A CYTOCHEMICAL INVESTIGATION OF OÖGENESIS AND DEVELOPMENT TO THE SWIMMING LARVAL STAGE IN THE CHITON, *CHITON TUBERCULATUM* L.¹

RONALD R. COWDEN^{2, 3}

*Bermuda Biological Station,⁴ St. George's West, Bermuda,
and
Department of Biology, Johns Hopkins University, Baltimore, Md.*

The chitons have received very little attention from modern developmental biologists. Older accounts dealing with the morphology of chiton development have been published by Heath (1899), Hammersten and Runnström (1925), and Grave (1932). Yet for the chemical embryologist this group offers a number of advantages. Chitons may be readily induced to shed gametes in the laboratory, and artificially inseminated eggs develop well under laboratory conditions. Large numbers of eggs are produced by each individual, and although these eggs are surrounded by a rather ornate chorion, only a thin layer of jelly is present. Development in the species used in this study is quite rapid, requiring only about 12 hours at 26° C. to produce a swimming trochophore. In addition, like all molluscs, chitons are mosaic and exhibit the classical spiral pattern of cleavage.

The objective of this investigation has been to examine the pattern of distribution of nucleic acids, proteins and mucopolysaccharides during the development of the gonadal oöcyte, and during the subsequent development of the egg into the swimming larva. Since the mosaicism of the chiton egg must be dependent upon its physico-chemical organization, by examining oögenesis cytochemically one might hope to identify those aspects of the organization of the egg which are causally related to the mosaic nature of its development.

Further, a consideration of the distribution of the macromolecular substances during development could yield information about the patterns of synthetic activity in molluscan mosaic development as compared to mosaic development in other phyla and also to regulative development.

MATERIALS AND METHODS

The chitons used in these studies were collected in the vicinity of the Bermuda Biological Station, St. George's West, Bermuda, and identified as *Chiton tuberculatum* L. Specimens were removed from intertidal rocks at low tide and maintained in moist but not filled sea water tanks until the second evening after their collection. Individual animals were then placed in fingerbowls and covered with

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³ Present address: Division of Cell Biology, Institute for Muscle Disease, 515 E. 71st Street, New York 21, N. Y.

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FIGS. 1-11.

sea water. Ovulation or the release of sperm ensued two to three hours later. Eggs were then inseminated with sperm suspension and allowed to develop. Development was observed with a binocular dissecting microscope, and embryos were removed for fixation at the desired stages.

For studies of oögenesis, the ovaries were removed from fresh specimens and fixed in appropriate fixatives. Acetic acid-ethanol (1:3) fixation was used for material to be stained for nucleic acids, Lillie's (1954) calcium acetate formalin for material to be stained for mucopolysaccharides and proteins, and Bouin's for material to be stained in mercuric bromphenol blue for proteins.

The pH 4.0 azure B bromide method of Flax and Himes (1952) was used for demonstrating RNA in sections which had been pre-treated with DNAase; Einarson's (1949) gallocyanin-chromalum method was used for staining DNA in sections which were pretreated with RNAase. Crawley *et al.*'s (1956) alcian blue-periodic acid Schiff (AB-PAS) method, which distinguishes between acid mucopolysaccharides and other polysaccharides or substances containing 1,2-glycol groups, was used for visualizing mucopolysaccharides. Some sections of embryos were stained with either alcian blue or with the PAS reaction alone. Some control sections were incubated in 0.1% α -amylase prior to staining with the PAS reaction to remove glycogen. Mazia, Brewer and Alfert's (1953) mercuric bromphenol blue (MBB) method for reactive groups of proteins was employed on Bouin-fixed sections. The picro-naphthol blue black-brilliant purpurin-azofuchsin (NBB-BP-Az) connective tissue method of Lillie (1954) was used without the conventional nuclear staining on calcium acetate formalin-fixed material in an attempt to distinguish between various protein components of the cells. Each of the three acid dyes used in this mixture stains only protein structures, probably by binding to the sites of dissociable basic groups. Some calcium acetate formalin-fixed sections were stained for protein tryptophan by Adam's (1957) dimethylaminobenzaldehyde method.

The eggs and embryos were partially dehydrated in 70% and 95% ethanol and then dehydrated in tertiary butanol. They were infiltrated with first a slush of

FIGURE 1. Chiton ovary, low power photomicrograph. Azure B bromide after DNAase. 300 $\times$.

FIGURE 2. Early stage one oöcyte with large but not heavily basophilic nucleolus. Azure B bromide after DNAase. 1920 $\times$.

FIGURE 3. Stage one oöcyte. Mercuric bromphenol blue. 1920 $\times$.

FIGURE 4. Early stage one oöcyte showing almost complete absence of cytoplasmic staining. Protein tryptophan method. 750 $\times$.

FIGURE 5. Smallest recognizable oöcyte stained for DNA; note nucleolus-associated heterochromatin and coarse nuclear chromatin. Gallocyanin-chromalum after RNAase. 4800 $\times$.

FIGURE 6. Larger stage one oöcyte surrounded by accessory cells. Gallocyanin-chromalum after RNAase. 1200 $\times$.

FIGURE 7. Stage two oöcyte with somewhat diminished cytoplasmic basophilia and relatively smaller nucleolus than stage one oöcytes. Azure B bromide after DNAase. 1200 $\times$.

FIGURE 8. Stage two oöcyte showing distribution of proteins. Mercuric bromphenol blue. 1200 $\times$.

FIGURE 9. Terminal oöcyte showing nucleolus and protein yolk granules stained by protein tryptophan method. 768 $\times$.

FIGURE 10. Stage two oöcyte nucleus in pachytene. Gallocyanin-chromalum after RNAase. 1200 $\times$.

FIGURE 11. Nuclei of oöcyte accessory cells. Gallocyanin-chromalum after RNAase. 4800 $\times$.

FIGS. 12-19.

equal parts of tertiary butanol and Tissuemat, and finally in pure Tissuemat. This treatment is preferable to conventional infiltration with xylol or toluol for yolky material. Eggs, embryos and ovaries were embedded in 56–58° C. m.p. Tissuemat and serially sectioned at 5 μ except for the specimens to be stained for DNA, which were sectioned at 15 μ .

OBSERVATIONS

Oögenesis. A germinal epithelium surrounds the chiton ovary. The smallest oöcytes were found on the periphery of the ovary; the oöcytes increase in size toward the inner portion of the ovary (Fig. 1). Three different stages could be recognized in the development of the chiton oöcytes. In the first stage the oöcyte underwent its greatest relative increase in size, and this was also the stage in which the principal elaboration of cytoplasmic RNA occurred; in the second stage yolk synthesis was initiated and a chorion was formed. Except for the nuclei which were in diplotene and diakinesis of meiosis, third stage oöcytes were indistinguishable from freshly ovulated eggs.

The cytoplasm of stage one oöcytes was intensely basophilic. The nuclei contained a single basophilic nucleolus which occupied a large proportion of the nuclear volume (Fig. 2). In sections stained with mercuric bromphenol blue for basic groups of proteins, the relative intensity and distribution of staining were similar to that of basophilia due to RNA (Fig. 3). Differential staining of various protein structures was obtained with the (NBB-BP-Az) method: nucleoli were stained red by azofuchsin, nuclear proteins brown by brilliant purpurin, and cytoplasmic proteins red by azofuchsin. Toward the end of the first stage, however, there was a change in the staining behavior of the oöcyte cytoplasmic proteins. Instead of binding the red dye, azofuchsin, they bound the brown dye, brilliant purpurin. Again the distribution of staining was similar to that of RNA basophilia. The protein tryptophan method produced only weak staining in the cytoplasm of stage one oöcytes (Fig. 4). The cytoplasm of these oöcytes also contained very little glycogen; the cytoplasm was faintly PAS-positive, and this could be abolished by prior digestion in α -amylase. The oöcyte nucleoli were slightly PAS-positive, and nucleolar staining persisted with this method through all three stages until the nucleolus disappeared. No alcian blue staining was observed in stage one oöcytes which could not be prevented by prior incubation in RNAase.

FIGURE 12. Chiton oöcyte showing distribution of alcian blue-stainable material (dark) and PAS-positive material (light) at stage of chorion formation. Alcian blue-PAS. 1920 $\times$.

FIGURE 13. Evagination of chorionic processes from chorion, pushing accessory cells away. Mercuric bromphenol blue. 3000 $\times$.

FIGURE 14. Chorionic processes fully developed with "stalk" and terminal "bud." Picro-naphthol blue black-brilliant purpurin-azofuchsin. 1200 $\times$.

FIGURE 15. Top view of "bud," showing six radially symmetrical points and a center point. Piconaphthol blue black-brilliant purpurin-azofuchsin. 4800 $\times$.

FIGURE 16. Stage three oöcyte, showing reduced cytoplasmic basophilia except around nuclear membrane and absence of nucleolus. Azure B bromide after DNAase. 1600 $\times$.

FIGURE 17. Stage three oöcyte; cytoplasm is filled with uniform protein yolk granules. Mercuric bromphenol blue. 600 $\times$.

FIGURE 18. Stage three oöcyte nucleus stained for DNA, showing postpachytene chromosomes adherent to nuclear membrane. Gallocyanin-chromalum after RNAase. 1920 $\times$.

FIGURE 19. Cortical layer of stage three oöcyte is stained by alcian blue, the endoplasm beneath is filled with PAS-positive protein yolk granules. Alcian blue-PAS. 4800 $\times$.

FIGS. 20-27.

The chromosomes were somewhat condensed in very small stage one oöcytes. The nucleolus was surrounded by nucleolus-associated heterochromatin which, like the chromosomes, was stained by the gallocyanin-chromalum method for DNA (Fig. 5). In the later phases of stage one, the oöcytes became surrounded by a layer of somewhat flattened oöcyte accessory cells (Fig. 6).

In the second stage of oöcyte growth the concentration of cytoplasmic basophilia was reduced and the nucleolus occupied a relatively smaller proportion of the nucleus (Fig. 7). In addition to the RNA-associated proteins which were present in stage one, small granules of protein yolk appeared which were deeply stained by mercuric bromphenol blue (Fig. 8). With the (NBB-BP-Az) method, the yolk granules were stained by azofuchsin while the RNA-associated proteins were stained by brilliant purpurin; both the nucleolus and nucleoplasm continued to stain as in stage one. The yolk granules were strongly stained by the protein tryptophan method (Fig. 9), and were also strongly PAS-positive. Again, within the oöcyte there was no alcian blue staining which was not removable by prior digestion in RNAase. The stage two oöcyte chromosomes were in pachytene, and were adherent to the nuclear membrane (Fig. 10).

The second stage oöcytes were chiefly concerned with yolk synthesis, but outside the oöcyte, the oöcyte accessory cells were involved in the construction of a chorion. While these cells were somewhat flattened in stage one, they proliferated and eventually formed a single layer of rounded cells around the stage two oöcyte (Fig. 11). A chorionic membrane was then laid down around the oöcyte beneath the oöcyte accessory cells. The chorion itself was PAS-positive (Fig. 12) and was lightly stained by both mercuric bromphenol blue and by azofuchsin in the (NBB-BP-Az) method. Then from beneath the oöcyte accessory cells, hollow hydranth-like processes evaginated from the chorion (Fig. 13). These processes grew outward from beneath the oöcyte accessory cells. When fully developed, the processes consisted of a distal hydranth-like "bud," a stalk, and a basal region which opened into the space between the chorion and the oöcyte (Fig. 14). The "bud" consisted of six radially symmetrical points and one center point (Fig. 15). When the development of the processes was completed, the accessory cells were no longer in evidence.

The cytoplasm of stage three oöcytes was considerably less basophilic than the cytoplasm of stage two oöcytes. The basophilia was distributed between the large protein yolk granules except in the region of the nuclear membrane where it seemed to be accumulated in a concentric pattern (Fig. 16). The nucleoli had disappeared

FIGURE 20. Ovulated egg. Picronaphthol blue black-brilliant purpurin-azofuchsin. 768 $\times$.

FIGURE 21. Polar body division stained for DNA. Gallocyanin-chromalum. 4800 $\times$.

FIGURE 22. Polar body division, showing staining of the meiotic apparatus and fine cytoplasmic granules surrounding the apparatus. Mercuric bromphenol blue. 4800 $\times$.

FIGURE 23. Mitotic apparatus of an early cleavage figure stained for protein. Mercuric bromphenol blue. 3840 $\times$.

FIGURE 24. Cleavage figure stained for DNA; note spindle is longer and narrower than polar body spindle. Gallocyanin-chromalum after RNAase. 4800 $\times$.

FIGURE 25. Spiral cleavage pattern. Gallocyanin-chromalum after RNAase. 800 $\times$.

FIGURE 26. Early cleavage embryo stained for protein; note equal density of protein yolk granules in all blastomeres. Mercuric bromphenol blue. 768 $\times$.

FIGURE 27. Intermediate cleavage stained for RNA; note equal distribution of cytoplasmic basophilia; dark nuclear zones are mitotic apparati. Azure B bromide after DNAase. 768 $\times$.

FIGS. 28-33.

in most stage three oöcyte nuclei. The cytoplasm was filled with protein yolk granules of uniform size which gave staining reactions identical to those observed in the smaller growing granules of stage two (Fig. 17). In the nucleus, the nucleolus had disappeared, and the post-pachytene chromosomes were observed adherent to the nuclear membrane (Fig. 18). Beneath the chorion, a granular cortical zone appeared which was stained by alcian blue (Fig. 19), presumably acid mucopolysaccharide material.

The chorionic processes had completed their outward growth by the beginning of stage three, and the oöcyte was coated with mucoprotein material. This material surrounded the oöcyte from the chorion to just beyond the distal "buds" of the chorionic processes (Fig. 20). This substance was lightly stained by both mercuric bromphenol blue and azofuchsin in the (NBB-BP-Az) method, faintly PAS-positive, and contained some granules which were deeply stained by alcian blue.

Early development. After ovulation, the first meiotic division occurred (Fig. 21). Shortly after fertilization, a second division followed, but it was not possible to determine the sequential relationship between the second polar body division, and the entrance of the sperm. The cytoplasmic distribution of both protein yolk granules and RNA-basophilia was identical with that observed in stage three oöcytes except in the region of the meiotic apparatus. Here yolk granules were pushed aside and the spindle was surrounded by smaller granular protein material (Fig. 22). Basophilia was associated with the spindle.

The mitotic apparati of cleaving embryos were stained by the mercuric bromphenol blue method for proteins (Fig. 23). Cleavage occurred by normal mitosis (Fig. 24), and exhibited the spiral pattern (Fig. 25). Both protein yolk and basophilia were evenly distributed within the cells (Figs. 26 and 27). Since the macromeres were larger they received more material, but not through any obvious differential segregation of material. No nucleoli were formed in the cleavage blastomere nuclei. However, there was considerable difference in the size of macromere and micromere nuclei (Fig. 28).

By the time of gastrulation the density of protein yolk granules was greater in the endodermal cells which had been derived from the invaginated macromeres (Fig. 29). No local increases in basophilia were observed (Fig. 30), but very small nucleoli appeared in the ectodermal cells which later became the prototroch or apical tuft. These nucleoli were only faintly basophilic in the gastrula (Fig. 31). The slight difference in degree of basophilia between the two primary layers is probably due to differences in cell size.

FIGURE 28. Intermediate cleavage stained for proteins; note equality of distribution of protein yolk granules and disparity in size of nuclei of the various blastomeres. Mercuric bromphenol blue. 768 $\times$.

FIGURE 29. Gastrula stained for protein; somewhat heavier concentrations of protein yolk granules in the endoderm. Mercuric bromphenol blue. 600 $\times$.

FIGURE 30. Gastrula stained for RNA; with the exception of division figures, basophilia not significantly different in the primary layer. Azure B bromide after DNAase. 768 $\times$.

FIGURE 31. Prototroch cell showing increased cytoplasmic basophilia and a small nucleolus. Azure B bromide after DNAase. 4800 $\times$.

FIGURE 32. Trochophore larva showing distribution of protein; note yolk in the larval gut. Picronaphthol blue black-brilliant purpurin-azofuchsin. 768 $\times$.

FIGURE 33. Trochophore larva showing distribution of RNA; the prototroch and apical tuft cells are basophilic. Azure B bromide after DNAase. 768 $\times$.

In the swimming larva, protein yolk was most dense in the endodermal cells of the trochophore gut (Fig. 32). The cytoplasm of the cells of the prototroch and apical tuft was intensely basophilic, and their nucleoli were somewhat more heavily stained than in the gastrula (Fig. 33). In the other cells of the trochophore, there was no local increase in basophilia, and no nucleoli were present.

DISCUSSION

The patterns of appearance and distribution of RNA in chiton oöcytes were similar to those described in other molluscan species (see Raven, 1958). The greatest relative volume increment occurred in the first stage of oöcyte growth. This was also apparently the stage in which the principal synthesis of cytoplasmic RNA occurred. In the successive stages of oöcyte growth cytoplasmic basophilia decreased, suggesting that either RNA synthesis had ceased entirely or that the rate of its synthesis was not commensurate with the oöcytes' growth in volume. The oöcyte nucleoli had also reached their maximum size and intensity of basophilia by the end of the first stage. Ranzoli (1953) reported a similar situation in the oöcytes of the gastropod *Patella*. RNA synthesis and nucleolar growth apparently reached a peak just prior to the initiation of protein yolk synthesis. In all of the published accounts of RNA distribution in relation to oöcyte growth in molluscs, a progressive decrease in cytoplasmic concentration has been noted as the oöcytes increased in size. RNA synthesis appears to be chiefly restricted to the first stage of oöcyte growth prior to protein yolk synthesis in *Chiton tuberculatum*, and if some cytoplasmic RNA synthesis continues after the nucleoli start to become depleted, as suggested by Swift *et al.* (1956), it is not sufficient to maintain cytoplasmic RNA concentrations at the level observed in the first stage of oöcyte growth during the subsequent stages. Further, the results of this investigation suggest that very little RNA synthesis occurs during the development of the chiton to the trochophore stage. The little synthesis that did occur was limited to a single kind of cell: namely, the cilia-bearing cells of the prototroch and apical tuft.

The single incidence of such a pattern of RNA distribution during oögenesis and early embryology would hardly represent sufficient grounds for proposing a general mechanism in mosaic development. Recently, however, Cowden and Markert (unpublished data) have investigated the distribution of RNA during the development of ascidian, *Ascidia nigra*, and here also no evidence of new synthesis of RNA was found at any stage from the second stage of gonadal oöcyte growth to the initiation of metamorphosis in the swimming larva. This is not true of all molluscan or ascidian embryos. Minganti (1950) reported that basophilia increased during the development of the gastropod *Lymnaea*, and according to Raven (1958), nucleoli initially appear in the nuclei of 24-cell stage embryos of *Lymnaea*. More recently Mancuso (1959) has described the appearance of nucleoli and increasing cytoplasmic basophilia in cells taking part in major morphogenetic events such as neural tube formation or myogenesis in the ascidian, *Ciona*. In mosaic development, however, primary differentiation does not occur at or just prior to gastrulation, but is fixed at the latest by the four-cell stage. While it is probable that the new synthesis of RNA may be initiated at any time during the later course of development, starting earlier in some species than in others, the production of new RNA does not appear to play any role in primary differentiation of molluscan or ascidian

embryos. To do so, synthesis would have to be initiated before the second cleavage, and none of the usual manifestations of RNA synthesis have been detected in early cleavage stages in any of the molluscan or ascidian embryos for which adequate cytochemical studies are available. If RNA has any function in primary differentiation, it must depend on RNA produced during oöcyte growth. Such a function could be possible if there were a heterogeneity in the distribution of qualitatively different kinds of RNA in the mature ovum. In this way the different kinds of RNA could be segregated into the blastomeres at cleavage. Many of the classical experiments on the effects of centrifugation on the development of mosaic eggs suggest that some form of cytoplasmic "structuring" is present in these eggs.

Protein yolk synthesis occurred primarily in the second stage of chiton oöcyte growth. These protein yolk granules were considerably richer in protein tryptophan than the other cytoplasmic proteins. Thus the synthetic activities of the oöcyte fell into two distinct phases: the elaboration of RNA-proteins and the production of specialized cytoplasmic proteins. Since the oöcyte is a very specialized cell, this dichotomy may not necessarily represent the patterns of synthetic activity of cells in general, but is probably true of other cell types which produce a particular kind of protein in quantity, *e.g.*, the mammalian reticulocyte. It is also interesting to note that the staining affinity for acid dyes of the cytoplasmic RNA-associated proteins was altered as the oöcytes approached the period of protein yolk synthesis. Since these dyes in acid solutions are generally bound at the sites of dissociated basic groups of proteins, such a shift in affinity would not be expected to depend on differences in kinds of reactive groups but rather on differences in the arrangement of dissociated reactive groups. According to Baker (1958), differential binding of acid dyes in mixtures probably depends on the size of the dye molecule, the spacing of its reactive groups, the degree to which the dye molecules tend to form aggregates, and the structural arrangement of the proteins in question. A shift in affinity, such as that observed in the later stage one chiton oöcytes, could indicate an alteration in the structure of the RNA-protein complex. Both Himes (1958) and Love and Liles (1959) have developed methods for detecting differences in the RNA-protein complex by alterations in the capacity of the RNA-moiety to bind basic dyes under certain conditions. Such alterations can also apparently be detected by staining methods designed to attach to the protein moiety. By the end of the first stage, the generation of cytoplasmic ribonucleoprotein (RNP) had essentially been completed, and an alteration in the RNP complex in preparation for protein synthesis might occur. This is particularly interesting in the light of recent electron microscope studies which have indicated that Golgi zones might be specializations of the endoplasmic reticulum. Bolognari (1960) has convincingly demonstrated that protein yolk synthesis in the oöcyte of two molluscan species occurs within the Golgi zones, and that these Golgi zones are composed of concentric lamellae which contain dark granules of about 300 Å diameter.

The functions of the oöcyte accessory cells and chorionic processes deserve some comment because of their unusual nature. The processes themselves seem to be hollow modifications of the chorion which presumably increase its surface area and facilitate exchange. The oöcyte accessory cells were involved in the construction of the process since each process grew outward into an accessory cell and the accessory cells were absent when the processes were completely formed. The oöcyte

accessory cells were also probably involved in the construction of the chorion. When the accessory cells achieved their maximum size and number, the chorion appeared between the accessory cells and the cortex of the oöcyte. The role of these cells may not necessarily involve the production of chorionic material since there was an abundance of cortical PAS-positive and protein material in the oöcyte, but they may produce a tanning agent which hardens the chorionic material. In other invertebrates which have tough chorions such a mechanism is involved. Monné (1955) and Smyth (1956) have investigated chorion formation in a number of invertebrates, and some form of quinone tanning was involved in each case.

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SUMMARY

1. Oöcyte growth and development and the subsequent development of the egg to the swimming larva stage were investigated in the chiton, *Chiton tuberculatum*, using topological cytochemical methods for nucleic acids, proteins and mucopolysaccharides.

2. The growing oöcytes exhibited two phases of synthetic activity: the first phase was chiefly concerned with RNA production and the second phase with protein yolk synthesis.

3. A probable alteration in the cytoplasmic RNA-protein complex prior to the initiation of yolk synthesis was detected by an alteration of the affinity of RNA-associated proteins for binding acid dyes in mixtures.

4. Oöcyte accessory cells were probably responsible in some way for chorion formation. After the chorion was formed, ornate hollow processes grew out of the chorion, each beneath an accessory cell. When development of the processes was completed, the accessory cells had disappeared.

5. There was no evidence of new synthesis of RNA in any of the cells of the developing embryo except the cells of the prototroch and apical tuft. This applies to the complete developmental history of the oöcyte from the cessation of RNA synthesis prior to protein yolk formation in the oöcyte to the swimming trochophore larva.

6. Since primary differentiation occurs in mosaic embryos in the absence of synthesis of new RNA, the possibility that primary differentiation is controlled by the RNA produced in the oöcyte was discussed.

7. Although there was no differential segregation of protein yolk into the blastomeres—both RNA-basophilia and protein yolk being evenly distributed in all blastomeres—the posterior blastomeres received a larger proportion of yolk material than the micromeres. This was particularly evident in the yolk-laden cells of the trochophore gut.

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