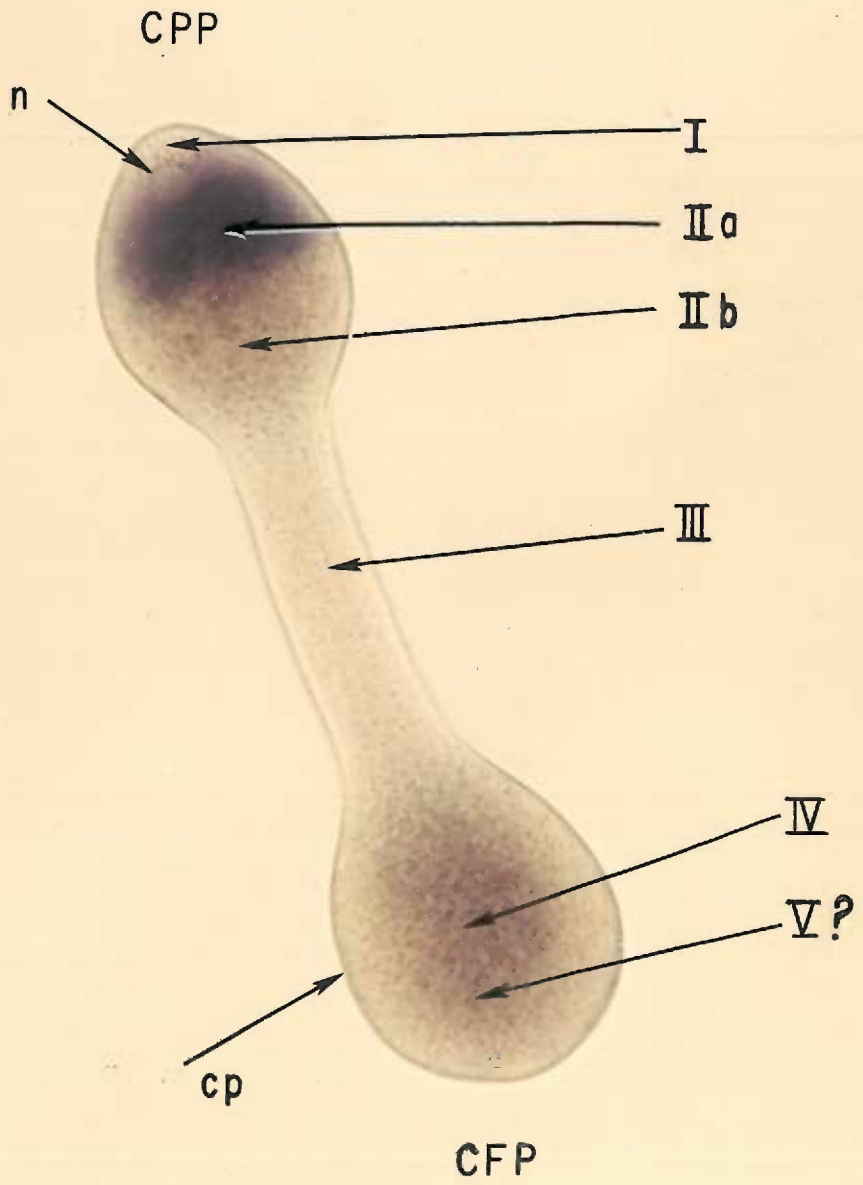

CPP
n
I
IIa
IIb
III
IV
V?
cp
CFP

Figure 9.

At low magnification the general features of both the corticoplasm and the medulloplasm in the glutaraldehyde fixed egg can be appreciated. Microvilli are relatively regularly placed upon the surface of the egg. The cortical ooplasm, with its characteristic cortical granules in two rows, lies immediately subjacent to the oolemma. Within the medulla the ooplasm is conspicuous in its content of yolk granules, mitochondria, macro- and microvesicles, and lipid bodies. Of particular note is the fact that between these organelles lies a complex endoplasmic reticulum. This membranous system is present both in circumferential arrays, as yolk nuclei, and linear arrays. Mag. 4,480x; unstained. cg: cortical granule; YN: yolk nucleus; YG: yolk granules; mv: microvesicle; MV: macrovesicle; L: lipid droplets; M: mitochondria; LL: linear lamellar array of endoplasmic reticulum; A: artifact.

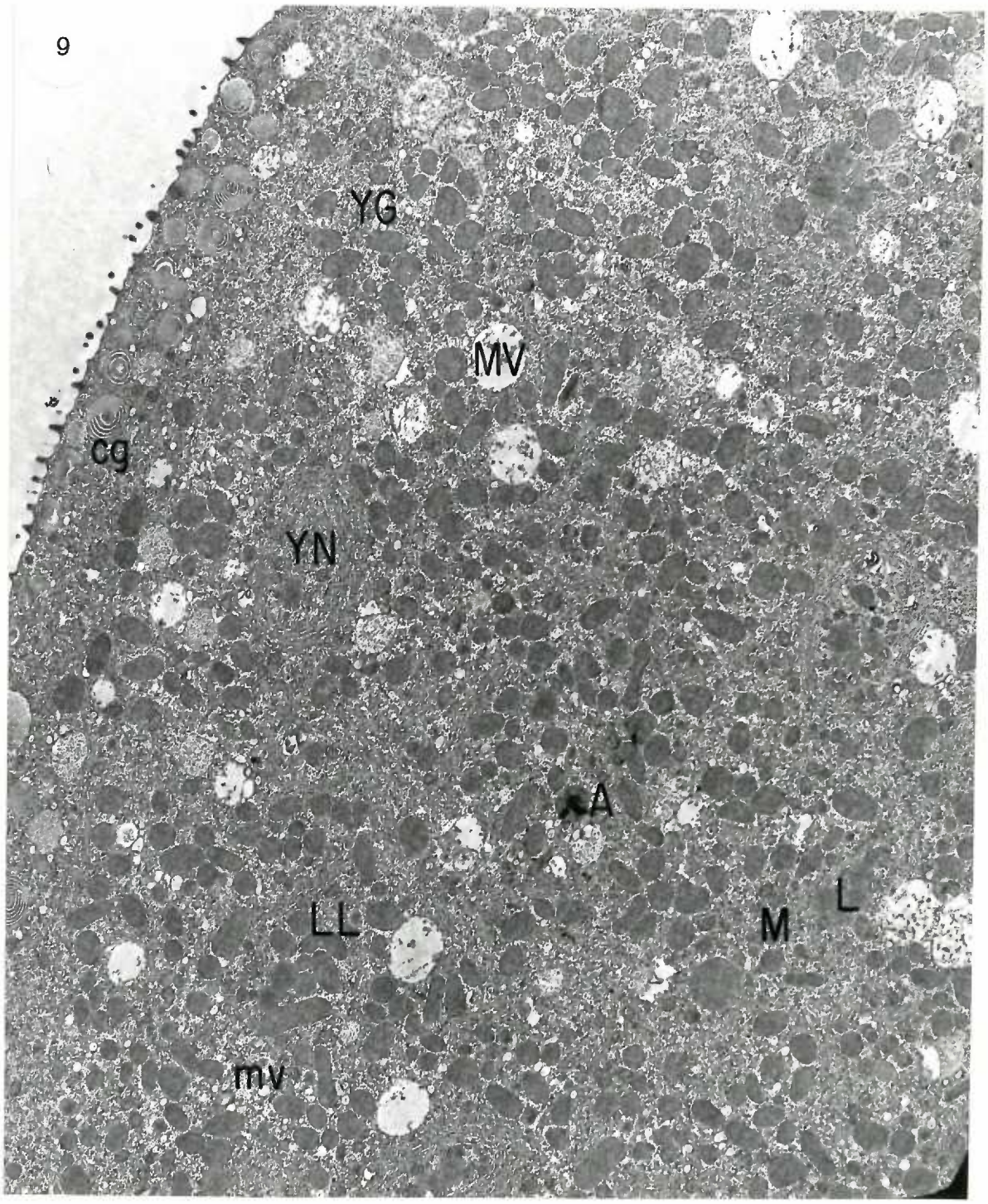

YG
MV
cg
YN
A
LL
M
L
mv

Figure 10.

The character of the microvilli and the evanescent fibrogranular layer which extends across and between them can be seen. In the cortical granule (cg1) to the left of center, the granular nature of the lamellae is apparent, while in the cortical granule (cg2) to the left and above the lamellae are more amorphous. In the former cortical granule (cg1) both the dense (a1) and the less dense (a2) components of the amorphous portion of the cortical granule appear. Between these two cortical granules (cg1 and cg2) their limiting membranes are clearly visible. Notice that at the medullary aspect of cg1 the membrane is discontinuous and the lamellae appear dispersed within the cytoplasm. There is a tangential section of a mitochondrion at the lower right center, while to its right a portion of a Golgi complex is present. Mag. 24,800x; osmium fixation; uranyl acetate (20 m.) and lead citrate (2 m.) stain. Pg: extra-oval fibrogranular layer; cg1 and cg2: cortical granules; L: lamellar component of cg1; a1: dense amorphous component of cg1; a2: less dense amorphous component of cg1; M: mitochondrion; GC: Golgi complex; YG: yolk granule.

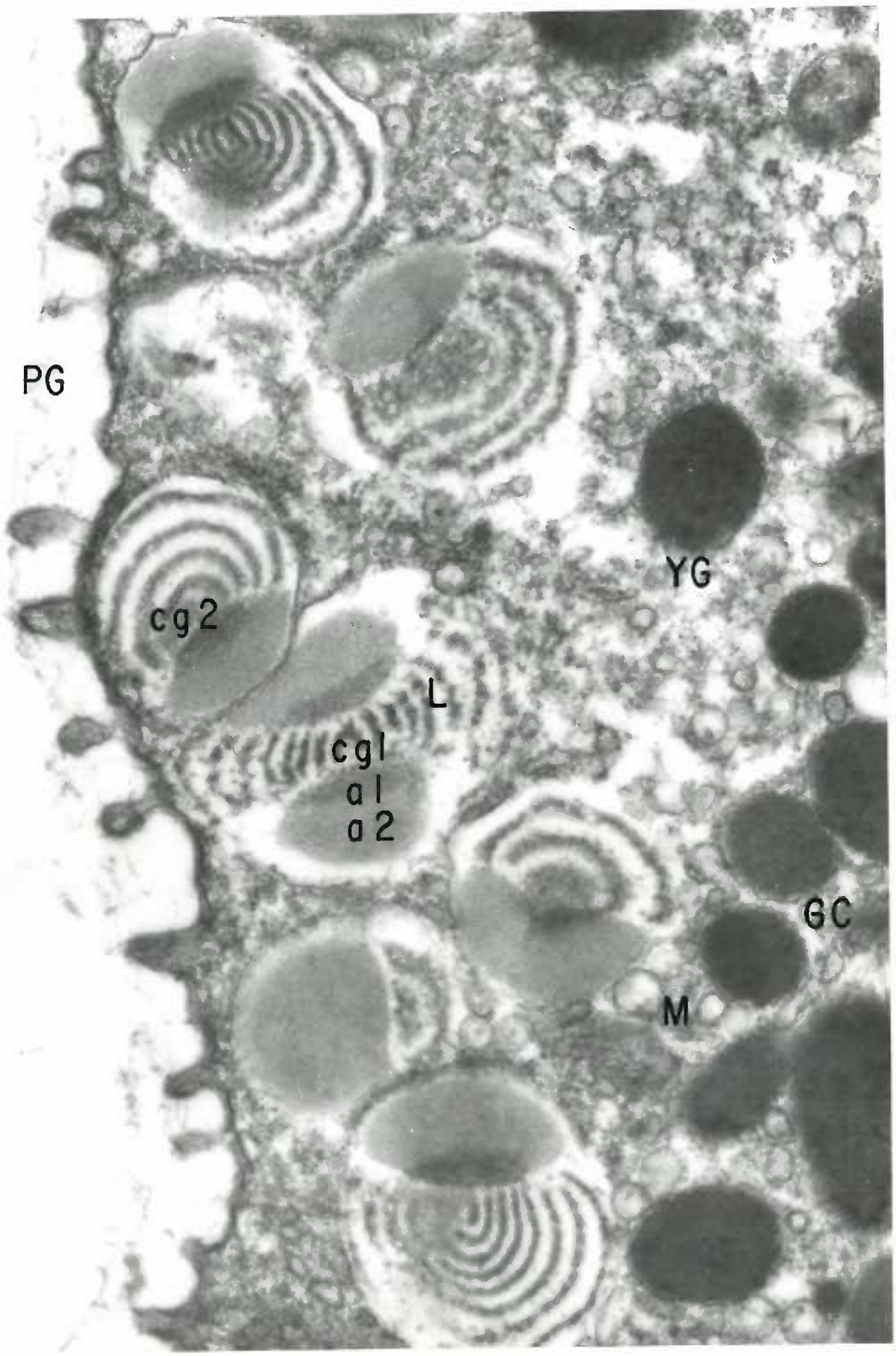

PG
YG
cg2
L
cgl
al
a2
GC
M

Figure 11.

Two heavy bodies with the characteristically triangular arrayed annulate lamellae are present. An individual annulate lamella is present above the lower heavy body which communicates with a granular vesicle at the arrow. The granular membrane bounded microvesicles are abundantly distributed. Several mitochondria are seen with characteristically cuneiform cristae mitochondriales. Mag. 36,000x; osmium fixation; uranyl acetate (20 m.) and lead citrate (2 m.) stain. AL: annulate lamella; HB: heavy body; M: mitochondrion; cm: cristae mitochondriales.

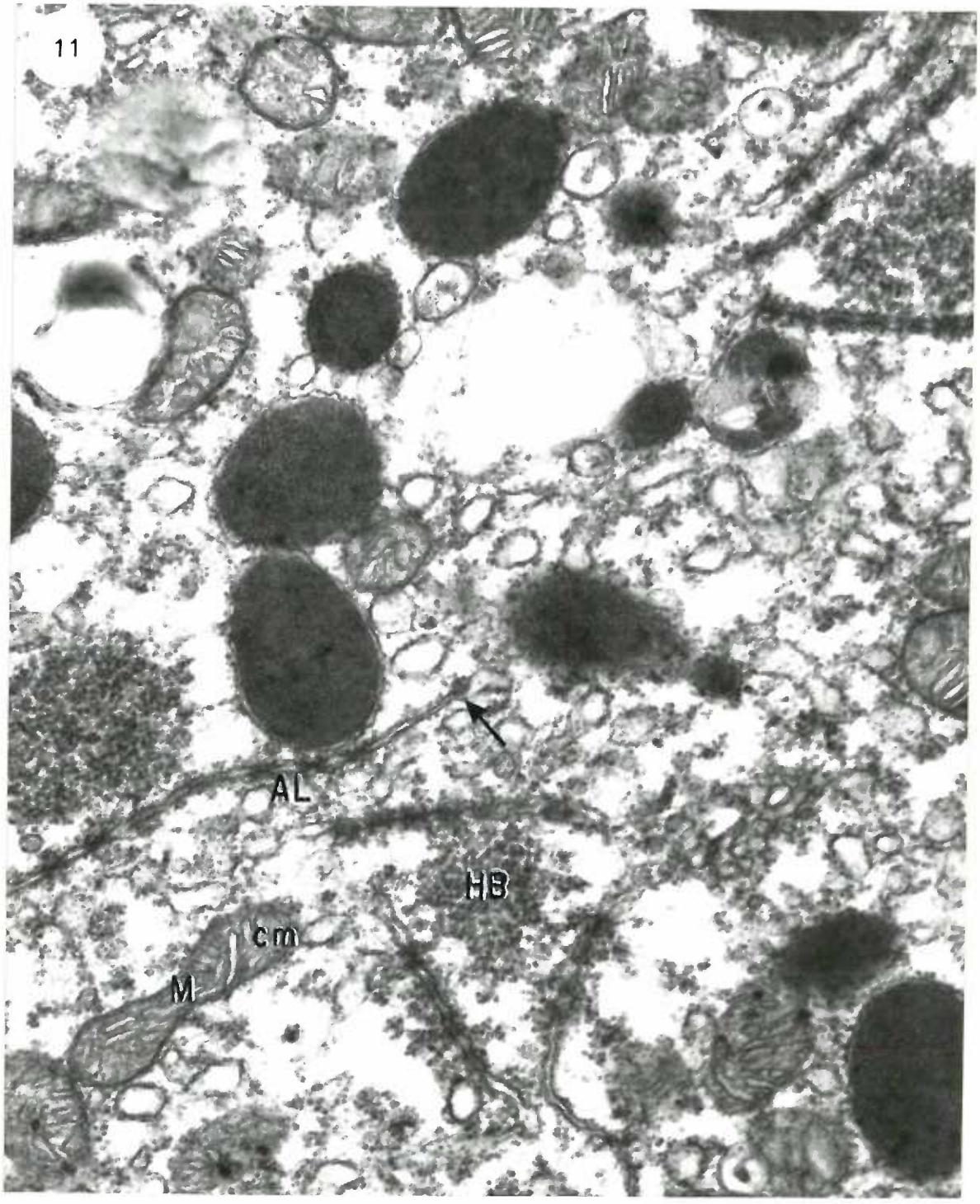

11
AL
HB
cm
M

Figure 12.

The Golgi complexes are frequently encountered within the medulloplasm. Whether the association with the yolk granule at the asterisk (*) is fortuitous or functionally significant is not clear. The arrow indicates where the interior of a microvesicle appears to be confluent with that of a yolk granule. Mag. 67,200x; osmium fixation; uranyl acetate (20 m.) and lead citrate (2 m.) stain.

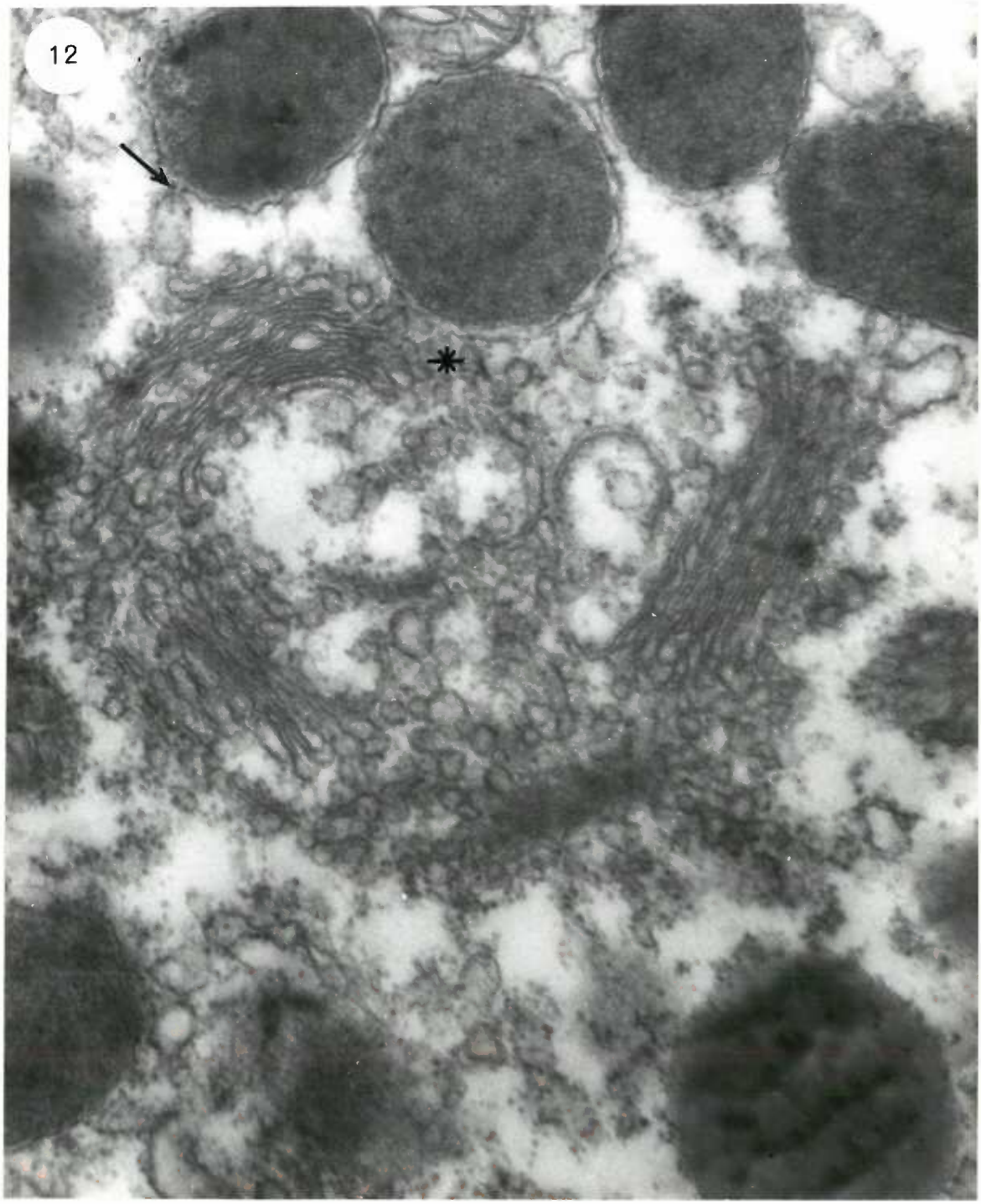

12

Figure 13.

The nuclei in osmium fixed material are conspicuously irregular in outline. Complex foldings of nuclear membranes often create the appearance of intranuclear membrane masses (arrows). The nucleoplasm contains an irregular fibro-granular network which is unevenly condensed along the inner surface of the inner nuclear membrane. Throughout the cytoplasm other frequently encountered elements of the medulloplasm are seen. Mag. 17,200x; uranyl acetate (20 m.) and lead citrate (2 m.) stain. A: artifact; m: mitochondrion; mv: microvesicle; MV: macrovesicle; N: nucleus; g: mass of ribosomes; L: lipid droplet; GC: Golgi complex.

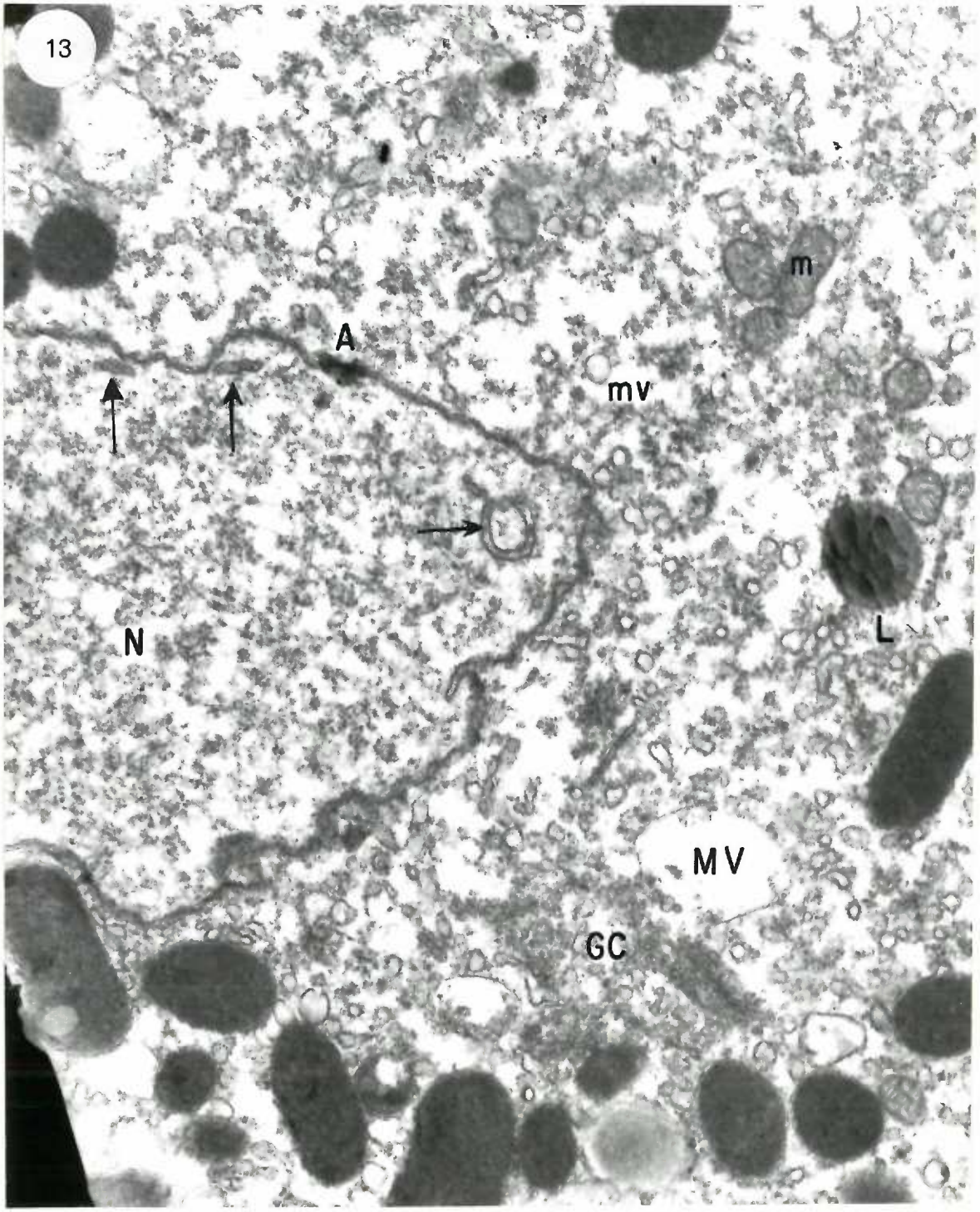

13
m
A
mv
N
L
MV
GC

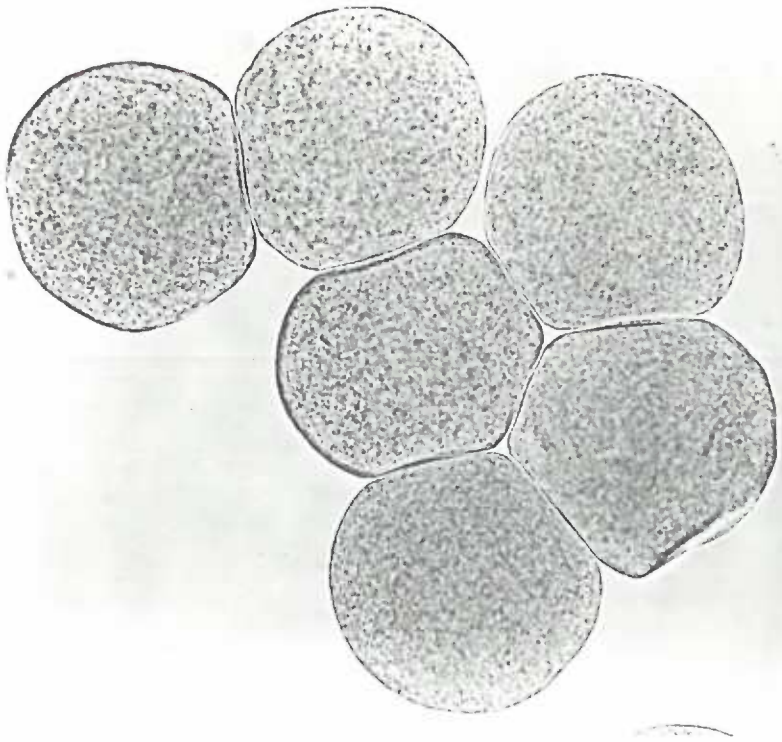
Figure 14.

95% ethanol denatured controls from an incubation with ATP as substrate. The eggs have the typical glassy translucent appearance after ethanol treatment and there is no FRP present. Mag. 375x; glutaraldehyde fixation; permanent whole mount; unstained.

Figure 15.

Controls reacted without substrate from an incubation with ATP as substrate. Notice that there is no FRP present, that the eggs are slightly more granular in appearance, and that they resemble unreacted eggs more than the denatured controls. Mag. 375x; glutaraldehyde fixation; permanent whole mount; unstained.

14



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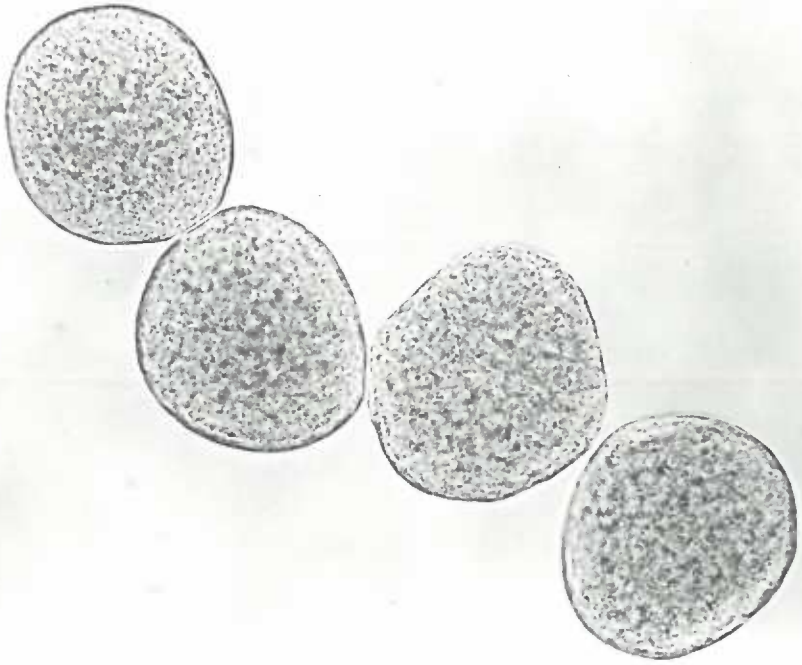


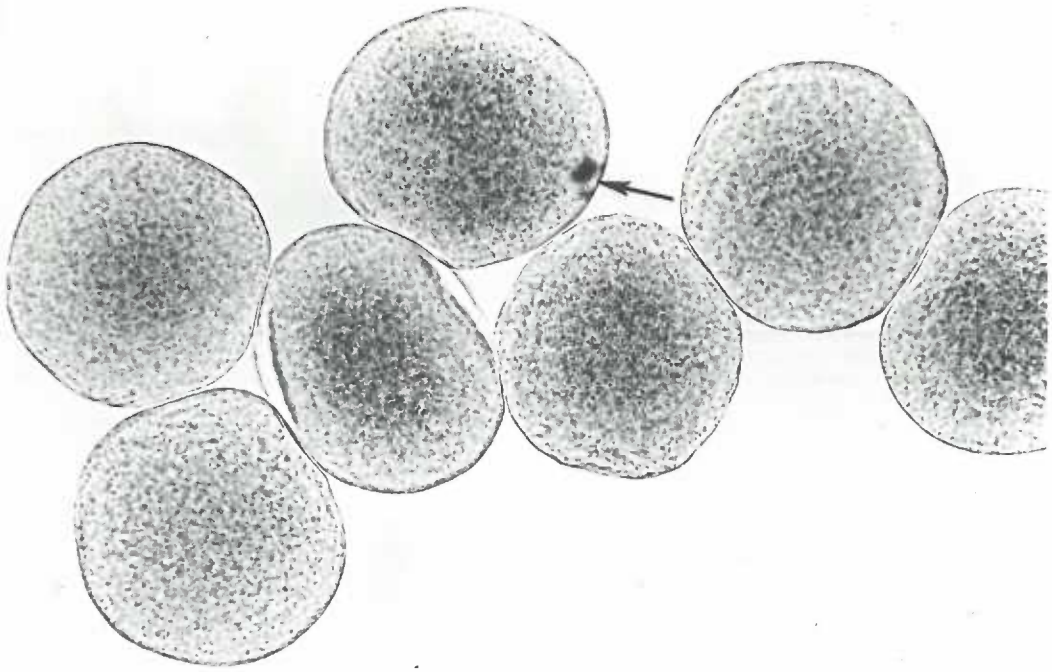
Figure 16.

Eggs reacted in the standard medium with ATP as substrate for 3 hours. The reaction is medullary in location and appears as an amorphous brown-black background with dense 0.5-2.0 μ granules. The arrow indicates a granular type cortiplasmic location of FRP. Mag. 375x; glutaraldehyde fixation; permanent whole mount; unstained.

Figure 17.

A higher magnification of Figure 16. The arrow points to the granular cortical FRP which, by changing the focal place, was demonstrated to be attached to the inner surface of the oolemma. The amorphous and granular components of the FRP can be clearly seen. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained.

16



17

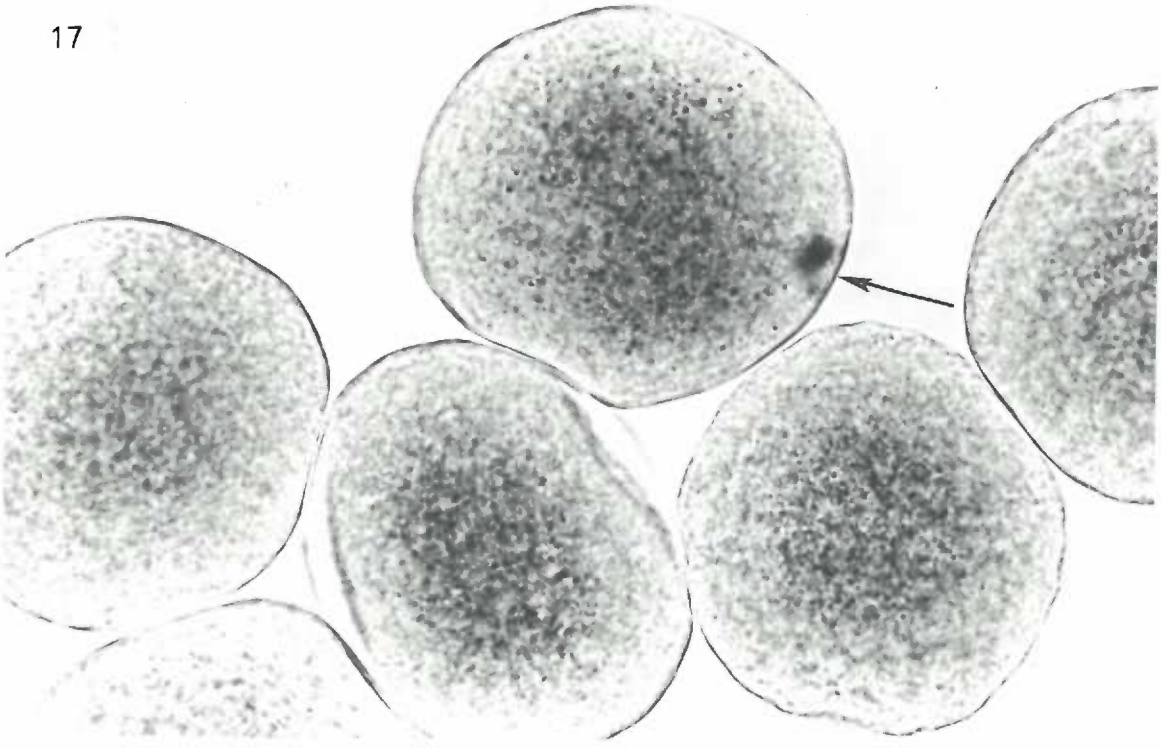


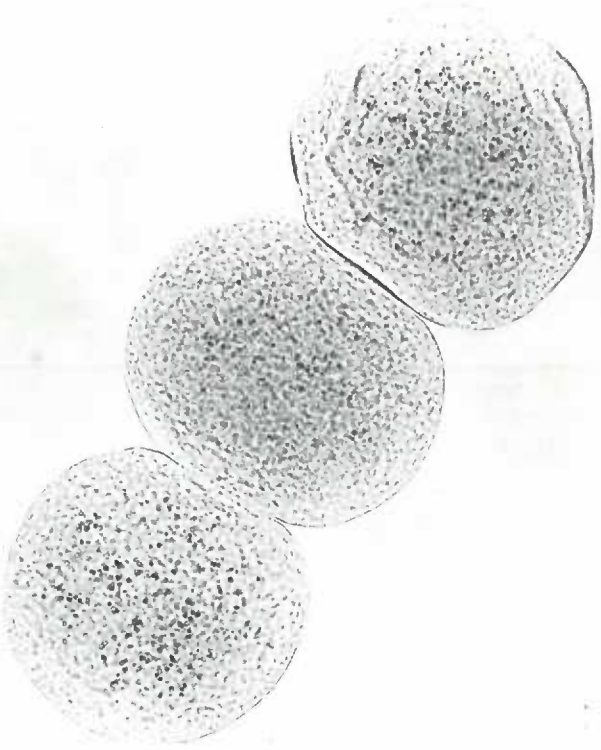
Figure 18.

Eggs from a 3 hour incubation with ATP as substrate. Here the granular component of the reaction was greater than the amorphous component. The amorphous component was present but is somewhat obscured by the granularity of the cytoplasm. Mag. 375x; glutaraldehyde fixation; permanent whole mount; unstained.

Figure 19.

Eggs from a 3 hour incubation with ATP as substrate. The egg labeled I is a type I reaction where granular but no amorphous FRP is present. The other two eggs have a mixture of the two types of FRP which is far more commonly encountered. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained.

18



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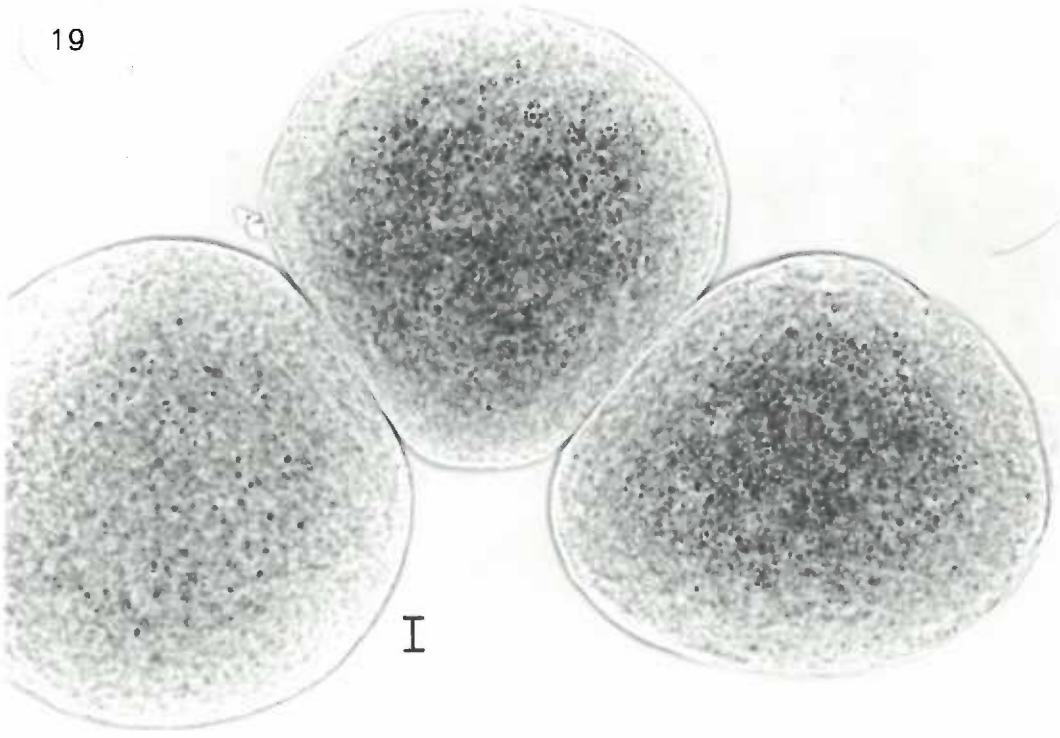


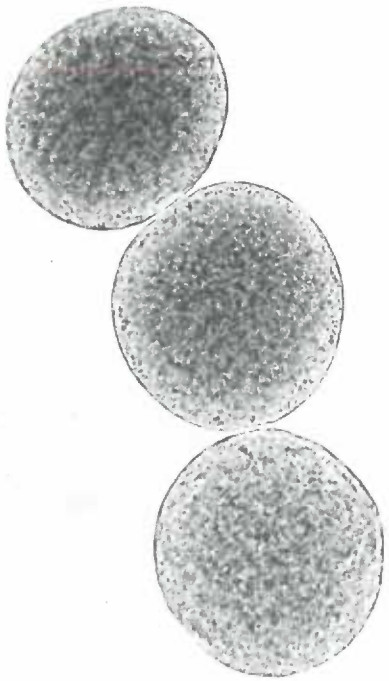
Figure 20.

Eggs from a 3 hour incubation with ATP as substrate. The reaction product is all amorphous and is primarily medullary in location. Mag. 375x; glutaraldehyde fixation; permanent whole mount; unstained.

Figure 21.

Eggs from a 3 hour incubation with ATP as substrate. The FRP is all amorphous and chiefly medullary in location, but there is some staining of the corticoplasm. The arrow indicates a region where the surface of the egg is indented and seen from the side. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained.

20



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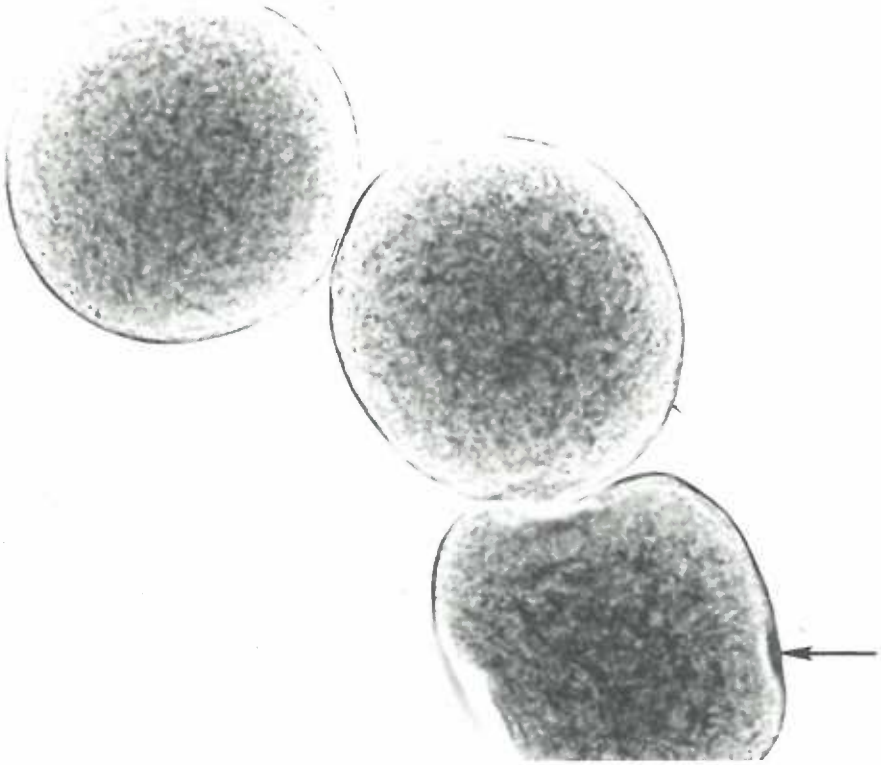


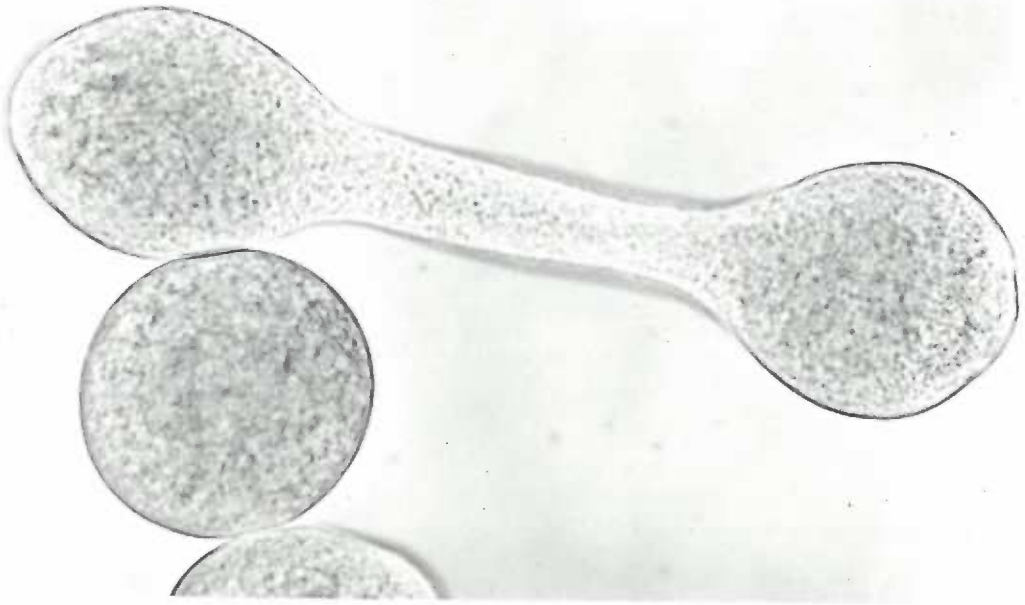
Figure 22.

Unreacted sucrose density-gradient centrifuged stretched egg. Although the ethanol denatured controls were more glassy and the controls reacted without substrate were slightly more granular, they were all alike in their absence of FRP. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained.

Figure 23.

Sucrose density-gradient centrifuged stretched egg after a 3 hour standard incubation with ATP as substrate. The FRP is localized to portions of the centripetal and centrifugal poles. The corticoplasm and lipid hood are unreacted. The cytoplasmic masses (f1 and f2) are portions of eggs fragmented completely by the centrifugation process. Notice that there was a 1+ reaction in both of these. Mag. 375x; glutaraldehyde fixation; permanent whole mount; unstained.

22



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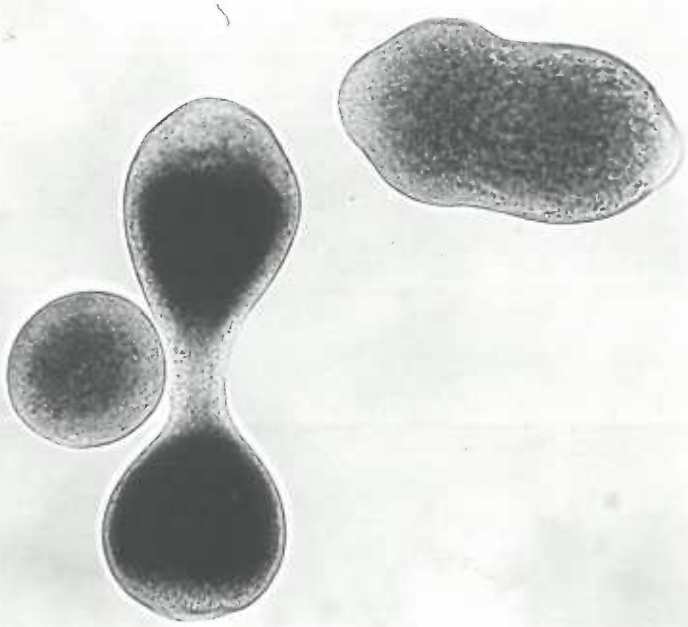


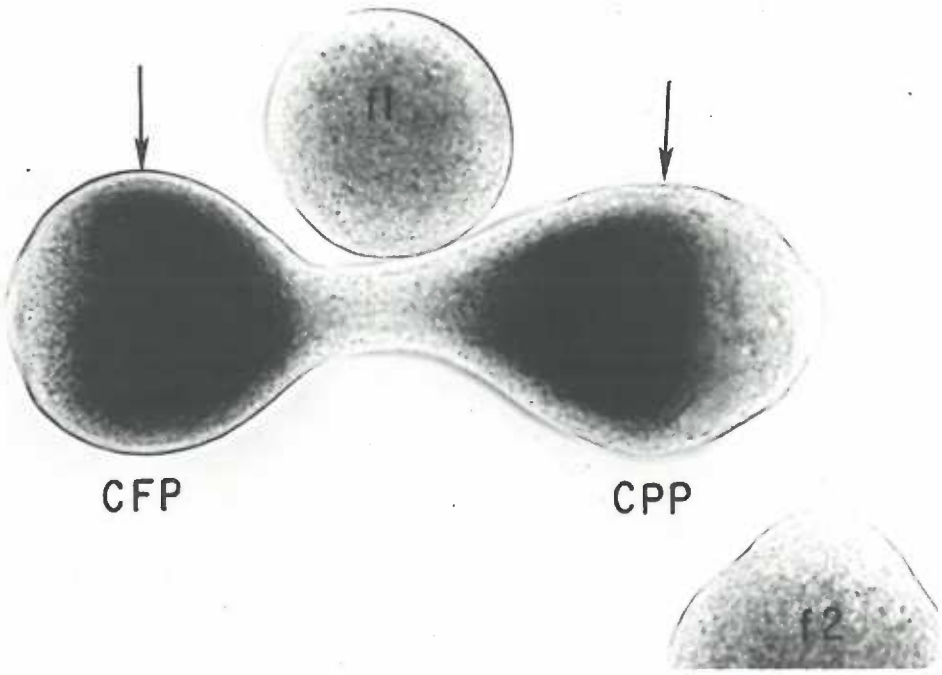
Figure 24.

A higher magnification of the reacted egg seen in Figure 23. The arrows indicate the interface between the third and second granular layer in the centrifugal pole (CFP) and the centrifugal and centripetal halves of the first granular layer in the centripetal pole (CPP). Notice the absence of FRP in the third granular layer, the centripetal half of the first granular layer, the cortico-plasm, and the lipid hood. The nucleus cannot be clearly distinguished in this focal plane. Cytoplasmic fragment f1 and a portion of f2 are also present. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained.

Figure 25.

Stretched eggs after a 3 hour incubation with ATP as substrate. On the left is an egg exhibiting atypical centrifugation properties in which the centripetal pole is remarkably larger than the centrifugal pole. On the right is a stretched egg as they usually appear. Notice the typical localization of FRP in this egg. The arrow points to the nucleus which is unreacted. Mag. 375x; glutaraldehyde fixation; permanent whole mount; unstained.

24

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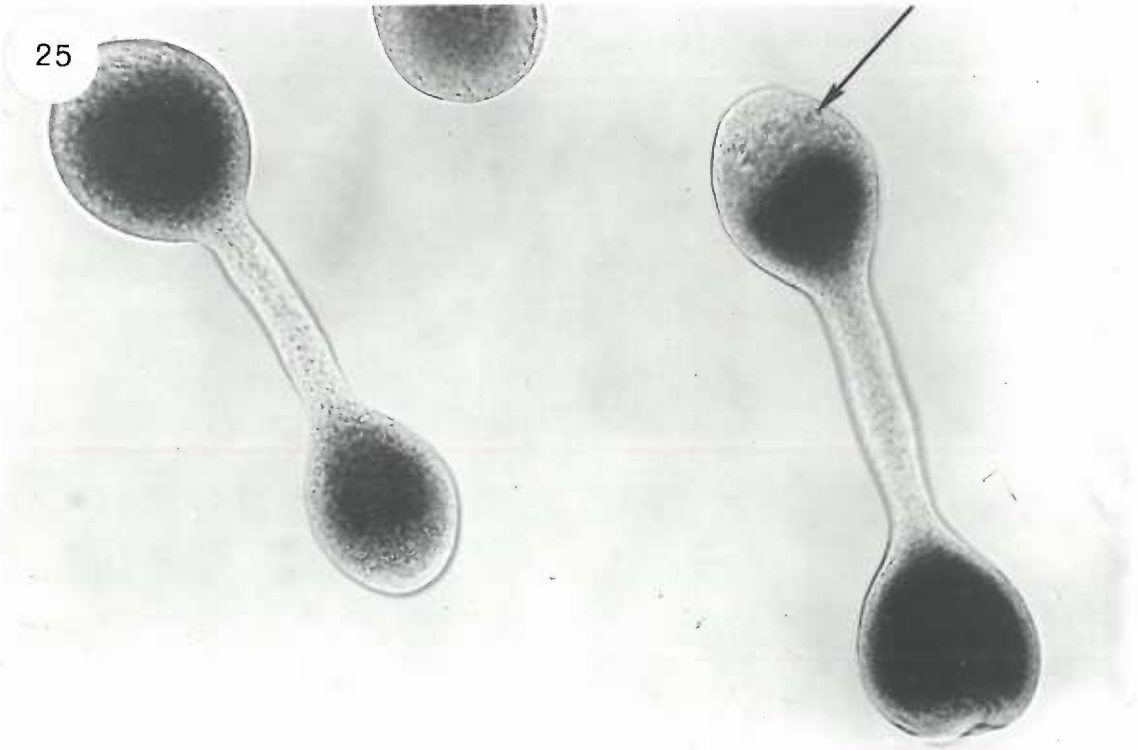


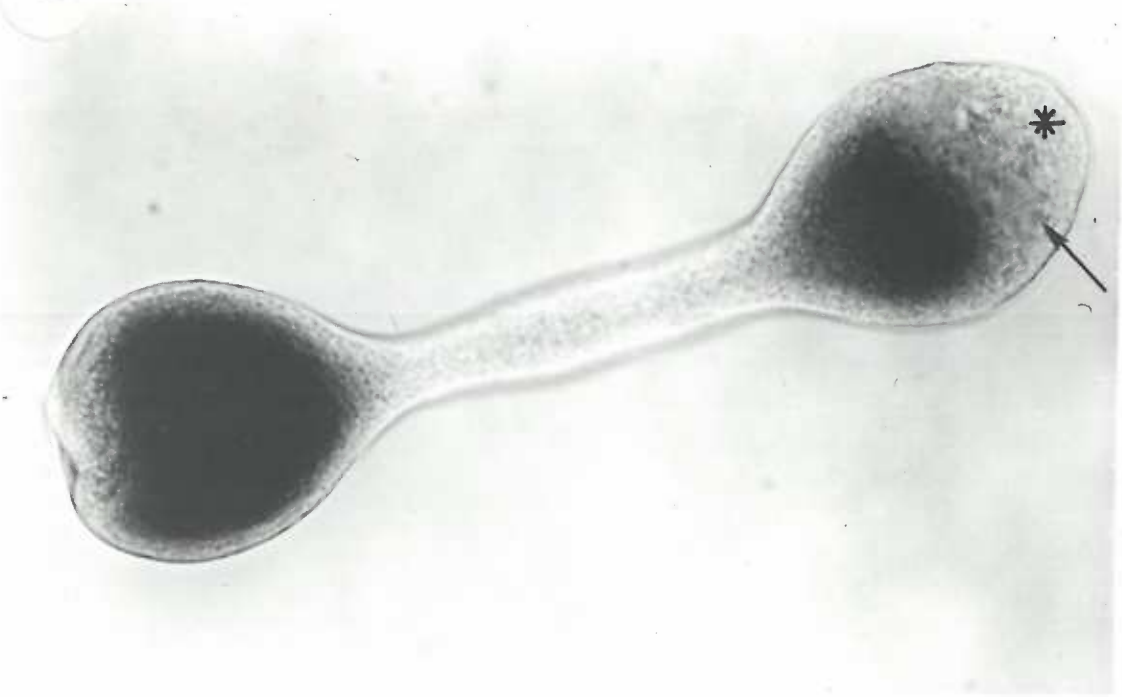
Figure 26.

A higher magnification of the stretched egg on the right in Figure 25. Notice that the nucleus (arrow) which lies within the centripetal half of the first granular layer and at the edge of the lipid hood, is not reacted. The lipid hood, indicated by the asterisk (*), is similarly unreacted. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained.

Figure 27.

A stretched egg incubated with ATP as substrate in the post-$R_t$ period after sea water reactivation. Notice that the FRP is limited to the centrifugal pole (CFP). The boundary between the second and third granular layers is less distinct and there appears to be more FRP in the corticoplasm around the second granular layer. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained.

26



27

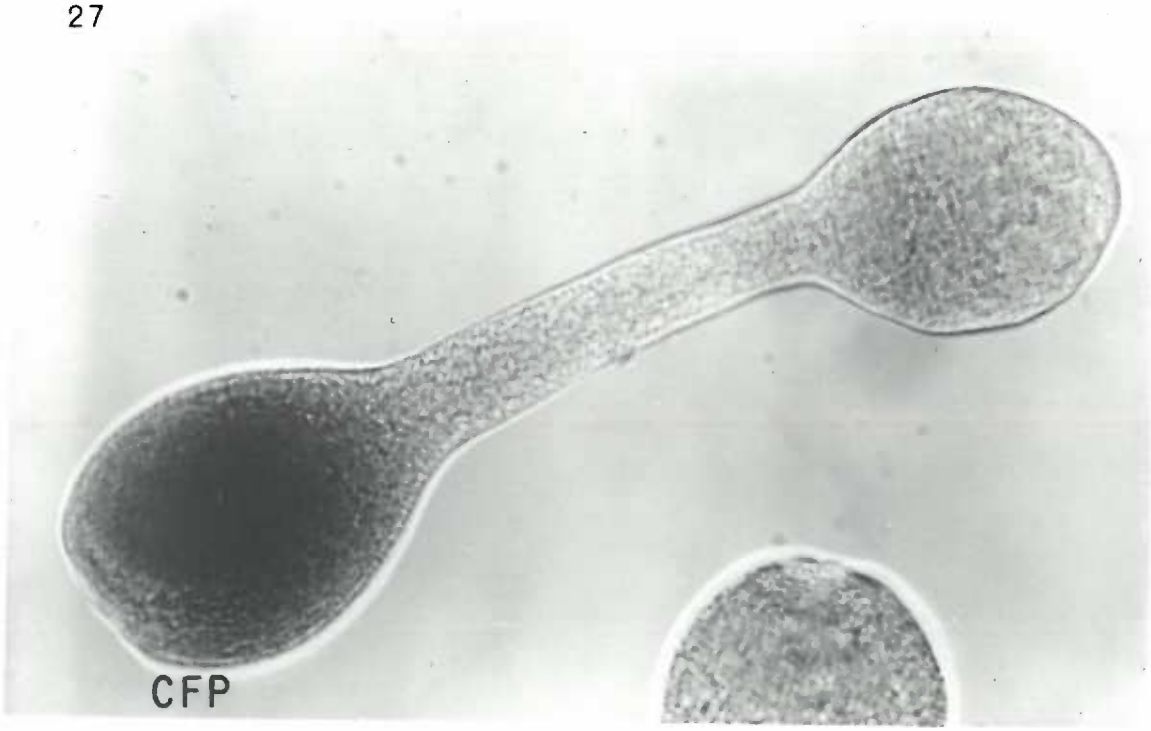


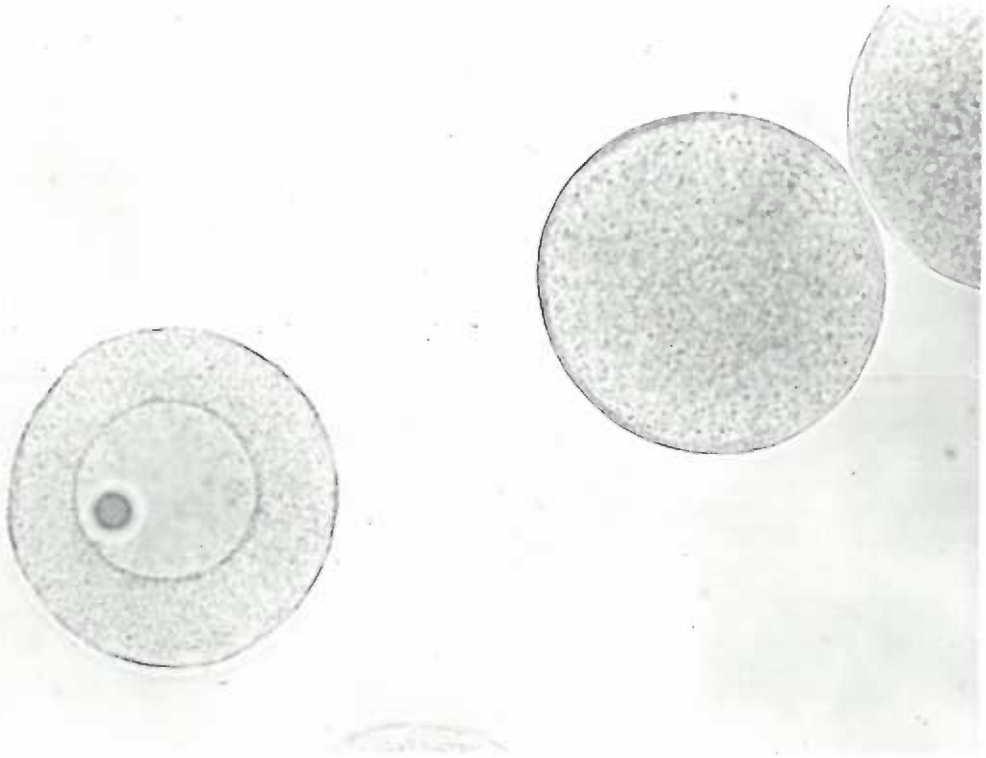
Figure 28.

Unreacted eggs of both mature and germinal vesicle stages. The denatured controls were slightly more glassy and translucent, while the controls without substrate were slightly more granular but all were similar in their distinct absence of FRP. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained. GVL germinal vesicle stage; ME: mature egg; n: nucleus.

Figure 29.

Reacted germinal vesicle stage after a 3 hour incubation with ATP as substrate. Reaction at 16 days post-fixation after $CaSO_4$ reactivation and DNP treatment. FRP is present as a crescentic amorphous mass, freely distributed small granules (0.5-1.0 μ), and large suboolemmal plaques. The arrow indicates such a plaque which is just below the plane of focus and which was attached to the inner surface of the oolemma. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained.

28



29

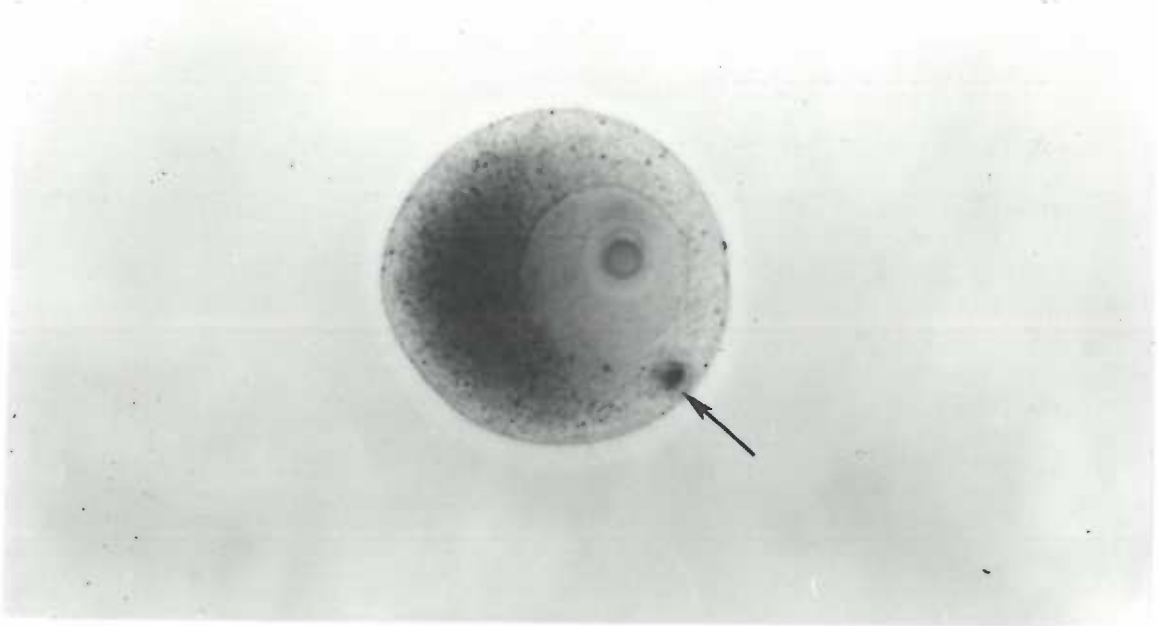


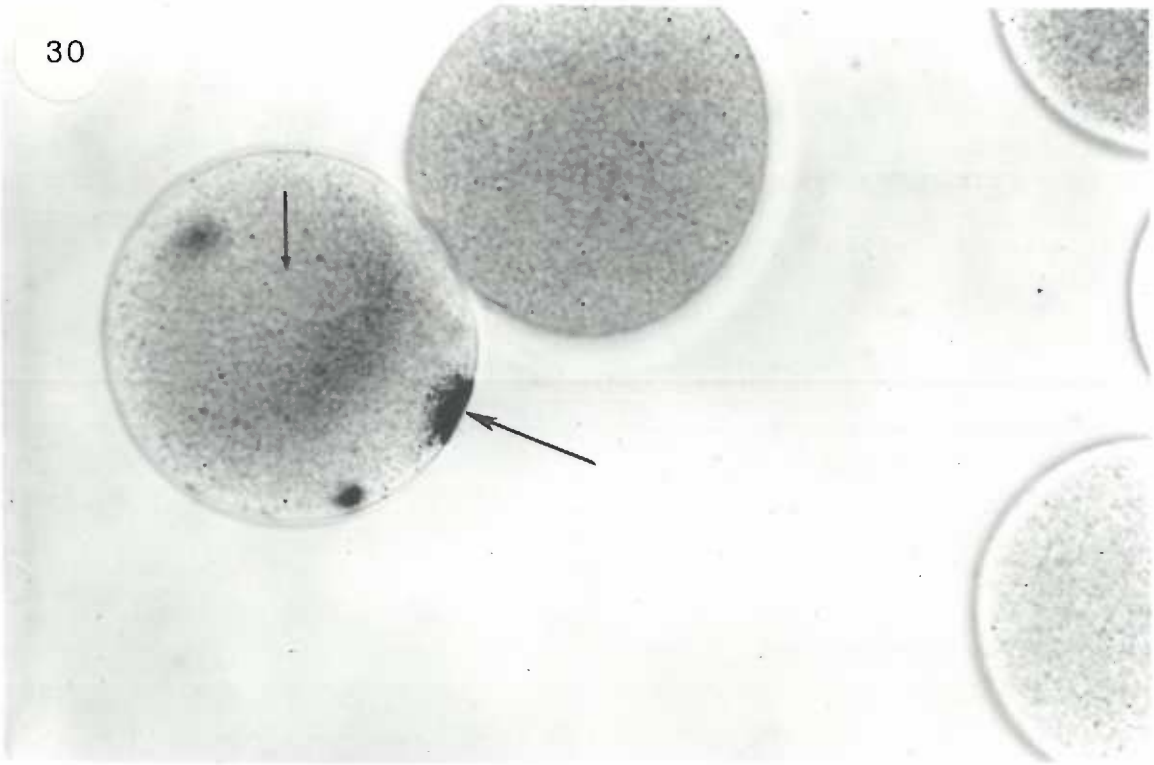
Figure 30.

A band type of reaction after a 3 hour incubation with ATP as substrate. The reaction took place at 16 days post-fixation with $CaSO_4$ reactivation and treatment with $NaN_3$. The small arrow indicates the nucleus lying between the two arms of the crescentric shaped mass of amorphous FRP. Small granules are distributed throughout the cytoplasm and the large arrow indicates a large suboolemmal plaque of FRP. Two other such plaques are present and are slightly out of focus. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained.

Figure 31.

A band type reaction after a 3 hour incubation under the same conditions as in Figure 30. At the left such a reaction is seen in "end-on" view (EV), while at the right the view is "side-on" (SV). The small arrow indicates the nucleus seen in the side on view. Between the nucleus and the suboolemmal plaque, the mass of FRP represents another such plaque below the plane of focus. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained.

30



31

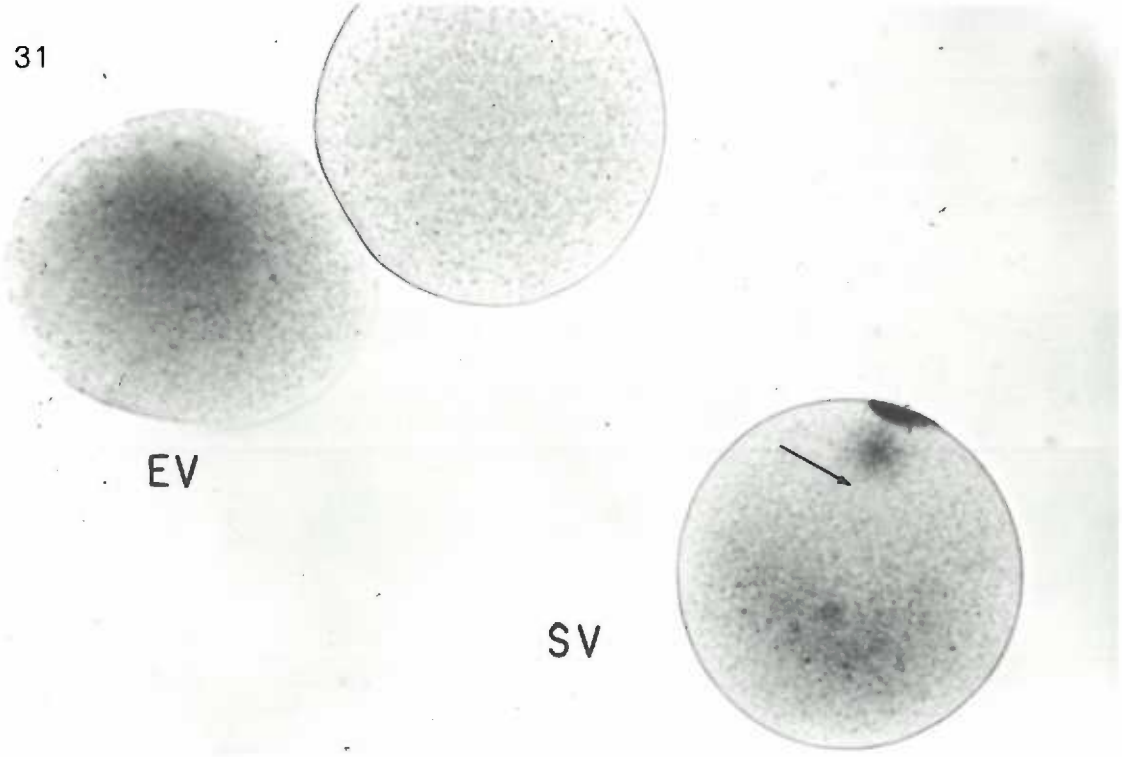


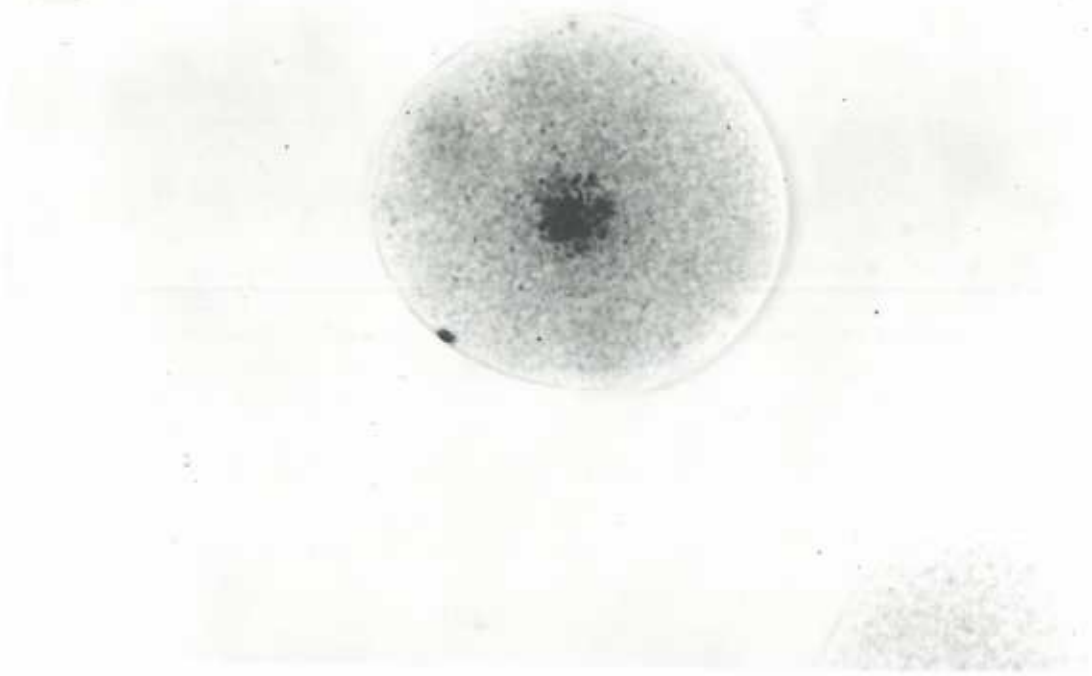
Figure 32.

A dot type reaction after a 3 hour incubation with ATP as substrate. The reaction occurred at 16 days post-fixation after $CaSO_4$ reactivation and $NaN_3$ treatment. There is a small subcolemmal plaque and a few randomly distributed small granules, but the majority of the FRP occurs within the medulloplasm. This larger mass consists of a condensation of the small granules into a region about 5.0-10.0 u in diameter. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained.

Figure 33.

A dot reaction after 3 hours of incubation under the same conditions as in Figure 32. The central mass of small granules is more eccentrically located here. There are two subcolemmal plaques which are out of focus and a remarkable absence of randomly distributed small granules. Mag. 600x; glutaraldehyde fixation; permanent whole mount; unstained.

32



33