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MOVEMENTS OF ECHINOCHROME PIGMENT GRANULES IN SEA URCHIN EMBRYOS

by

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MOVEMENTS OF ECHINOCHROME PIGMENT GRANULES
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Abstract

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Characteristic movements of echinochrome pigment granules are known to accompany specific events in the embryonic development of the sea urchin Arbacia punctulata. Pigment granules in unfertilized eggs exhibit randomly directed saltatory movements. At fertilization, the majority of these granules migrate to the egg cortex and become embedded therein. During cleavage, the echinochrome granules tend to accumulate in the cortex of the furrow region and decrease in concentration at the poles; this may reflect cortical activities rather than individual granule movements. At the 4-cell stage, pigment granules begin to migrate out of the region which will become the cortex of the micromeres, leaving a "clear area" on each blastomere. This investigation has been concerned with processes involved in each of these movements, with particular emphasis on clear area formation and its relationship to mitosis and cleavage.

Prefertilization saltation, migration to the cortex at fertilization and formation of clear areas in the presumptive micromere region occurred normally in eggs treated with colcemid or vinblastine, but were inhibited by cytochalasin B. This suggests that these movements do not require microtubules, but may involve microfilaments or other cytochalasin B-sensitive structures.

Inhibition of cleavage by cytochalasin B did not prevent the accumulation of pigment granules in the furrow region. Ruffling of the hyaline layer above the resulting band of pigment was also observed in these eggs. Thus, certain processes associated with cleavage can continue in the absence of furrowing.

Migration of pigment granules out of the presumptive micromere region to form clear areas is the earliest visible sign of differentiation of micromeres. In the present study, this process occurred at the normal time without previous mitosis or cleavage in eggs which were blocked before pronuclear fusion or at metaphase with colcemid or vinblastine, or during prophase with Y-radiation or puromycin. This suggests that formation of clear areas is part of a cycle of cortical events involved in differentiation which is independent of mitosis and cleavage.

Movements of individual pigment granules leading to clear area formation in colcemid-treated, non-dividing eggs were oriented predominantly toward the perimeter of the presumptive micromere region. No long saltatory movements were observed in this region and rates of granule movement were comparable with those in other por-

tions of the cortex of eggs at the same stage or in the cortex of fertilized eggs prior to division.

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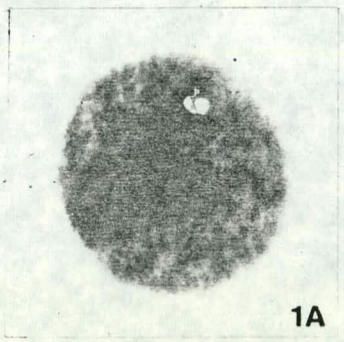
INTRODUCTION

Eggs of the sea urchin Arbacia punctulata contain echinochrome pigment granules which undergo characteristic movements during early embryonic development. These movements provide a system for investigating both the process of pigment granule movement and the cortical phenomena involved in cleavage and cellular differentiation, particularly the formation of micromeres.

1. Movements of echinochrome granules in normally-developing eggs

In unfertilized Arbacia eggs, the membrane-bound echinochrome granules (0.5 - 2.0u in diameter) are distributed throughout the cytoplasm and exhibit randomly directed saltatory movements. Each saltation consists of a non-Brownian, "jump-like" movement along a straight path of 1 - 5u at a speed within the range of 0.3 - 0.8u/sec (Parpart, 1953; 1964). Within about ten minutes after fertilization, nearly all of the pigment granules migrate to the cortex of the egg (McClendon, 1909) (Figure 1) and cease to saltate (Parpart, 1964). The echinochrome granules which do not reach the cortex (up to 15%) continue to display saltatory movements and later become associated with the asters of the mitotic apparatus (Rebhun, 1972). Pigment granules in the cortex of fertilized, non-dividing eggs show a slow, seemingly random migration (Scott, 1960). During the first cleavage, the concentration of pigment granules increases in the furrow region

Figure 1. Unfertilized and fertilized Arbacia eggs. In unfertilized eggs, pigment granules are scattered throughout the cytoplasm [A) surface and B) equatorial views]. After fertilization, the majority of pigment granules have migrated to the cortex [C) surface and D) equatorial views]. x 480.



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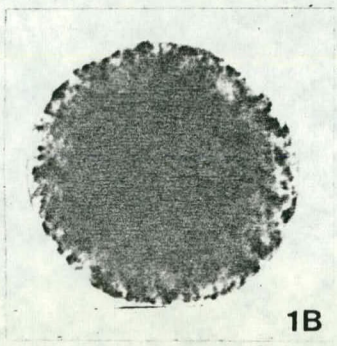

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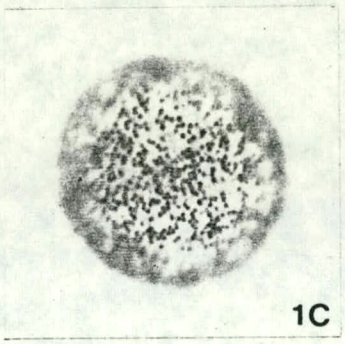

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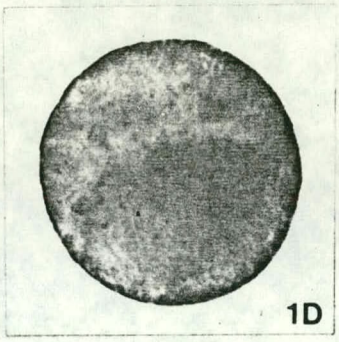


1D

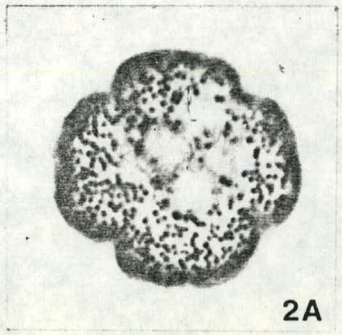
and decreases at the poles. Thereafter, pigment granules become evenly distributed in the cortex of the resulting blastomeres, then accumulate preferentially at the poles, and finally return to a uniform distribution before the next cleavage (Dan, 1951; 1954). These echinochrome granule movements associated with the division cycle are thought to reflect the activities of portions of the cortex rather than the independent movement of granules (Dan, 1954; Scott, 1960).

The fourth cleavage of the sea urchin embryo is unequal, giving rise to a ring of eight mesomeres at the animal pole, a quartet of macromeres near the equator and a quartet of micromeres at the vegetal pole. In Arbacia, the micromeres contain few, if any, pigment granules (Figure 2). During the 4-cell stage, pigment granules begin to migrate out of the region that will become the micromeres, leaving a "clear area" on each blastomere (Figure 3). Occasionally, this movement can be detected as early as the 2-cell stage (Morgan, 1893). The formation of micromeres is one of the earliest visible steps in the differentiation of the embryonic axis. The prior disappearance of pigment from that region suggests that the cortex differentiates at an even earlier stage (see Section 4). The vegetal pigment-free zone is maintained through the blastula stage (Figure 4). At the beginning of gastrulation, the unpigmented micromere derivatives migrate into the blastocoele forming primary mesenchyme cells, which will eventually secrete the larval skeleton. Pigment granules remain evenly distributed over the outer surface of the embryo until the early pluteus stage, at which time groups of twenty to thirty

Figure 2. Sixteen-cell embryo [A) vegetal and B) lateral views]. The concentration of echinochrome granules is much lower in the micromeres (MI) than in either the macromeres (MA) or the mesomeres (ME). x 480.

Figure 3. Four-cell embryo. Echinochrome granules have begun to migrate out of the region which will become the micromeres at the 16-cell stage, leaving a "clear area" on each blastomere. x 480.

Figure 4. Blastula. The derivatives of the micromeres can be identified by the low concentration of echinochrome granules. x 480.



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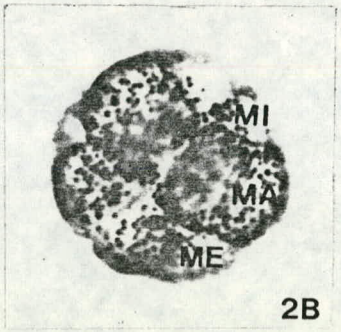

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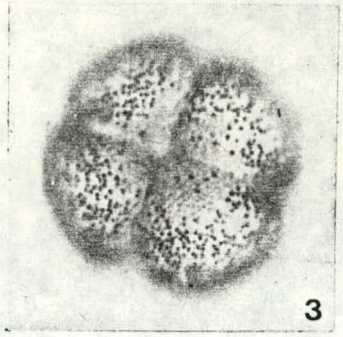

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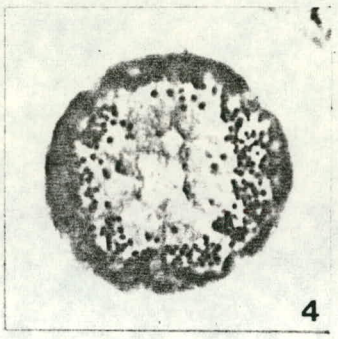

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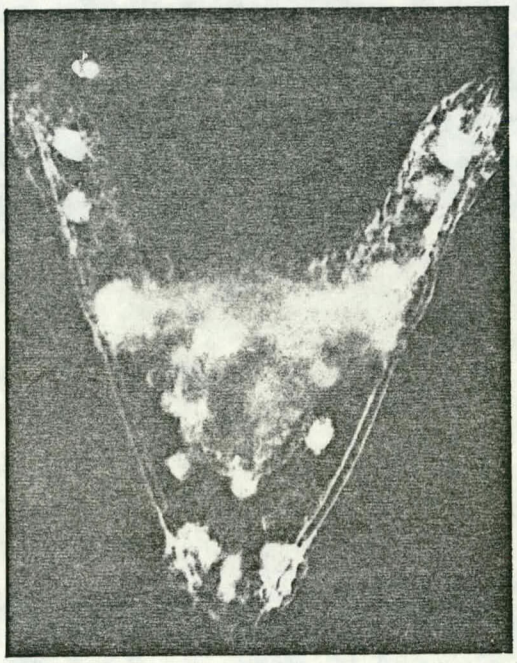
granules coalesce, forming large pigment spots (Figure 5). In the adult Arbacia, echinochrome pigment is located in the red amoebocytes of the perivisceral fluid and in the test (exoskeleton) as well as in the eggs (Harvey, 1956).

2. Properties of echinochrome pigment

The functions of echinochrome pigment during both embryonic development and adult life are unknown. The name "echinochrome" (MacMunn, 1885) designates not one, but a family of polyhydroxynaphthoquinone pigments, which occur in a number of echinoid species (Goodwin, 1969). Friedheim (1932) claimed that exogenously applied echinochrome increases respiration sixteen-fold in Paracentrotus lividus and Sphaerechinus granularis, but Tyler (1939) showed that this does not occur in Strongylocentrotus purpuratus. Hartmann and his colleagues (1939) proposed that echinochrome is a sperm activating agent in Arbacia lixula; this was disputed by Tyler (1939) for S. purpuratus and by Cornman (1941) for A. punctulata. Echinochrome from A. punctulata amoebocytes seems to be the oxidant in a reversible oxidation-reduction reaction, but does not appear to form a dissociable compound with molecular oxygen (Cannan, 1927). Vlès and Vellinger (1928) demonstrated that echinochrome from A. punctulata eggs is an indicator of pH, appearing orange below pH 4, violet between pH 4 and pH 6, and yellow above pH 6. Echinochrome is apparently bound to protein in Arbacia lixula eggs (Kuhn and Wallenfels, 1939).

Figure 5. Pluteus larva. Echinochrome granules have aggregated into groups, forming large pigment spots, which appear white in this negative image. x 480.



Since eggs of many sea urchin species contain no echinochrome, it seems probable that echinochrome granules are unnecessary for normal development and that their movements are primarily indicative of other developmental processes. The present investigation has been particularly concerned with the migration of echinochrome granules out of the region which will become the micromeres of the 16-cell stage.

3. The role of micromeres in sea urchin development

The small, vegetal cells known as micromeres are not only the progenitors of the primary mesenchyme cells responsible for skeleton formation (see Section 1), but are also capable of exerting an inductive influence on surrounding blastomeres. As mentioned previously, the micromere derivatives migrate into the blastocoele just before the formation of the archenteron (gastrulation). A secondary archenteron can be induced by transplantation of a quartet of micromeres from a 16-cell stage onto a complete 32-cell embryo. An archenteron can also be induced by implantation of a quartet of micromeres onto the equatorial surface of an isolated animal half-embryo, which normally does not gastrulate (see Horstadius, 1939). Micromeres have been extensively studied in an attempt to find specific cellular characteristics which might explain their ability to induce formation of the archenteron.

In ultrastructural studies, only the micromeres have been shown to form cytoplasmic connections with the other blastomeres of the 16-cell stage. Portions of the cell membranes between micromeres

and adjoining blastomeres apparently disappear during interphase, leaving rows of vesicles (Pucci-Minafra et al., 1968; Hagström and Lönning, 1969). This occurs both in situ and after implantation of a micromere onto a macro- or mesomere (Hagström and Lönning, 1969; Lönning and Hagström, 1971). Cytoplasmic streaming and movement of granules between micromeres and macromeres have been observed in time lapse films (Hagström and Lönning, 1965; 1969), demonstrating that these intercellular bridges are open connections which allow cytoplasmic exchange between blastomeres. Isolated micromeres in culture exhibit formation of pseudopodia, amoeboid activity, cytoplasmic streaming and nuclear pulsation. These phenomena have not been observed in macromeres or mesomeres and are most evident in aggregates of micromeres during interphase, when open connections exist between the cells (Hagström and Lönning, 1965; 1969; Pucci-Minafra et al., 1968). The increased cytoplasmic mobility of the micromeres as well as their ability to form syncytia with neighboring blastomeres may facilitate transport of substances involved in induction of the archenteron.

In histological sections of 16-cell embryos stained with Giemsa, Agrell (1958) observed a transient, preferential staining of the micromeres during interphase. [Interphase is prolonged in the micromeres, which thus cleave at a slower rate than either macro- or mesomeres (Theel, 1892; Kuhl and Kuhl, 1949; Zeuthen, 1951).] Treatment with ribonuclease removed the cytoplasmic staining from all blastomeres. Thus, Agrell concluded that only the micromeres active-

ly synthesize RNA during the 16-cell stage. His observation stimulated a series of investigations of the relationship of RNA synthesis and transport to specific micromere functions. When 16-cell stages were pulse-labelled with ^{3}H-uridine, the label was found almost exclusively over the micromeres in autoradiographs (Czihak, 1965), thus supporting Agrell's conclusion. However, if embryos were pulse-labelled with ^{3}H-uridine at the 16-cell stage and then incubated in sea water for an hour before fixation, label was observed in the other blastomeres as well. When micromeres labelled with ^{3}H-uridine were transplanted onto unlabelled animal half-embryos, label appeared in the cytoplasm of the animal blastomeres (Czihak and Hörstadius, 1970). If 16-cell stages were treated with 8-azaguanine, an antimetabolite known to produce defective RNA (Bamberger et al., 1963), no archenteron formed, even though the primary mesenchyme migrated normally and a skeleton was subsequently produced (Czihak, 1965). These observations suggest that most of the RNA produced at the 16-cell stage is synthesized in the micromeres and can be transported to adjoining blastomeres. RNA produced at this time seems to be specifically involved in formation of the archenteron in whole embryos, and thus, micromere RNA might be the agent responsible for the induction of an archenteron by transplanted micromeres.

In contrast to the earlier observations, Hynes and Gross (1970) have found that micromeres incorporate ^{3}H-uridine into RNA at only five to eight times the rate (per milligram protein) of mesomeres or macromeres respectively. Incorporation was measured in

suspensions of the three cell types separated on Ficoll gradients after pulse-labelling of 8- to 16-cell embryos. Using a similar technique, Spiegel and Rubinstein (1962) found three- to five-fold differences in the rates of incorporation between micromeres and mesomeres or macromeres respectively. These authors maintained that such differences in incorporation rates of micromeres as compared with other blastomeres cannot account for the autoradiographic observations described above (i.e., Czihak, 1965; Czihak and Hörstadius, 1970). In addition, Spiegel and Rubinstein (1972) observed no transport of labelled RNA from micromeres to other blastomeres in reaggregation experiments. They suggest that changes in precursor pools and inadequate dilution of excess labelled precursor may explain these discrepancies.

In summary, it appears that RNA is not synthesized exclusively in the micromeres of the 16-cell stage, but is produced there at a higher rate than in the other blastomeres. Migration of labelled material from the micromeres to adjoining blastomeres has been demonstrated, but it is not certain whether this consists of RNA or RNA precursors. Although some species of RNA produced during the 16-cell stage seems to be involved in the induction of the archenteron, the possibility that micromere RNA is the specific inducer requires further investigation.

Electron microscopy has failed to reveal any organelle or inclusion which is unique to the cytoplasm of the micromeres (Pucci-Minafra et al., 1968; Hagström and Lönning, 1969; Lönning and Hagström,

1971). Pucci-Minafra and colleagues (1968) reported that the cytoplasm of isolated micromeres appeared to be richer in vesicles of the endoplasmic reticulum than that of macro- or mesomeres. They also suggested that the concentration of mitochondria is higher in the micromeres, but direct counts were not reported. Gustafson and Lenique (1952) have reported increasing numbers of Nile blue sulfate-staining granules, presumably mitochondria, in cells nearer to the animal pole in sea urchin gastrulae. However, in ultrastructural studies of both 16-cell stages and gastrulae of several sea urchin species, Berg and his colleagues (1962) observed no differences in the concentration of mitochondria between animal and vegetal blastomeres. Vegetal pole mitochondria appeared larger in thin sections of gastrulae, but no differences in mitochondrial size were observed in the blastomeres of the 16-cell stage (Berg and Long, 1964). In a recent ultrastructural study, Lönning and Hagström (1971) found that the ratio of the number of mitochondria to the number of yolk granules is twice as high in the micromeres as in the macro- or mesomeres. However, they did not calculate the number of mitochondria per unit volume in the three cell types. As yet, investigations into the possible role of mitochondria in micromere specialization have failed to demonstrate any pronounced differences in the size or concentration of these organelles between micromeres and other blastomeres of the 16-cell stage.

One cytological feature which distinguishes the micromeres, at least in Arbacia embryos, is the near absence of certain granular inclusions from these blastomeres. Most of the granules which stain

metachromatically with toluidine blue (meta-granules) seem to be excluded from the cytoplasm of the micromeres. In macromeres, mesomeres and all blastomeres of earlier stages, the meta-granules are found in a region which encircles the asters or the nucleus (Burchill, 1963). Breakdown of these granules appears to be necessary for artificial induction of furrowing in Arbacia eggs (Marsland, Zimmerman and Auclair, 1960) and their presence seems to determine which fragments of centrifuged eggs are able to cleave (Kojima, 1959a; 1959b). Meta-granules appear to exhibit acid phosphatase activity and are believed to be lysosomes (Burchill, 1963). If these interpretations are correct, the absence of meta-granules could have a profound effect on the physiology of the micromeres.

In Arbacia, the cortex of the micromeres is nearly devoid of echinochrome pigment granules, which were evenly distributed in the cortex of fertilized eggs before cleavage. Migration of echinochrome granules out of the micromere cortex begins as early as the 2-cell stage (see Section 1). This was regarded by Morgan and Spooner (1909) as an indication of concurrent influx of other materials. Such a process could introduce specific substances into the micromere region; alternatively, exclusion of materials alone might confer functional specificity on the micromeres. In any case, the movement of pigment out of the micromere cortex suggests that this cytoplasmic layer is involved in early events leading to the differentiation of micromeres.

4. The role of the egg cortex in micromere differentiation

The mechanical properties and behavior of the egg surface appear to be determined not only by the plasma membrane, but also by an underlying layer of cytoplasm known as the cortex. This region is often characterized by the presence or absence of specific granules or organelles. Cortical granules in unfertilized sea urchin eggs (Mercer and Wolpert, 1962; Gross et al., 1960) and pigment granules in Paracentrotus (Harvey, 1933) and fertilized Arbacia eggs (Brown, 1934) are localized in the cortex and cannot be dislodged by centrifugation. On the basis of micromanipulation studies, Chambers (1917) suggested that the cortex is a region of gelated or high viscosity cytoplasm. When fertilized Arbacia eggs were centrifuged at high hydrostatic pressures, thought to liquify certain types of gels, Brown (1934) observed that pigment granules formerly embedded in the cortex could be displaced. This led to the hypothesis of a "cortical gel" which is liquified by exposure to high pressures and may account for some of the structural properties of the egg surface (Marsland, 1939; 1950; 1956). The concept of the cortex as a distinct morphological entity was further supported by the discovery of several methods for the isolation of cortical fragments and intact cortical shells (Motomura, 1954; Allen, 1955; 1957; Sakai, 1960).

The thickness of the cortex, based on measurements of the peripheral granular layer observed in the hyaline zone of centrifuged eggs, is between 1.5μ (Mitchison, 1956) and 6.0μ (Marsland and Landau, 1954). The apparent width of the cortical gel, determined as

the distance between the edge of an injected oil droplet and the plasma membrane of the advancing furrow, is 4 - 5μ (Chambers and Kopac, 1937). When a microneedle was drawn through the cytoplasm, a narrow cortical layer remained immobile. Its thickness was estimated to be 3μ (Hiramoto, 1957). Electron microscopy has revealed no structural specialization of the cortical cytoplasm which might correspond to a gel layer of these dimensions. A much narrower layer (0.1 - 0.2μ) of 40 - 60 Å filaments has been observed just under the plasma membrane, primarily in the furrow region during cleavage (Mercer and Wolpert, 1958; Weinstein, 1964; Schroeder, 1972). Bundles of similar filaments, which form the cores of surface microvilli, also extend down into the cortical region of Strongylocentrotus eggs (Harris, 1968).

Since the extent and character of the cortical layer remains somewhat uncertain, the term cortex will be used here to refer simply to the layer of undetermined thickness in which the echinochrome granules become embedded at fertilization.

Changes in the birefringence and the rigidity of the cortex occur at fertilization and continue in cycles associated with the cleavage process. Cortical birefringence disappears at fertilization, returns during the sperm aster stage, and reaches a high level during metaphase and telophase (Monroy and Montalenti, 1949; Mitchison and Swann, 1952). The rigidity of the cortex increases just after fertilization, then returns to a low level until mitosis, and finally reaches a maximum during anaphase (Mitchison and Swann,

1954). Although these cortical changes appear to be involved in cleavage, they also occur in colchicine-treated, non-dividing eggs, demonstrating the independence of certain cortical events from mitosis and cleavage.

Regional differences in the properties of the cortex have also been observed during the cleavage process. Eggs burst preferentially in the polar regions when cytolysis was induced by dilute sea water (Just, 1922; Chambers, 1938), and several other agents (Kuno, 1954). However, cytolysis occurred in the furrow region alone when cleaving eggs were treated with sodium tungstate or acidified sea water. The formation of rows of blisters in the furrow region was observed in eggs exposed to alkali or heat shock (Kuno, 1954). The furrow cortex appeared to bulge out in eggs placed in hypertonic sea water after heat shock or in eggs treated with dinitrophenol or sodium azide (Kuno-Kojima, 1957).

Cyclic changes and regional differences in the cortex of the sea urchin egg also appear to determine both the time and the site of micromere formation. Driesch (1893; 1906) observed that micromeres appeared at the normal time after fertilization in eggs developing in dilute sea water. Since mitosis was delayed by this treatment, micromeres were produced when the embryos had only reached the 8-cell stage. When phenyl urethane was used to induce mitotic delay, Painter (1915) observed the formation of micromeres as early as the 4-cell stage. He proposed that

> . . . at the time of fertilization progressive changes, which go on independently of the nucleus and of cleavage, are initiated in the cytoplasm of the eggs . . . and . . . differentiation, as far as the formation of the micromere is concerned in the sea urchin egg, is dependent upon cytoplasmic oxidation, the nucleus and the cleavage process playing no direct part here. (Theophilus Painter, 1915)

Micromeres can also form as early as the 2- or 4-cell stage in embryos in which mitotic delay has been induced by X-radiation (Rustad, 1960), UV-radiation (Ikeda, 1965) or mustard gas (Harvey, 1956). Thus, the time of micromere formation seems to be independent of the number of mitoses or cleavages which have occurred.

In eggs centrifuged before fertilization, Morgan and Spooner (1909) noted that the site of micromere formation is unrelated to the axis of stratification. Micromeres always appeared at the intersection of cleavage planes nearest to the vegetal pole. This suggests that the position of the micromeres is determined by some cytoplasmic component (such as the cortex) which cannot be displaced by centrifugation.

It appears that a series of cortical changes is initiated at fertilization, continues independently of mitotic and cleavage cycles and may be involved in the differentiation of micromeres. The movement of pigment granules in Arbacia embryos represents another series of cortical events during development. A portion of this investigation has been devoted to observations of these pigment granule movements and their relationships to other embryonic processes such as mitosis and cleavage.

5. Mechanisms of intracellular organelle movement

The movement of echinochrome granules in Arbacia eggs is one example of selective translocation which can result in the unequal distribution of a particular organelle within a cell. Such differential movement is essential for the normal functioning of many adult cell types and also appears to be involved in localization processes in developing embryos. Organelle movement (primarily saltation) has been directly observed in a number of systems. Metachromatic β-granules in Spisula eggs (Rebhun, 1959; 1963; 1964) and yolk granules in Pectinaria eggs (Rebhun, 1964) undergo saltatory movements in the astral regions of the mitotic apparatus. Pigment granules move to and from the center of chromatophores in the skin of Fundulus, causing changes in the color of the fish (Bikle et al., 1966; Green, 1968). In cultured cells, a variety of particles including pinosomes, lysosomes, lipid vesicles and ingested carbon particles display saltatory movements (see Freed and Lebowitz, 1970). Observation of axonal flow in cultured neurons has revealed independent movements of certain granules (Burdwood, 1965; Pomerat et al., 1967; Chang, 1972). Isolated droplets of cytoplasm from the internodal cells of the algae Nitella and Chara also contain particles which exhibit saltatory movements (Kamiya, 1959).

The kinetics, degree of orientation and specificity of granule movement vary in different systems. Although many hypotheses have been proposed to explain these movements, in no case is the mechanism of movement thoroughly understood (see review by Rebhun,

1972). The occurrence of Brownian processes in low viscosity channels has been proposed as one possible explanation. Another hypothesis involves the generation of local electric fields by waves propagated along cytoplasmic filaments (Hejnowicz, 1971). An interaction resembling that of actin and myosin in muscle has also been postulated between the particle and some cytoplasmic fiber. On the basis of electron microscope studies, microtubules (250Å) and microfilaments (50Å) have been suggested as linear elements which might be involved in particle movements in several systems (reviewed by Rebhun, 1972).

Colchicine, which seems to disrupt microtubules, does not interfere with prefertilization saltation of echinochrome granules or with their migration to the cortex at fertilization (Rebhun, 1967). Cytochalasin B, a mold metabolite which is thought to disrupt microfilaments, does inhibit saltatory movements of pigment granules in unfertilized Arbacia eggs (Van Wie, 1969). However, saltation of the echinochrome granules which do not migrate to the cortex at fertilization continues in the presence of cytochalasin B, but is inhibited by colchicine (Rebhun, 1972b). These observations suggest the possibility that echinochrome granule movements can involve either microfilaments or microtubules, depending on the location of the granules and the stage of development of the embryo. However, no direct association between pigment granules and microfilaments or microtubules other than spindle microtubules has been observed in electron micrographs of Arbacia eggs. This might be a result of the difficulty in preserving the structure of the cytoplasm of the Arbacia egg during

fixation.

Part of the present investigation has been devoted to further observations of pigment granule movements in normal eggs and in those exposed to colcemid (N-desacetyl-N-methyl colchicine) and cytochalasin B.

6. Purpose

This investigation has been concerned with the movements of echinochrome granules in Arbacia embryos both as examples of intracellular organelle movements and as a series of cortical changes related to embryonic development. Using colcemid and cytochalasin B, an attempt was made to determine whether microtubules and/or microfilaments might be involved in these movements. In the course of this work, it was observed that the migration of pigment granules out of the presumptive micromere region occurred at the normal time in colcemid-treated, non-dividing eggs. This observation suggested that pigment granule migration out of the micromere region, which is an early cortical event related to micromere differentiation, might be independent of the mitotic and cleavage cycles. In order to test this hypothesis, the occurrence of this phenomenon was studied in eggs in which mitosis and/or cleavage had been inhibited and in eggs exposed to a variety of other experimental treatments. An additional objective has been the characterization of the movements of individual pigment granules during their migration out of the presumptive micromere region, by means of time-lapse photomicrography. Movement

during this process has been compared with those of echinochrome granules in other stages and regions of the embryo.

MATERIALS AND METHODS

1. Obtaining and handling gametes

Adult Arbacia punctulata were collected by the Supply Department of the Marine Biological Laboratory, Woods Hole, Massachusetts, or purchased from Glendle Noble, Panama City, Florida. Animals were maintained in running sea water or in aerated, refiltered Instant Ocean in a circulating aquarium (Model 110 C, Aquarium Systems, Eastlake, Ohio).

Spawning was usually induced by application of 10 - 12 volts (AC) across the test of the animal by means of a Powerstat variable transformer (Superior Electric Company, Bristol, Connecticut) (Harvey, 1952). Occasionally, gametes were obtained by injection of 0.53M KCl into the coelom (Tyler, 1949). Eggs were shed into a beaker of filtered (Whatman #1 filter paper) sea water or Instant Ocean; sperm were collected "dry" using a Pasteur pipette. Prior to fertilization, eggs were washed three times with filtered sea water. Dilute suspensions of eggs in petri or stender dishes were fertilized with a few drops of a dilution of sperm in sea water approximately equal to 1:1000. Embryos were allowed to develop at room temperature which was constant for any one experiment and varied between 20° and 26°C in different experiments.

2. Experimental treatments

Observations of pigment granule movements were made on both normal eggs and those exposed to a variety of agents. All chemicals used were dissolved in filtered sea water, Millipore-filtered sea water or Instant Ocean. Results of experiments using Instant Ocean for maintenance of animals, culture of embryos or preparation of solutions were always verified using sea water. Pigment granule movements were studied in eggs treated with agents which inhibit mitosis by interfering with microtubule function. Eggs were exposed to 10^{-6} - 10^{-2}M colcemid (CIBA Pharmaceutical Company, Summit, New Jersey) or to 10^{-5} - 10^{-4}M vinblastine sulfate (Velban, Eli Lilly and Company, Indianapolis, Indiana). Observations of pigment granule movements were also made on eggs in which mitotic delay had been induced by exposure to up to 250kR of γ-radiation from a cesium^{137} source. The dose rate used was approximately 4kR/minute. In order to investigate the movement of pigment granules in eggs in which cleavage (cytokinesis), but not mitosis (karyokinesis) was inhibited, eggs were treated with 0.5 - 5.0μg/ml cytochalasin B (a generous gift of S. B. Carter, Imperial Chemical Industries Limited, Macclesfield, England). This mold metabolite is thought to act by selective destruction or disorientation of 40 - 60Å microfilaments, but this interpretation has been questioned (see **DISCUSSION**). Cytochalasin B (CB) was dissolved in dimethyl sulfoxide (DMSO) and sea water was added to make a stock solution of 5μg/ml CB and 0.5 per cent DMSO. Parallel observations were made on eggs treated with a solution of

0.5 per cent DMSO in sea water. Observations of pigment granule movements were made on eggs in which protein synthesis was inhibited by treatment with 10^{-4}M puromycin (Nutritional Biochemicals Corporation, Cleveland, Ohio). Pigment granule movements were also investigated in eggs which had been incubated at temperatures of -2° - 8°C for various periods of time.

Eggs were exposed to concentrations of sea water ranging from 50 to 200% in an attempt to determine if the pigment-free cortex of the micromeres differs in mechanical strength from the rest of the egg surface. Equal volumes of distilled water and filtered sea water were combined to make 50% sea water. Sodium chloride (0.3g/ml) was added to filtered sea water to make 200% sea water. In 50% sea water, localized evaginations of the egg surface were formed. At concentrations approaching 200% sea water, one or several invaginations were produced. At the time of migration of pigment granules out of the micromere region, both normal and colcemid-treated eggs were placed in concentrations of sea water which produced single evaginations or invaginations on most eggs. The location of the evagination or invagination with respect to that of the "clear area" was noted. For some of these experiments, fertilization membranes were removed with 1M glycerol according to the method of Kane (1970).

3. Light microscope observations and time lapse photomicrography

Eggs were observed and photographed in sealed chambers, which consisted of a glass slide and a coverslip separated by a ring of a

vaseline-paraffin mixture. All observations were made with a Zeiss GFL microscope, using either a 16X or a Neofluar 40X objective with 10X oculars. A 100 watt lamp in a Zeiss large lamp housing was used with a Kodak Wratten #11 filter, a Zeiss Grünfilter VG 9 and a Zeiss Wärmeschutzfilter. Photomicrographs were taken on Kodak High Contrast Copy film, High Speed Ektachrome B film or Kodachrome II film, using a Leitz MIKAS attachment with a 35mm camera back.

For analysis of pigment granule movements, single eggs were photographed every thirty or sixty seconds for up to fifty minutes using the 40X objective. Each frame in a given series was enlarged approximately twelve times and printed on a single sheet (8 x 10 in or 8½ x 11 in) of Kodabromide F-5 paper. The overall magnification of the prints was approximately 4800 times life size (i.e., 1mm = 0.2μ).

4. Procedures for analysis of pigment granule movements

The purpose of this analysis has been to characterize the migration of pigment granules out of the presumptive micromere region, with respect to the following questions: (1) Are the movements of individual granules directed toward the perimeter of the micromere region, or are they random in direction? (2) Are the movements of pigment granules leading to the formation of a clear area quantitatively similar to pigment granule movements observed at other stages? The measurements to be described were made on time lapse photomicrographs of colcemid-treated, non-dividing eggs during

the formation of a clear area. For comparison, the same measurements were made on another region of an egg at the same stage and on both normal and colcemid-treated, fertilized eggs, in which the pigment granules had migrated to the cortex, but in which cleavage had not yet begun.

In order to trace the movements of individual pigment granules, a number was assigned to each of the granules in focus on a print of the first frame of a series (approximately 100 to 200 granules per egg). These granules were numbered accordingly in successive frames. The following records were kept to determine which granules could be followed throughout the period of filming and would thus be suitable for the analysis of individual movements. For each frame, the numbers of any granules which could not be identified with certainty were recorded. A record was also kept of which granules disappeared from the focal plane or became indistinguishable from surrounding granules (usually at the edge of the region in focus) in each frame.

a. Paths of individual granules during clear area formation

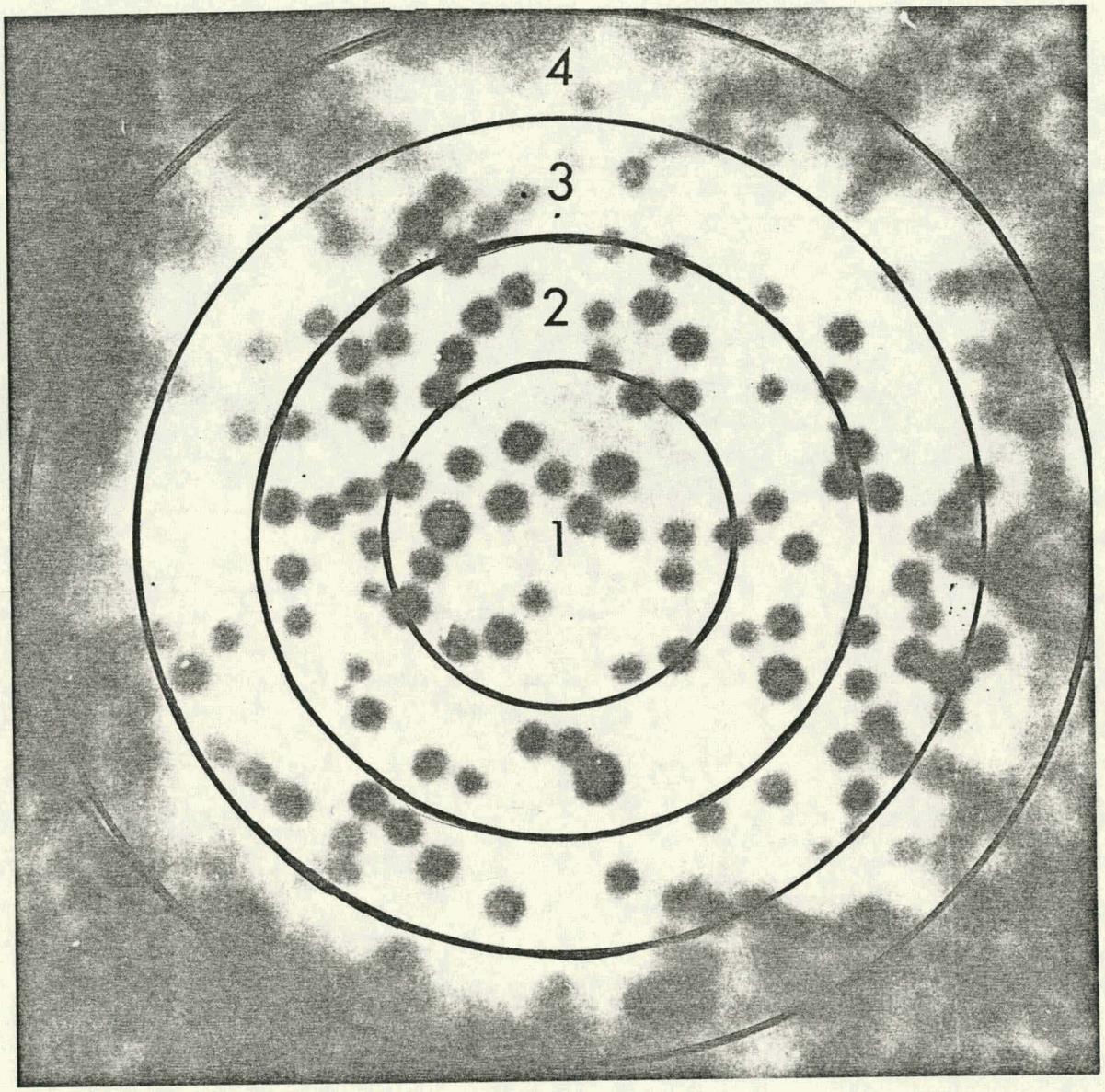
The paths of individual granules were traced in order to obtain a qualitative description of pigment granule movements during the stages considered. A grid of 0.5 cm squares drawn on a sheet of transparent plastic was placed over each frame in a given series and the coordinates of the centers of particular granules were recorded. The coordinates were rounded to the nearest 0.25cm and measurements were made on frames taken at 2 minute intervals. In each series, the

paths of a total of twenty to thirty granules were traced. Granules were chosen to provide an approximately even distribution over the region photographed. This sample included granules which moved relatively long distances and others which remained nearly stationary. Several pairs of granules which were initially very close together were also traced in each series.

b. General orientation of pigment granule movements during clear area formation

To estimate the degree to which pigment granule movements are oriented toward the perimeter of the micromere region, the number of granules which moved toward the edge was compared to the number which moved nearer to the center during the formation of a clear area. For recording the positions of pigment granules relative to the center of the clear area, a circle, divided into four concentric rings, was drawn on a sheet of transparent plastic (Figure 6). The diameter of this circular grid was slightly smaller than that of a completed clear area in the enlargements used. The grid was centered with respect to the center of the completed clear area in the final frame of a series. Using the perimeter of the egg as a frame of reference, the grid was then transferred to the first frame of the series. The ring in which each granule was located was recorded for both the first and last frame of a given period of filming. If a granule lay on the borderline separating two rings and was equally distributed between them, the number recorded was that of the ring nearer to the center. Calculations were then made of the percentages of the gran-

Figure 6. Illustration of the use of a circular grid to record positions of individual granules with respect to the center of the clear area being formed. x 4800.


4
3
2
1

ules initially in a given ring which had remained in that ring or moved nearer to, or farther from the center of the clear area.

c. Quantitative comparison of pigment granule movements in different stages and regions

Measurement of changes in distances between members of pairs of granules was chosen as a method of comparing movement of pigment granules in different stages and regions. Values obtained for changes in inter-granule distance over a given period of time will reflect the rates of movement of a population of granules within a region. However, these values cannot be used to calculate exact rates for individual granules within that region, since integral values can result from movement of one or both members of a pair and zero values may represent parallel movement of both granules as well as lack of movement by either one.

For each series of frames, ten granules were chosen to provide an approximately even distribution over the region photographed. The center-to-center distances (± 1mm) between each granule and the other nine were measured in each photomicrograph of a series of ten, taken at one minute intervals (Figure 7, columns A, B, . . . J). The differences between measurements for one frame and corresponding measurements for the next frame were then computed (Figure 7, columns 1, 2, . . . 9). The relative frequencies of occurrence of values obtained in this manner were calculated for each series. The average change in inter-granule distance was also calculated. These frequency distributions and average values were used to compare the movements

Figure 7. Measurement of changes in distances between pairs of granules. Columns A, B, ... J are measurements (mm) of the distance between members of 45 granule pairs in enlargements of frames taken at one minute intervals. Columns 1, 2, ... 9 are the absolute values of the differences between corresponding measurements from successive frames. Figures in Row X are the average changes in inter-granule distance for each minute interval.

Column	A	1	B	2	C	3	D	4	E	5	F	6	G	7	H	8	I	9	J
Frame	1		3		5		7		9		11		13		15		17		19
Granule pairs:																			
12-29	44	1	45	2	47	4	43	3	40	0	40	1	41	2	39	4	43	1	44
12-35	80	1	81	1	82	5	87	0	87	3	84	3	81	3	78	1	77	3	74
12-47	37	1	36	1	37	2	39	0	39	0	39	4	35	1	34	0	34	0	34
12-50	30	0	30	2	32	0	32	2	30	3	27	2	29	1	30	3	27	1	26
12-57	61	2	59	4	63	2	65	1	64	3	61	1	62	0	62	1	61	2	59
12-62	42	6	36	2	38	3	41	1	42	4	38	4	42	2	44	1	44	2	42
12-79	86	3	83	1	82	5	87	1	86	2	84	2	86	1	85	0	85	2	87
12-105	80	3	83	1	82	1	83	1	82	3	79	2	81	1	80	2	78	0	78
12-114	57	4	53	4	57	0	57	7	64	3	61	2	59	3	62	3	59	1	58
.	.						.						.						.
.	.						.						.						.
.	.						.						.						.
Average change in inter-granule distance	1.36		2.71		2.24		2.67		2.96		2.69		2.64		3.13		4.13		

of pigment granules during clear area formation in colcemid-treated eggs with movements in another portion of colcemid-treated eggs at the same stage and in both normal and colcemid-treated, fertilized eggs prior to the time of division.

RESULTS

1. Inhibitors of microtubules and microfilaments

a. General technique

In experiments involving exposure of eggs to inhibitors, the exact time and duration of a given treatment was varied, in ordor to adjust to differences in the timing of developmental events between eggs obtained from different females and at different times during the breeding season. Several concentrations or inhibitory treatments were investigated concurrently, so the time of each specific observation was also variable. Whenever possible, observations have been reported here in terms of the developmental stage of control eggs. (A timetable of sea urchin development is presented in Table 1.) Unless specifically noted otherwise, the longest time interval recorded for a given effect signifies termination of the experiment, rather than cessation of the effect.

b. Colcemid

Concentrations of colcemid greater than, or equal to 2.7×10^{-5}M are known to prevent migration and subsequent fusion of pronuclei when applied to sea urchin eggs 5 minutes after fertilization (Zimmerman and Zimmerman, 1967). If eggs are treated at metaphase with the same concentrations, the mitotic spindle is destroyed and cleavage is blocked (Sauaia and Mazia, 1961).

TABLE 1

SCHEDULE OF EVENTS IN THE EARLY DEVELOPMENT OF ARBACIA PUNCTULATA EMBRYOS*

Event	Time after Fertilization (at 23°C)
Completion of fertilization membrane	2 min
Sperm aster	8 min
Pronuclear fusion (monaster stage)	10 min
Streak stage (early prophase)	20 - 35 min
Metaphase	40 min
First cleavage (2-cell stage)	50 min
Second cleavage (4-cell stage)	78 min
Third cleavage (8-cell stage)	103 min
Fourth cleavage (16-cell stage - micromere formation)	130 - 135 min
Blastula (hatched)	7 - 8 hrs
Gastrula	12 - 15 hrs
Pluteus larva	1 day

* Adapted from Harvey, 1956.

In the present experiments, eggs were exposed to 2.7×10^{-6}M to 2.7×10^{-2}M colcemid for various intervals, in order to investigate whether the occurrence of pigment granule movements depends upon the presence of microtubules and/or upon the mitotic cycle. Treatment was initiated during colcemid-sensitive mitotic stages and during, or prior to, specific pigment granule movements.

Unfertilized eggs were treated with colcemid for 5, 15, or 60 minutes, then either washed or allowed to remain in the drug during and after fertilization. In other experiments, eggs were placed in colcemid after fertilization but before pronuclear fusion, at metaphase, or just after division 1, 2 or 3. In an attempt to delete single divisions, pulses of 2 - 15 minutes were sometimes applied, usually during metaphase.

Mitosis and cleavage.--During the course of these experiments, no cleavage was noted during the period of observation of pigment granule movement in eggs remaining in concentrations of 2.7×10^{-5}M or greater. Occasional irregular furrows were observed in these eggs after several hours of treatment. A concentration of 2.7×10^{-6}M colcemid was often insufficient to prevent mitosis. When colcemid-blocked eggs were returned to sea water after short treatments, mitosis and cleavage resumed, but furrowing was usually quite irregular.

Saltatory movement.--Colcemid concentrations as high as 2.7×10^{-4}M had no effects on saltatory movements of echinochrome granules in unfertilized eggs. Pigment granules in eggs observed

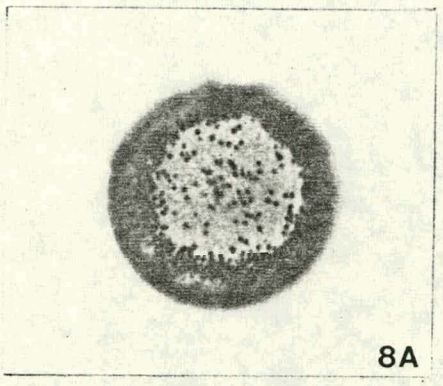
after 90 minutes of colcemid treatment exhibited apparently normal saltations. Higher concentrations did inhibit saltatory movement. In eggs treated with 2.7×10^{-3}M colcemid, saltation was still apparent after 15 minutes, but by 90 minutes the pigment granules were swollen and clumped together. After 12 minutes of exposure to approximately 10^{-2}M colcemid, no saltatory movements were observed in unfertilized eggs. In these eggs, pigment granules were also swollen and subsequently lysed.

Migration to the cortex and "slow shifting".--Echinochrome granules completed their migration to the cortex at the normal time in eggs exposed to up to 2.7×10^{-4}M colcemid. Normal migration was sometines observed in 2.7×10^{-3}M colcemid as well. However, at this concentration, pigment granules often tended to accumulate in the center of the egg rather than in the cortex. If these eggs were washed, the central aggregation disappeared and movement to the cortex was completed.

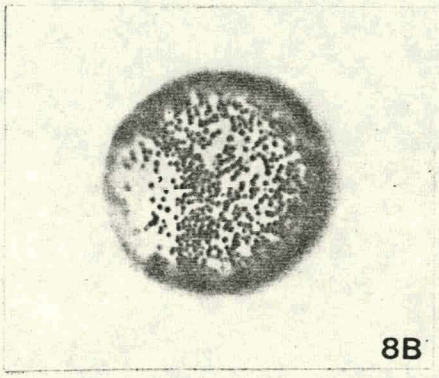
The slow migration of echinochrome granules which occurs in the cortex of fertilized eggs prior to division was unaffected by colcemid concentrations as high as 2.7×10^{-3}M.

Clear areas.--During the 4-cell stage of normally-cleaving eggs, echinochrome granules begin to migrate out of the presumptive micromere region, leaving a "clear area" at the vegetal pole. (See INTRODUCTION, Section 1.) In undivided eggs, which had been exposed to colcemid continuously from 60 minutes before fertilization, clear areas were formed at the same time as in controls (Figure 8). This

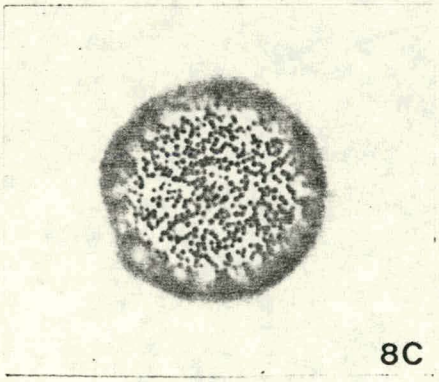
Figure 8. Clear areas in colcemid-treated eggs (A and B). C) Another region of the cortex of an egg at the same stage and from the same female as (B). x 480.



8A



8B



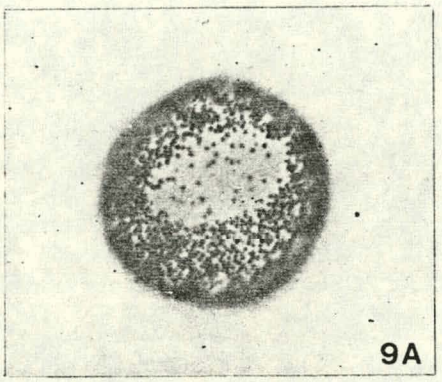
8C

phenomenon occurred consistently in colcemid concentrations as high as 2.7×10^{-4}M, sometimes in 2.7×10^{-3}M, but was not observed in 10^{-2}M colcemid. From morphological similarities, it seems reasonable to assume that the formation of a clear area marks the location of the vegetal pole in a colcemid-treated egg as well as in a cleaving egg.

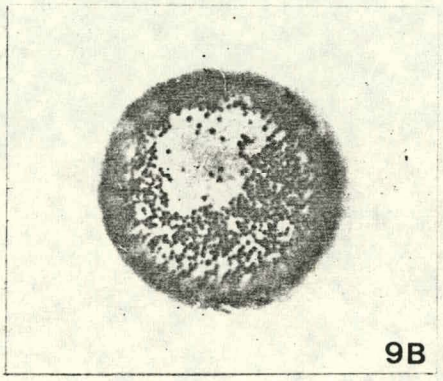
Clear areas in undivided eggs were generally circular in shape, but some variation was observed among eggs from a single female or between those from different females (Figure 9). These pigment-free regions ranged from 20 to 40μ in diameter (Figure 10), corresponding to approximately 7 - 29% of the surface area of the undivided egg. There appeared to be some variation in the proportion of pigment granules which migrated out of the region (Figure 11). Occasionally, a small, densely pigmented region remained in the center of the clear area (Figure 12). In rare instances, more than one clear area was observed on a single egg (Figure 13). These uncommon patterns appeared most often in eggs which had been exposed to colcemid for several hours.

Summary.--Treatment with concentrations of colcemid which completely inhibited mitosis and cleavage (2.7×10^{-5} - 2.7×10^{-4}M) had no apparent effects on any of the echinochrome granule movements studied. Very high colcemid concentrations (approximately 10^{-2}M) did inhibit these movements, but toxic effects including aggregation, swelling and lysis of pigment granules were also observed at these concentrations. From an embryological standpoint, the observation

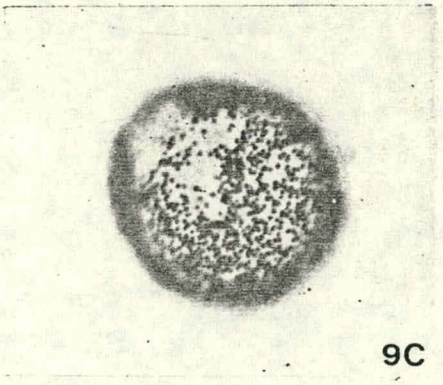
Figure 9. Clear areas with aberrant shapes. x 480.



9A



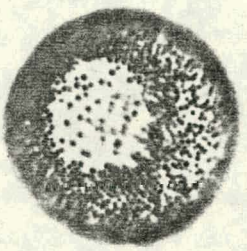
9B



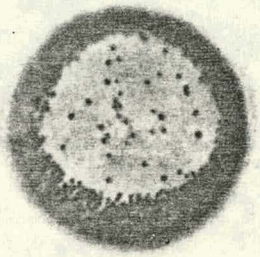
9C

Figure 10. Differences in the size of clear areas. These eggs represent a rare observation of this degree of size variation among eggs from a single female. x 480.

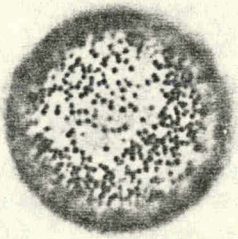
Figure 11. Unusually high concentration of pigment granules remaining within a clear area. x 480.



10A



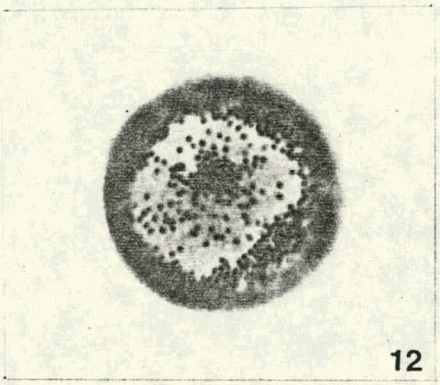
10B



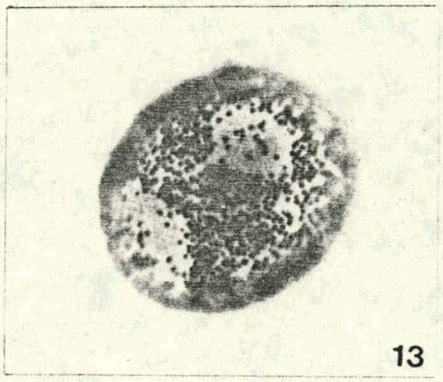
11

Figure 12. Accumulation of pigment granules in the center of a clear area. This egg was photographed at 143 minutes after fertilization; clear areas were reasonably complete by 120 minutes. x 480.

Figure 13. An egg containing two clear areas. This egg was elongated, suggesting some abnormality. However, two clear areas were occasionally observed on eggs which appeared normal in all other respects. x 480.



12



13

that migration of pigment granules out of the presumptive micromere region occurs at the normal time in colcemid-treated eggs, which have never undergone mitosis or cleavage, appears to be especially significant.

c. Vinblastine

Treatment with 10^{-5}M to 10^{-4}M vinblastine has been shown to disrupt spindle microtubules, thus blocking mitosis in mammalian cells (Krishan, 1968) and meiosis in oocytes of Pectinaria (Bensch and Malawista, 1968) and of the starfish Pisaster (Malawista and Sato, 1969). Observations of pigment granule movements were made on unfertilized eggs placed in 10^{-4}M vinblastine and on fertilized eggs placed in either 10^{-5}M or 10^{-4}M vinblastine before pronuclear fusion or at first metaphase.

No inhibition of saltatory movement of echinochrome granules was observed in unfertilized eggs after 38 minutes of vinblastine treatment. Migration of pigment granules to the cortex and subsequent formation of clear areas occurred in eggs which had been placed in vinblastine 2 minutes after fertilization. Pronuclear migration and fusion were inhibited in these eggs and cleavage was not observed. Cleavage did occur in some of the eggs treated with vinblastine at metaphase, but normal clear areas were formed whether or not the eggs had cleaved. Clear areas in vinblastine-treated eggs appeared to be smaller than those in colcemid-treated eggs, and formed slightly later than those in the controls.

d. Cytochalasin B

In Arbacia eggs treatment with cytochalasin B (CB) just before or during cleavage, inhibits the formation or completion of the cleavage furrow, while mitosis continues normally (Schroeder, 1972). This has been interpreted as the result of selective destruction of a band of 40 - 60Å microfilaments in the furrow region. In order to determine whether the cleavage process and/or microfilaments are required for pigment granule movements, eggs were exposed to CB during cleavage and at stages which exhibit specific pigment granule movements. For most experiments, eggs were exposed to 1 - 5µg/ml CB, but concentrations as low as 0.05µg/ml were also used. Concentrations required to produce the effects reported here varied between eggs from different females. Attempts were made to reverse the effects of CB by washing the drug out after a short exposure. Even though some processes which had been inhibited by CB did resume after washing (see below), these eggs rarely survived to undergo normal development. In the following experiments, no differences were observed between normal eggs and those treated with concentrations of DMSO equivalent to those present in CB solutions.

Saltatory movement.--Saltatory movements of echinochrome granules ceased within 5 - 10 minutes after unfertilized eggs were placed in CB. This effect was observed consistently at concentrations of 2.5 - 5.0µg/ml and sometimes even in 1µg/ml. Lower concentrations had little or no effect. If eggs were subsequently washed, saltatory movements could again be observed.

Activation.--Treatment of unfertilized eggs with CB also resulted in the elevation of fertilization membranes. After these activated eggs were washed, the migration of pigment granules to the cortex was completed, but no cleavage was observed. Some clear areas appeared after several hours.

Migration to the cortex.--Migration of pigment granules to the cortex was inhibited when eggs were treated with 2.5 - 5.0µg/ml CB within 1 to 5 minutes after fertilization. As soon as these eggs could be observed after washing, the distribution of pigment granules appeared to be the same as in controls.

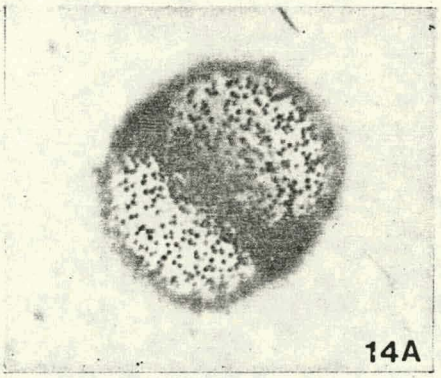
Mitosis.--It has been reported that CB blocks cleavage without interfering with mitosis in a number of cell types (Carter, 1967; Ridler and Smith, 1968; Krishan and Ray-Chaudhuri, 1969; Schroeder, 1970; Bluemink, 1971; 1972; Estensen, 1971; Hammer et al., 1971), including Arbacia eggs (Schroeder, 1972). However, in the present experiments, delay of mitotic stages or complete arrest of the cycle often occurred when eggs were treated at early stages with concentrations of CB sufficient to inhibit cleavage. The monaster and the early prophase "streak" stages were delayed or prolonged, when eggs were exposed to 1.5 - 5.0µg/ml CB beginning between 3 and 10 minutes after fertilization. At the time of first cleavage in the controls, most of these CB-treated eggs were still in the streak stage and no pigment bands had formed. Most eggs never progressed beyond this stage, but a small percentage (especially at lower concentrations) later formed spindles and an equatorial band of pigment. Since the

exposure times required to inhibit mitosis were longer than those which allowed any degree of recovery, it is possible that this inhibition represents a general toxic effect rather than a specific mitotic block.

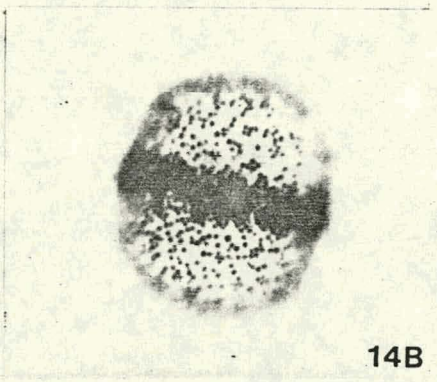
Cleavage.--If fertilized Arbacia eggs are exposed to CB just before or during cleavage, furrows either form and regress, or fail to form (Schroeder, 1972). In the present experiments, accumulation of pigment granules at the equator was observed in eggs in which furrowing had been inhibited by CB treatment (2.5 - 5.0µg/ml). This resulted in the formation of a concentrated band of pigment (Figure 14) accompanied by ruffling of the hyaline layer in the same region (Figure 15). The morphology of these equatorial pigment bands was somewhat variable. In addition to continuous rings of pigment, bands with a striped (Figure 16) or splotchy (Figure 17) appearance were observed in some eggs. Occasionally, pigment disappeared completely from the equatorial region (possibly because of preferential lysis of granules), leaving a clear band (Figure 18). The occurrence of these variations depended to some extent on the CB concentrations: continuous rings at low concentrations, striped and splotchy bands at intermediate concentrations and clear bands at high concentrations. The character of a single band often changed with time (Figure 20) and movement of individual granules could be observed within this region (Figure 21). In some eggs remaining in CB after bands have formed, the pigment granules returned to an even distribution over the egg cortex. However, bands sometimes persisted until the next

Figure 14. Concentrated bands of pigment in the furrow region of eggs in which cytokinesis was inhibited by cytochalasin B. x 480.

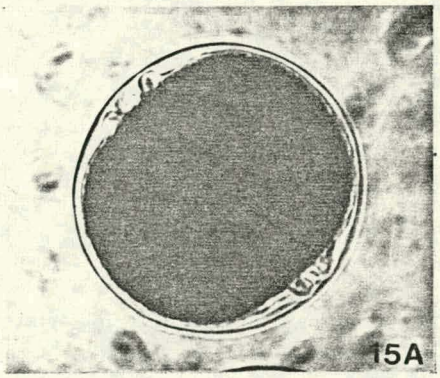
Figure 15. Ruffling of the hyaline layer in the furrow region of eggs in which cytokinesis was inhibited by cytochalasin B. The eggs shown in Figure 14 were photographed here with the condenser diaphragm closed down to show the hyaline layer. x 480.



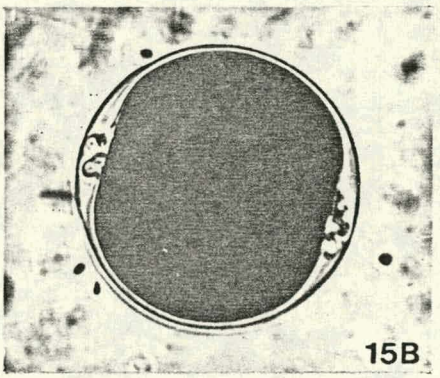
14A



14B



15A



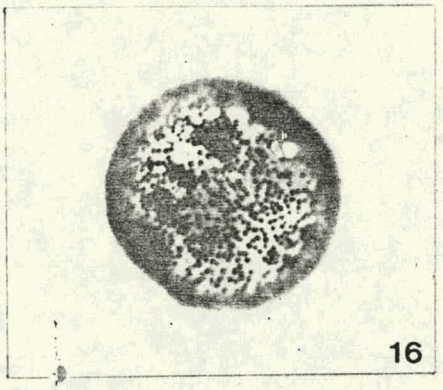
15B

Figure 16. "Striped" band of pigment in a cytochalasin B-treated egg. x 480.

Figure 17. "Splotchy" band of pigment in a cytochalasin B-treated egg. x 480.

Figure 18. Clear, or pigment-free band in a cytochalasin B-treated egg. x 480.

Figure 19. Bands of pigment corresponding to both the first and the second cleavage furrows. This egg was placed in cytochalasin B prior to first division and formed a pigment band at the equator. The band was retained until the next cleavage when a second band formed perpendicular to the first. Photographed at 90 minutes after fertilization. x 480.



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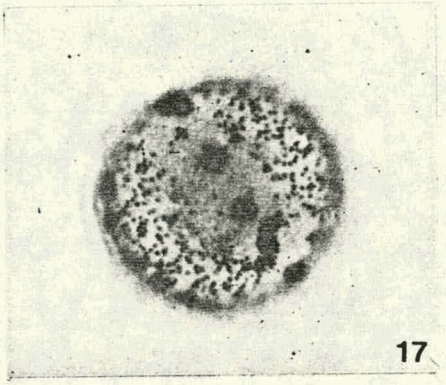

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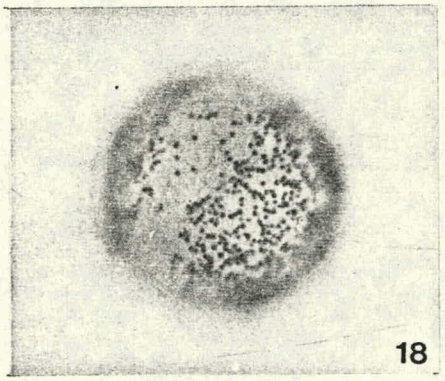

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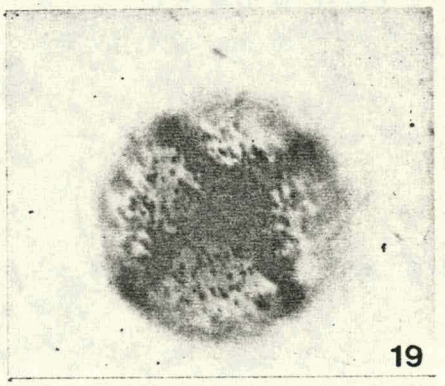
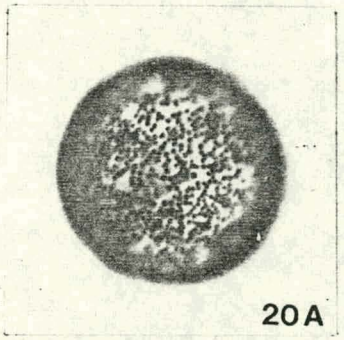
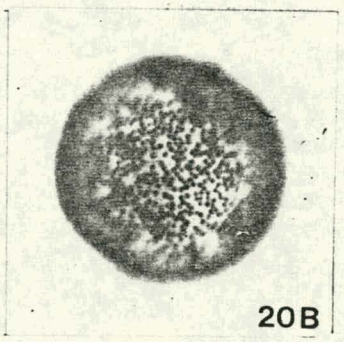


19

Figure 20. Formation of a pigment band and changes in its appearance. This egg was placed in CB at 43 min 30 sec after fertilization. A) 48 min 45 sec, B) 52 min 15 sec, C) 52 min 45 sec, D) 53 min 15 sec, E) 54 min 15 sec, F) 55 min 30 sec, G) 56 min, H) 60 min 15 sec. x 480.



20A



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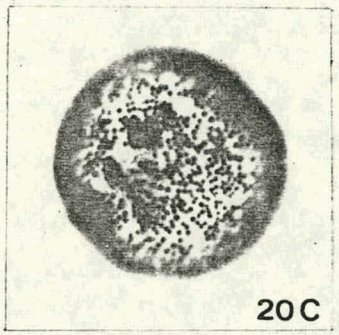

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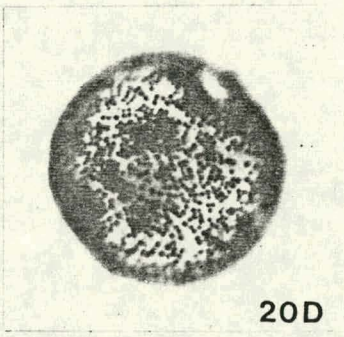
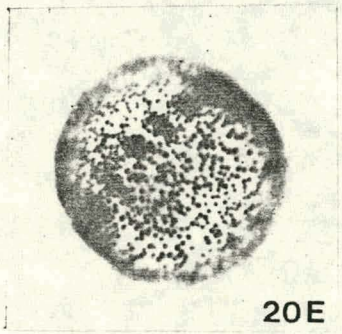


20D



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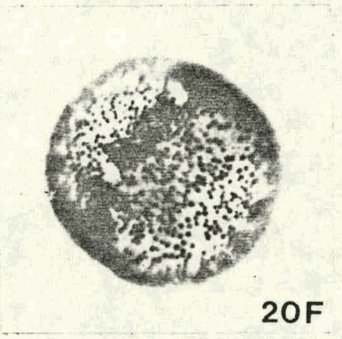

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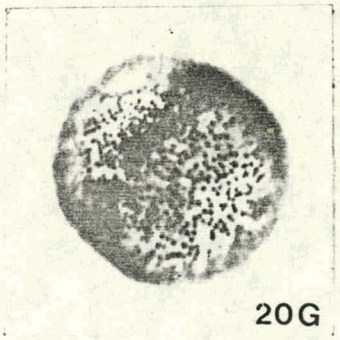
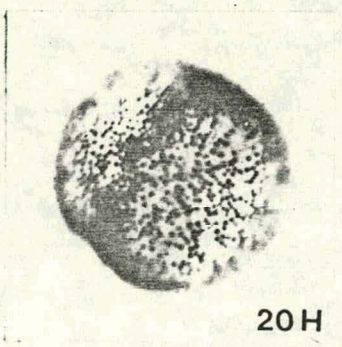
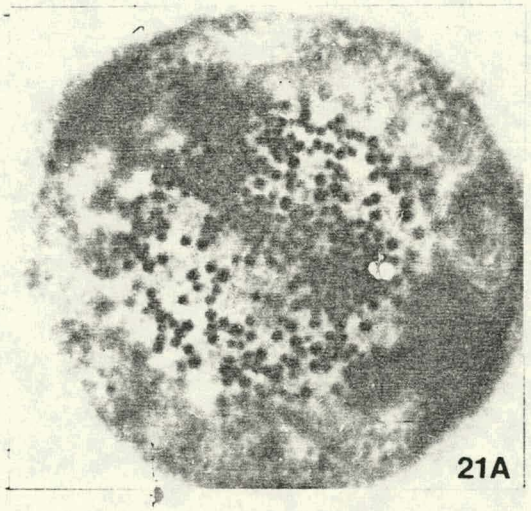


20G

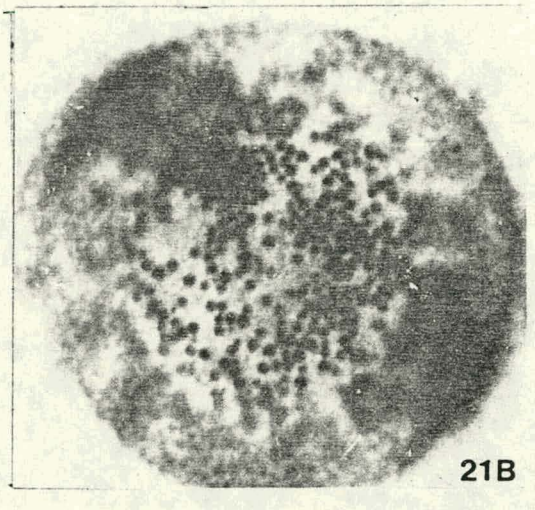


20H

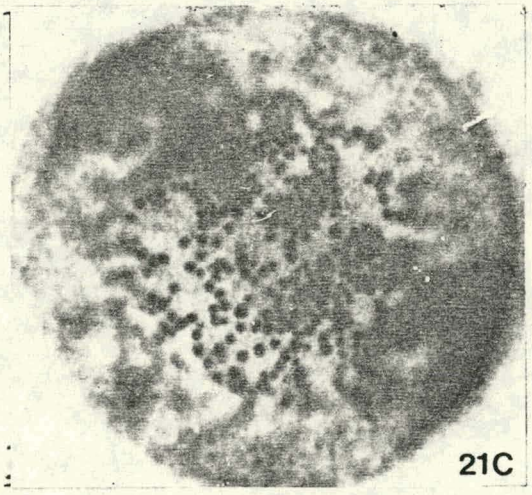
Figure 21. Movement of pigment granules within a pigment band. Eggs were placed in cytochalasin B at 58 minutes after fertilization. A) 66 min 4 sec, B) 66 min 43 sec, C) 67 min 6 sec, D) 67 min 35 sec. x 1200.



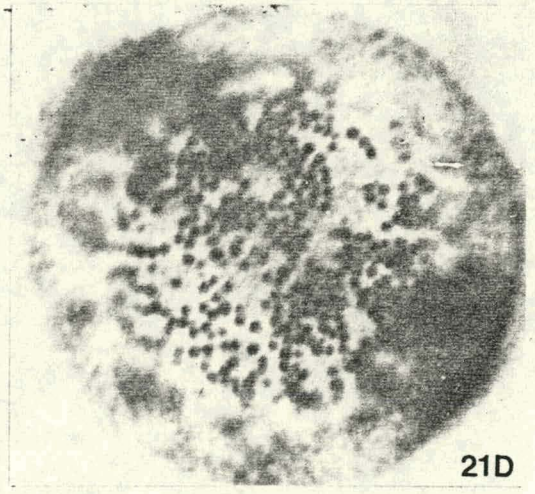
21A



21B



21C



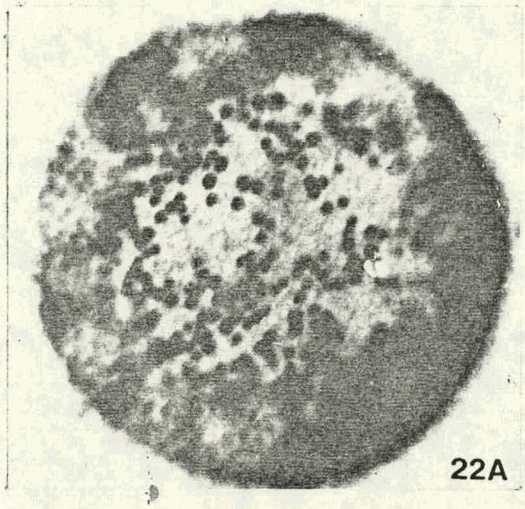
21D

cleavage, when a second band was formed (Figure 19), or until the eggs began to show the adverse effects of prolonged CB treatment.

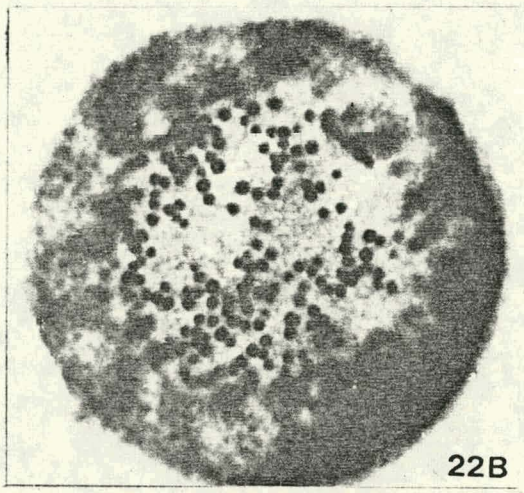
Clear areas.--Since prolonged exposures to CB resulted in a number of effects on the distribution of pigment granules (see below) it has been impossible to determine whether or not the process of clear area formation can occur in eggs treated with CB from fertilization onward. A few complete clear areas were observed after normal and colcemid-treated eggs were placed in 1 - 5µg/ml CB at the beginning of clear area formation. However, because of variation in the time of clear area formation among eggs from the same female, these clear areas might have been nearly complete at the time of exposure to CB. In order to determine whether the individual granule movements resulting in clear area formation were inhibited by CB, time lapse photomicrographs were made of a colcemid-treated egg exposed to 5µg/ml CB as a clear area was forming. Little, if any, movement of pigment granules was observed in this egg as compared with eggs treated with colcemid alone (Figures 22 and 23).

Effects of prolonged exposures to CB.--Several apparent changes at the cell surface or in the cortex were observed in eggs treated with 1 - 5µg/ml CB for longer than 10 minutes. In both unfertilized and fertilized eggs, the cell surface became wrinkled or bumpy (Figure 24). The hyaline layer of fertilized eggs appeared to increase in width and acquired a granular texture (Figure 25). Pigment granules in the cortex of fertilized eggs appeared to aggregate in small groups, giving the eggs a mottled appearance (Figure 26).

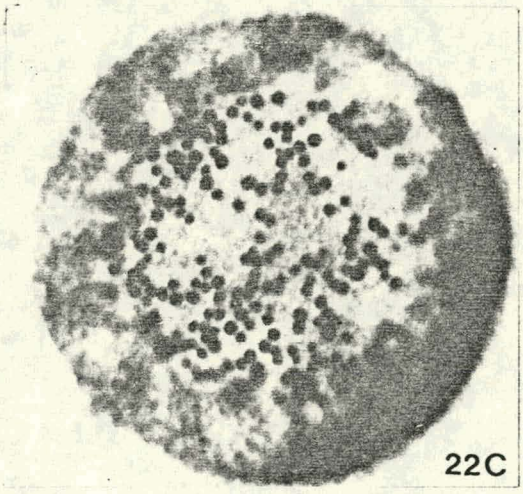
Figure 22. Colcemid-treated egg placed in cytochalasin B at the beginning of clear area formation (120 minutes after fertilization). A) 131 min, B) 132 min, C) 133 min, D) 134 min. Pigment granules remain close to their original positions. Compare with granules in the egg in Figure 23 which was treated with colcemid alone. x 1200.



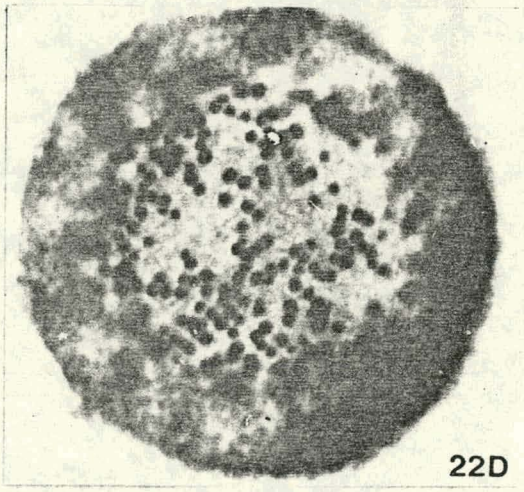
22A



22B

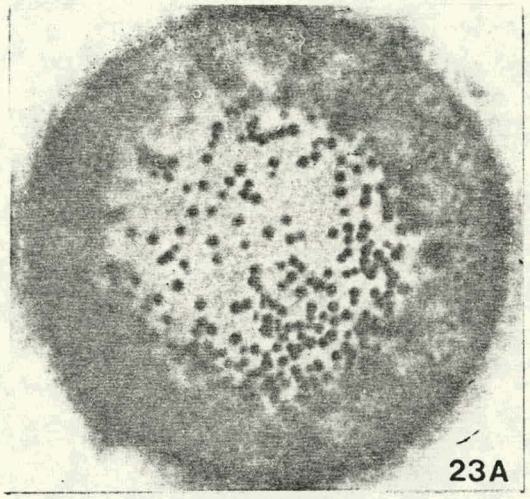


22C

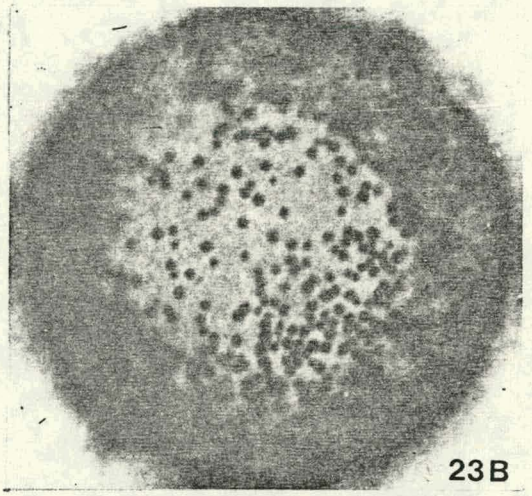


22D

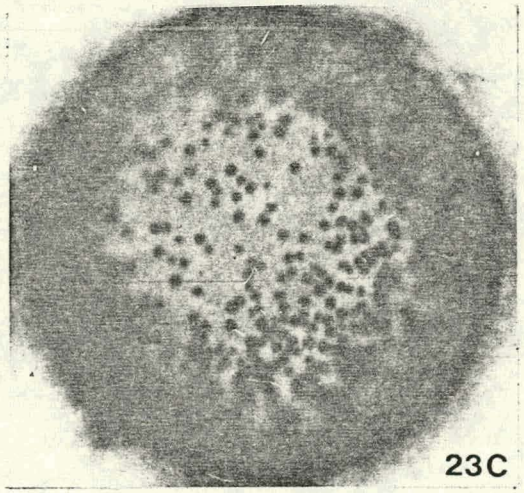
Figure 23. Movement of pigment granules during formation of a clear area in a colcemid-treated egg. A) 118 min, B) 119 min, C) 120 min, D) 121 min. x 1200.



23A



23B



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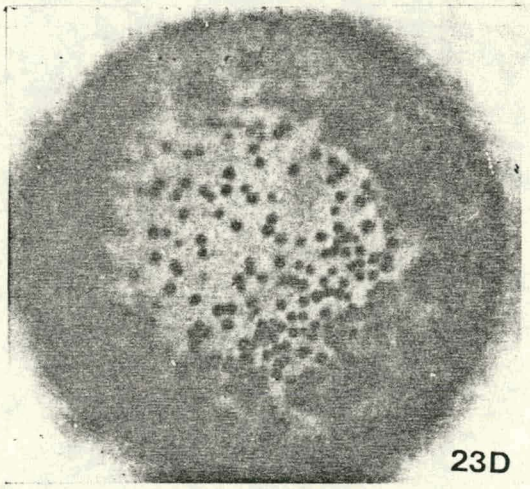


23D

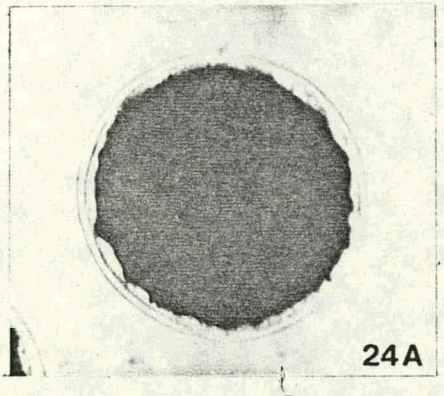
Figure 24. Surface wrinkling in a cytochalasin B-treated egg (A). B) Control egg. Hyaline layer (HL) and fertilization membrane (FM). x 480.

Figure 25. Hyaline layer hypertrophy in a cytochalasin B-treated egg. Compare with Figure 24B. x 480.

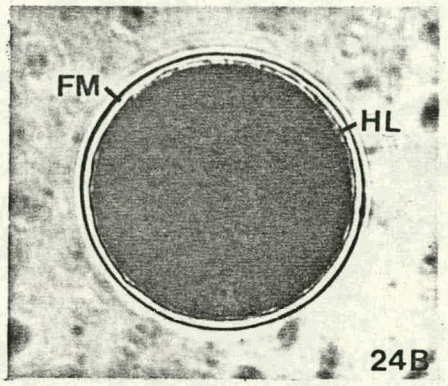
Figure 26. Aggregation of pigment granules into clusters in a cytochalasin B-treated egg. x 480.

Figure 27. Lysis of the majority of the pigment granules in a cytochalasin B-treated egg. x 480.

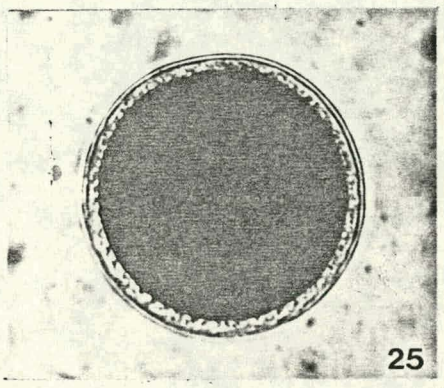
Figure 28. Extrusion of pigment granules into the hyaline layer of a cytochalasin B-treated egg. x 480.



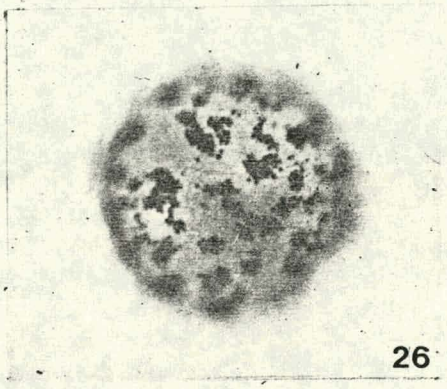
24A



24B



25



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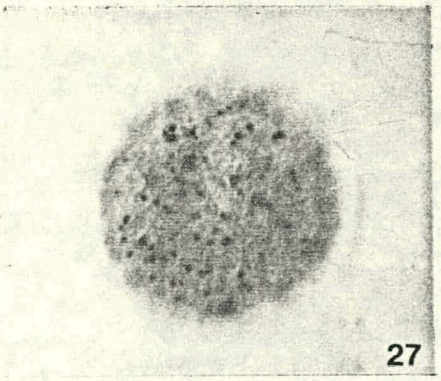
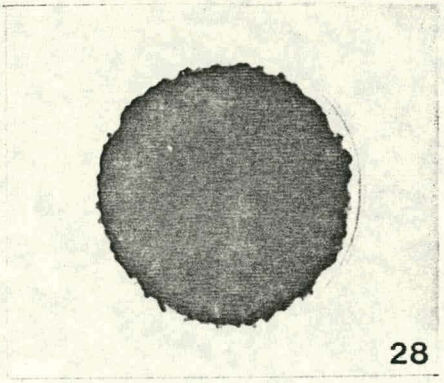


27



28

When eggs were placed in CB just before cleavage, a similar, but transient, aggregation of pigment often occurred over the whole egg surface. Frequently the majority of pigment granules in the cortex of fertilized eggs appeared to lyse, leaving the eggs nearly unpigmented (Figure 27). Apparent extrusion of pigment granules from the cortex out into the hyaline layer was also observed (Figure 28).

Summary.--Cytochalasin B reversibly inhibited saltatory movements of echinochrome granules in unfertilized eggs and their migration to the cortex at fertilization. Movements of individual pigment granules involved in clear area formation were also inhibited. When furrowing was inhibited by CB, a concentrated band of pigment granules appeared in the furrow region, where blebs of the hyaline layer also occurred. Exposure to CB often resulted in activation of unfertilized eggs. Prolonged exposures to CB resulted in mitotic delay and arrest, wrinkling of the cell surface, hyaline layer hypertrophy and the aggregation, lysis and extrusion of pigment granules.

2. Other treatments

a. Puromycin

Puromycin (10^{-4}M) inhibits mitosis in the early prophase "streak" stage and also substantially inhibits protein synthesis in fertilized eggs of Paracentrotus lividus (Hultin, 1961). In order to determine whether pigment granule movements could occur under these conditions, observations were made on unfertilized eggs exposed to 10^{-4}M puromycin. Saltatory movements of echinochrome granules in unfertilized eggs were unaffected by 127 minutes of puromycin treat-

ment. Migration of pigment granules to the cortex occurred normally in eggs placed in puromycin 30 minutes prior to, or immediately after fertilization. In these puromycin-treated eggs, no cleavage was observed at the time of division in the controls, but a few eggs escaped the mitotic block and cleaved about 50 minutes later. Normal clear areas were formed in non-dividing, puromycin-treated eggs. Sometines clear area formation was delayed in puromycin-treated eggs as compared with colcemid-treated eggs. Delays in clear area formation were longer in eggs placed in puromycin prior to fertilization than in those exposed immediately after fertilization.

b. Irradiation

γ-irradiation of unfertilized and fertilized eggs produces a dose-dependent delay of cleavage (Henshaw and Francis, 1936; Henshaw and Cohen, 1940). The initial delay is expressed in the early prophase "streak" stage of the mitotic cycle (Henshaw, 1940). In order to examine the effects of irradiation on pigment granule movements, eggs were exposed to 50 to 250kR of γ-radiation either before or immediately after fertilization. No inhibition of saltatory movements of echinochrome granules was observed in unfertilized eggs 90 minutes after the end of irradiation with as much as 250kR. Migration of pigment granules to the cortex occurred at the normal time in eggs irradiated before fertilization. Cleavage was observed in some eggs irradiated with less than 200kR before or after fertilization. At all dose levels, clear areas appeared on undivided eggs as well as on

those which were able to cleave.

c. Low temperatures

Unfertilized and fertilized eggs were incubated at temperatures between -2° and 8°C for periods of 1 to 24 hours to investigate whether normal pigment granule movements could continue at these temperatures. Eggs were placed in the cold just after fertilization or after pigment granules had migrated to the cortex. Mitosis and cleavage were greatly delayed or blocked at these temperatures. If eggs were placed in the cold between 1 and 3 minutes after fertilization, migration of pigment granules to the cortex was often still incomplete after 2 - 3 hours. Restoration of these eggs to room temperature allowed this process to occur, followed by nearly normal cleavage and development. When eggs were placed in the cold after pigment migration to the cortex, clear areas had not begun to form within 5 - 8 hours. Clear areas appeared within an hour after these eggs were returned to room temperature and mitosis and cleavage resumed at approximately the same time. Complete clear areas and occasional cleaving eggs were observed between 10 and 20 hours of incubation between 4°and 8°C. Eggs exposed to low temperatures for 10 to 20 hours also displayed large, localized evaginations or bulges, which were relatively free of pigment. However, normal clear areas were observed in other portions of the cortex of eggs exhibiting evaginations. After these eggs had been returned to room temperature, the evaginations disappeared, leaving pigment-free regions, which could not subsequently be distinguished from normal clear areas.

d. Treatment with hypo- and hypertonic sea water

To investigate whether the cortex of the clear area differed in mechanical strength from the remainder of the cortex, eggs were exposed to hypo- or hypertonic sea water. Normal and colcemid-treated eggs, with or without fertilization membranes, were placed in hypo- or hypertonic sea water at the time of clear area formation. Eggs swelled uniformly under mild hypotonic treatment, but formed localized evaginations or bulges when the concentration of sea water approached 50%. These evaginations were always relatively free of pigment, making it difficult to determine whether the evagination coincided with the normal clear area. Nonetheless, clear areas were often observed at other locations in eggs with pronounced evaginations. Eggs shrank evenly in slightly hypertonic solutions, but formed one to several invaginations or depressions in concentrations of sea water close to 200%. In groups of eggs which had each formed a single invagination, there was no correlation between the location of the invagination and that of the clear area.

3. Pigment granule movements during clear area formation

During the 4-cell stage of normally-cleaving Arbacia eggs, echinochrome granules begin to migrate out of the region which will become the micromeres, leaving a pigment-free zone or clear area on each blastomere (see INTRODUCTION, Section 1). Since this migration also occurs in colcemid-treated, non-dividing eggs (see RESULTS, Section 1), it has been possible to investigate the pigment granule movements involved in clear area formation in the absence of cleavage,

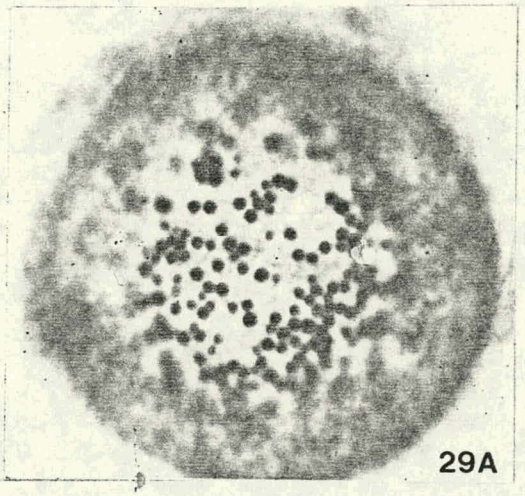
which itself involves movement of pigment granules. The following observations and measurements were made on time lapse films of colcemid-treated eggs forming clear areas. These analyses have been made in order to answer the following questions: (1) Are pigment granule movements specifically oriented toward the perimeter of the micromere region? (2) Are these movements quantitatively similar to those in regions of the cortex not forming a clear area, or in the cortex of fertilized eggs prior to division.

a. General observations

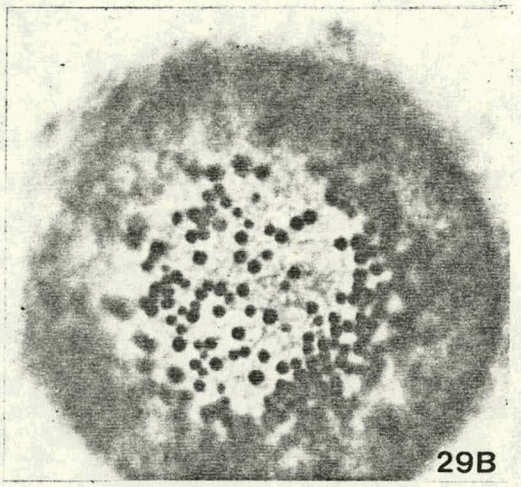
A clear area first became evident as a circular region containing a lower concentration of pigment granules than the remainder of the egg cortex. As granules continued to migrate out of this region, they accumulated at its perimeter, forming a ring in which pigment granules were highly concentrated or even contiguous (Figure 29). A clear area could first be detected 40 - 50 minutes before its completion. Since both the time of appearance and completion were subjective measurements, this interval represents an approximation of the time required for clear area formation.

When individual pigment granules were visually observed during the formation of a clear area, it was difficult to detect movement. Saltations of the magnitudes described for unfertilized eggs (1 - 5μ jumps at 0.3 to 0.8μ/sec, Parpart, 1953; 1964) were never observed. Comparison of frames taken 1 minute apart revealed that granules had moved, but usually only 2μ or less during this interval. Occasionally, displacements of 3μ had occurred within a 1 minute

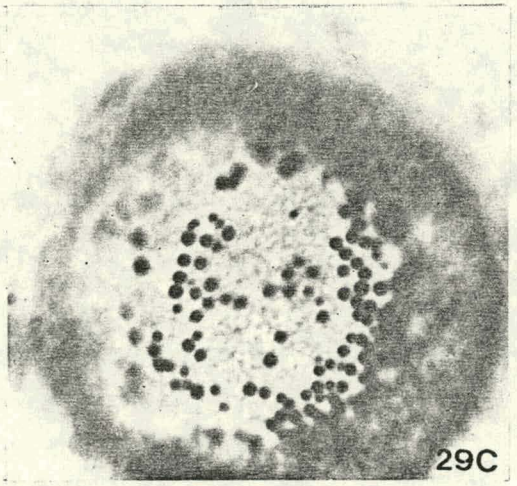
Figure 29. Clear area formation in a colcemid-treated egg. Photographed at A) 84, B) 90, C) 111 and D) 131 minutes after fertilization. Note the concentration of pigment granules at the rim of the clear area in B. x 1200.



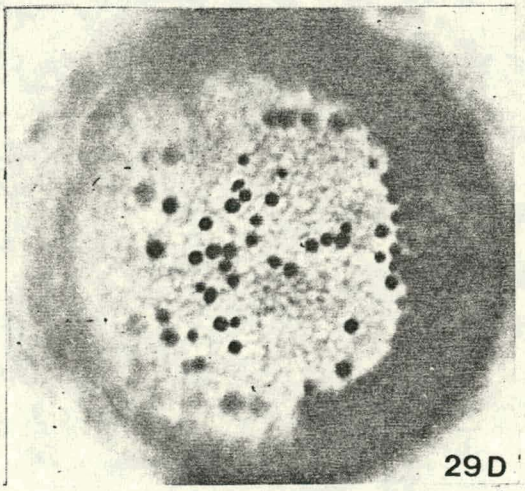
29A



29B



29C



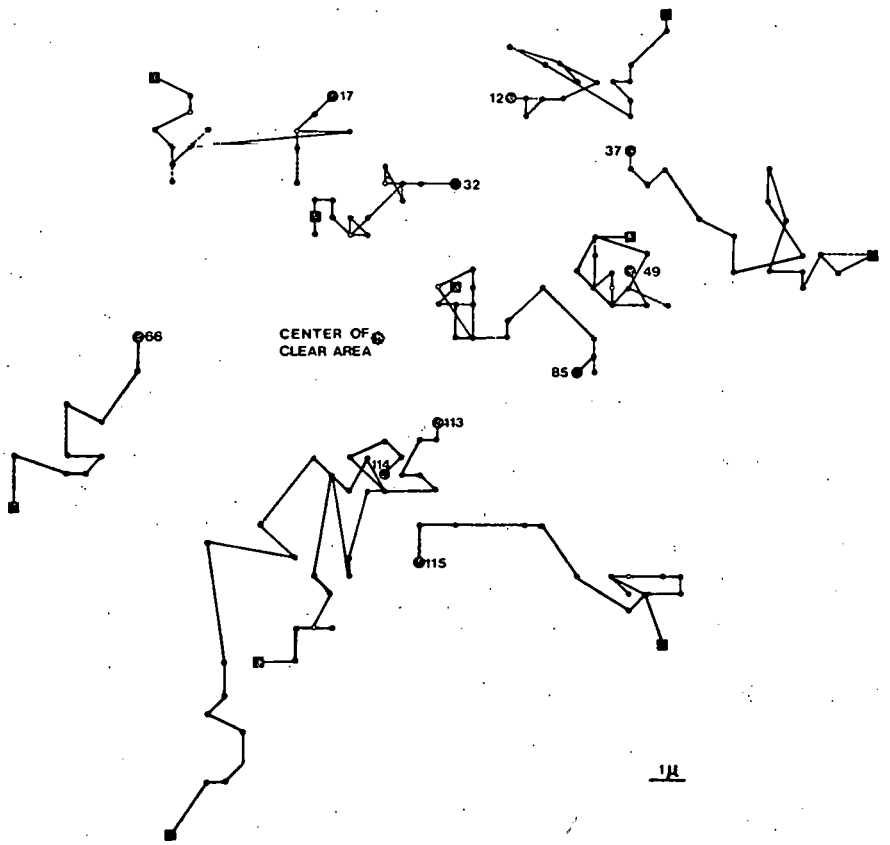
29D

time period.

b. Paths of individual granules during clear area formation

The coordinates of a number of granules were recorded from each photomicrograph in a series taken at 2 minute intervals. These points were then plotted to give approximations of the paths of individual granules. The paths of a group of granules during the formation of a typical clear area (one of two analyzed in detail) in a colcemid-treated egg are presented in Figure 30. Each granule was followed for 38 minutes, or until it reached the perimeter of the region and left the focal plane. The majority of pigment granules moved toward the perimeter of clear area being formed. However, a few granules did not display this oriented movement. For example, granules 32 and 85 moved toward the center of the region. Also, granules such as 49 exhibited movement, but were not displaced significantly from their original position. Regardless of the general direction of movement, granules moved along erratic rather than straight paths. Even granules which were moving toward the perimeter occasionally moved in the opposite direction for short distances (see especially granules 12, 17, and 113). No consistent relationship was observed between the patterns of movement of initially adjacent granules. Sometimes neighboring granules remained close to one another and followed similar paths (113 and 114). Other pairs of granules moved in widely divergent directions (compare 114 and 115). Occasionally, one granule, such as 49, remained nearly stationary, while another, 37, nearby, moved away.

Figure 30. Paths of individual granules involved in clear area formation. Granule positions were recorded from frames taken at 2 minute intervals beginning at 76 minutes and ending at 114 minutes after fertilization. x 4800.



KEY

- ● INITIAL GRANULE POSITION
- ■ FINAL GRANULE POSITION
- • INTERMEDIATE POSITIONS
- ◦ POSITION OF GRANULE IN SEVERAL CONSECUTIVE FRAMES
- --- PATHS RETRACED

A group of granules in another region of the cortex of a colcemid-treated egg at the time of clear area formation was followed over a 14 minute period (Figure 31). Movements of the majority of these pigment granules showed no predominant orientation. During the entire period of filming, granules rarely moved further than 2μ from their initial positions. However, several granules (especially 20, 35 and 180) moved in a specific direction over total distances comparable with those during the same period of time within a clear area. Since the clear area in this egg was located at the top of the photomicrograph, no obvious relationship was evident between these oriented movements and formation of the clear area. In general, granules moved shorter net distances during 2 minute intervals in this region than in a clear area.

The paths of a number of pigment granules were also traced from photomicrographs of a fertilized colcemid-treated egg prior to the time of first cleavage in the controls (Figure 32). Granule movements were followed for 22 minutes. The movements of most pigment granules at this stage were not oriented in a specific direction and granules rarely moved further than 2μ from their original positions. However, at least two granules (80, 112) moved total distances comparable to those observed within the same time period during clear area formation. The net distances moved during 2 minute intervals in this egg were approximately the same as those in the region outside of the clear area (Figure 31) and less than those within the clear area in Figure 30.

Figure 31. Paths of individual granules in another region of the cortex of an egg during clear area formation. Granule positions were recorded from frames taken at 2 minute intervals beginning at 76 minutes and ending at 90 minutes after fertilization. x 4800.

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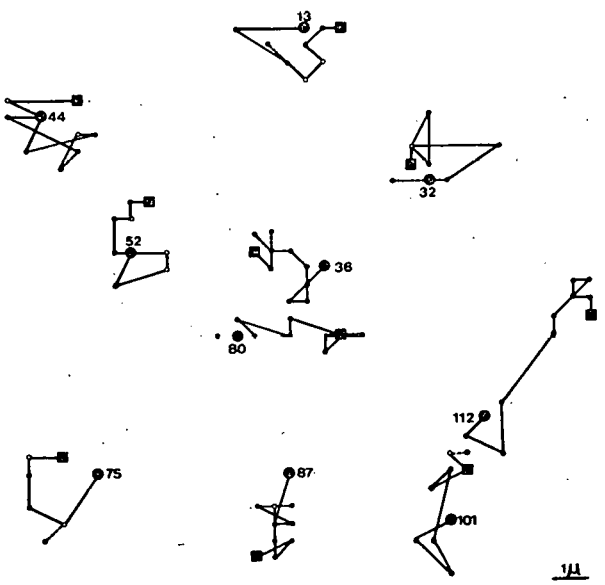
Figure 32. Paths of individual granules in the cortex of a fertilized egg prior to division. Granule positions were recorded from frames taken at 2 minute intervals beginning at 19 minutes and ending at 41 minutes after fertilization. x 4800.



KEY

- ● INITIAL GRANULE POSITION
- ⊠ FINAL GRANULE POSITION
- • INTERMEDIATE POSITIONS
- ◦ POSITION OF GRANULE IN SEVERAL CONSECUTIVE FRAMES
- ---PATHS RETRACED

Thus, most pigment granules in the cortex of fertilized eggs prior to division and in other regions of the egg cortex during clear area formation do not display the oriented movements characteristic of granules within the clear area, and remain within 2μ of their initial positions. However, a few granules in these eggs do display movement in a specific direction over distances comparable with those during clear area formation. Net granule displacements during 2 minute intervals are somewhat larger within the clear area than in the other region and stage studied. This may reflect orientation rather than a difference in rates of movement. (See Section d for a quantitative comparison of pigment granule movement in different stages and regions).

c. General orientation of pigment granule movements during clear area formation

Although the majority of pigment granules migrated toward the perimeter of the clear area and left the region entirely, a few granules moved toward the center of the region (see Section b, above). A small number of granules could still be observed near the center of the clear area after several hours.

Therefore, the following procedure was used to determine the proportions of pigment granules from different regions of the clear area which moved toward its center or toward its perimeter. The incipient clear area was divided into concentric rings, numbered 1 through 4 from the center to the periphery. The number of the ring in which each granule was located was recorded for the first and last

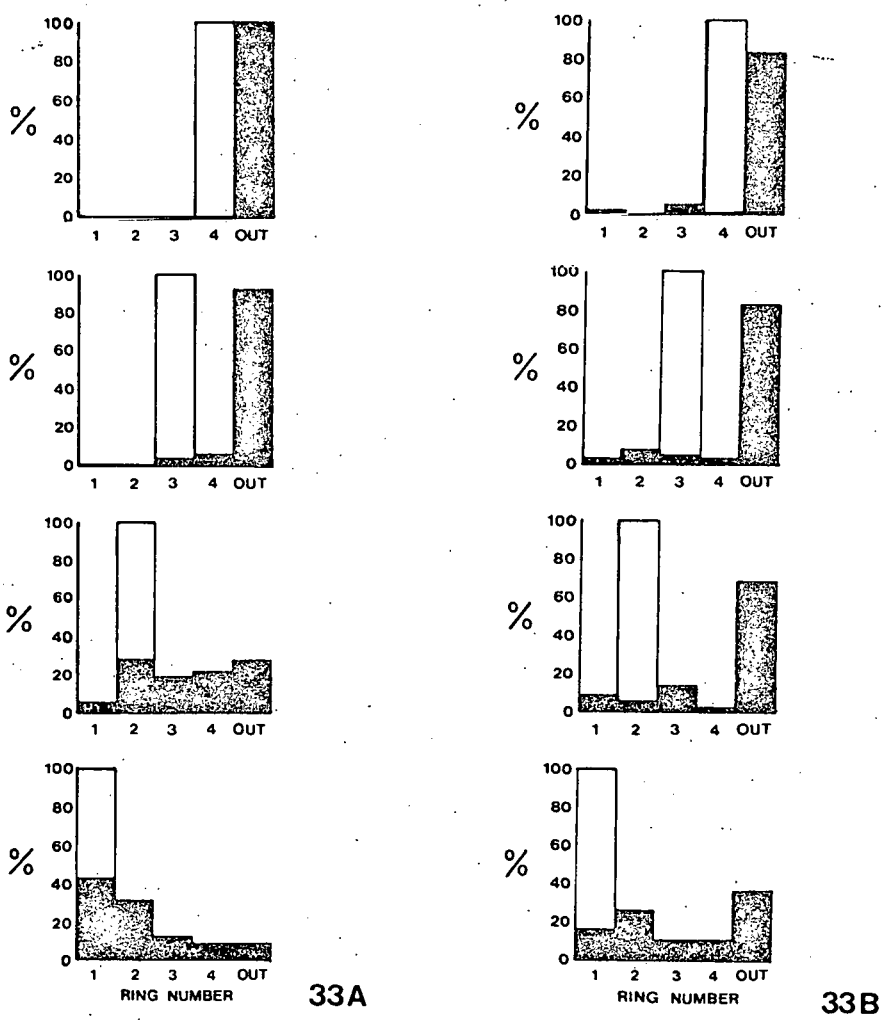
photomicrograph of a series taken over a 40 - 50 minute period during clear area formation (see MATERIALS AND METHODS, Section 4, especially Figure 6).

A comparison of the initial and final distributions of pigment granules for two typical clear areas is presented in Figure 33. The proportion of pigment granules which moved toward the center ranged from 1.4% (2 granules out of 139 - Figure 33a) to 7.5% (10 granules out of 134 - Figure 33b). In the clear area represented by Figure 33a, the only granules which moved toward the center were initially located in ring 2. Several granules from rings 2, 3 and 4 had moved toward the center of the clear area represented by Figure 33b. The proportion of granules which moved outside the region covered by the circular grid increased with increasing initial distance from the center of the region in both examples. These observations support the conclusion, based on individual granule paths, that the majority of pigment granules are moving toward the perimeter of the clear area. The presence of small numbers of granules which do not display this orientation suggests that some granules can move independently of whatever factor determines the predominant direction of movement.

d. Quantitative comparison of pigment granule movements in different stages and regions

The preceding measurements suggest that clear area formation involves oriented movements of pigment granules over longer distances than those travelled by most granules in other regions of the cortex at the same stage or in the cortex of fertilized eggs prior to

Figure 33. Distribution of pigment granules at the end of clear area formation as a function of their initial location. The ring in which each granule was located was recorded for the first and last frames of a series taken over A) a 38 minute interval and B) a 50 minute interval. This illustration presents the final location of all granules which were initially in each ring.


100
80
60
40
20
0
%
1 2 3 4 OUT
RING NUMBER
33A
33B
KEY
RING NUMBERS:
4
3
2
1
RIM OF CLEAR AREA
INITIAL LOCATION OF A GROUP OF GRANULES
FINAL DISTRIBUTION OF THOSE GRANULES

division. The granule displacements observed during 2 minute intervals in the latter eggs (Section b) were smaller than those for granules within a clear area. This difference might represent increased rates of granule movement within the clear area or might be a consequence of the oriented movement of granules. To differentiate between these possibilities, the interval over which displacements are measured must be small enough so that granules moving randomly do not move back and forth with small net displacements while granules moving in one direction at the same rate display large displacements. Visual observation of pigment granules in the stages and regions to be compared revealed no Brownian motion. When successive frames at 1 minute intervals were compared, most granule positions had not changed substantially and displacements were rarely as great as 2μ. Thus, the following measurements were made on frames taken at 1 minute intervals. To eliminate the errors inherent in measuring displacements using a grid, changes in distances between the members of pairs of granules were used as an indirect method of comparing the rates of granule movement within different stages and regions. For each one minute interval in a series, changes in inter-granule distance were measured for 45 granule pairs, representing all combinations of a group of 10 granules (see MATERIALS AND METHODS, Section 4c). Measurements were made on films of two clear areas, three other regions of eggs at the same stage, and four fertilized eggs prior to division (two normal and two colcemid-treated eggs).

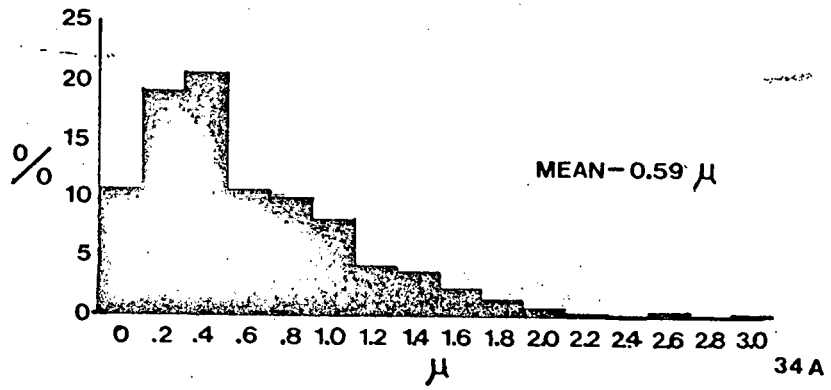
Frequency distributions of changes in inter-granule distance

in each of the above regions or stages of eggs from a single female are presented in Figure 34. The distribution of values for granules within a clear area differs from the others only in slightly higher frequencies of values between 1.2 and 2.0μ and in the presence of several values greater than 2.0μ. Significantly, the measurements resulting in values greater than 2.2μ within the clear area (representing only 0.6% of the total) all involved a particular granule at the edge of the region. The mean value for the change in inter-granule distance within this clear area differed by less than 0.2μ from those for the other region and stage measured. Since this figure represents the experimental error of the measurements, this difference is not significant. Thus, granules within clear areas do not appear to move at greater rates than those in other regions of the cortex of eggs at the same stage or in the cortex of fertilized eggs prior to division. Since these measurements were made on colcemid-treated eggs, values for a colcemid-treated, fertilized egg were compared with those for a normal egg at the same stage and from the same female. The frequency distributions which are presented in Figure 35 demonstrate no differences between these two eggs.

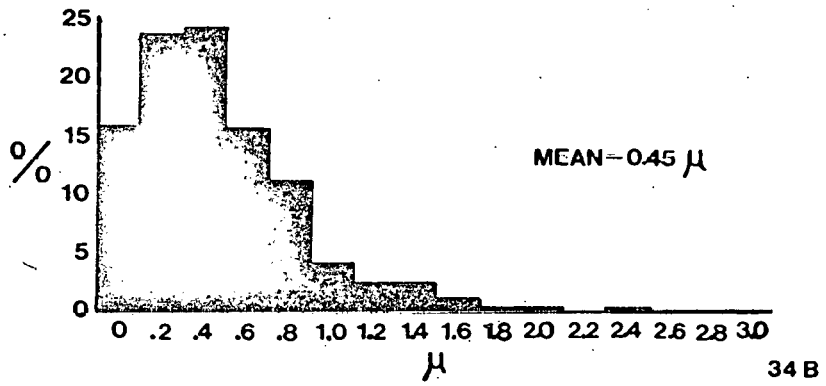
e. Summary

Migration of pigment granules out of the presumptive micromere region of colcemid-treated eggs involves oriented movement of the majority of pigment granules toward the perimeter of the region. However, a small percentage of granules move from the periphery to the center of the region. The rates of pigment granule movement

Figure 34. Frequency distributions of changes in inter-granule distance: A) within a clear area, B) in another region of an egg at the same stage and C) in the cortex of a fertilized egg prior to division. All eggs were obtained from the same female.


25
20
15
10
5
0
%
MEAN – 0.59 μ
0 .2 .4 .6 .8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8 3.0
μ

34 A


25
20
15
10
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0
%
MEAN – 0.45 μ
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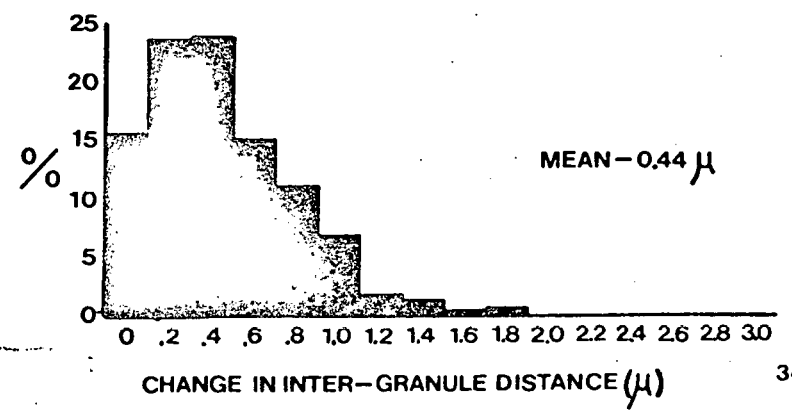
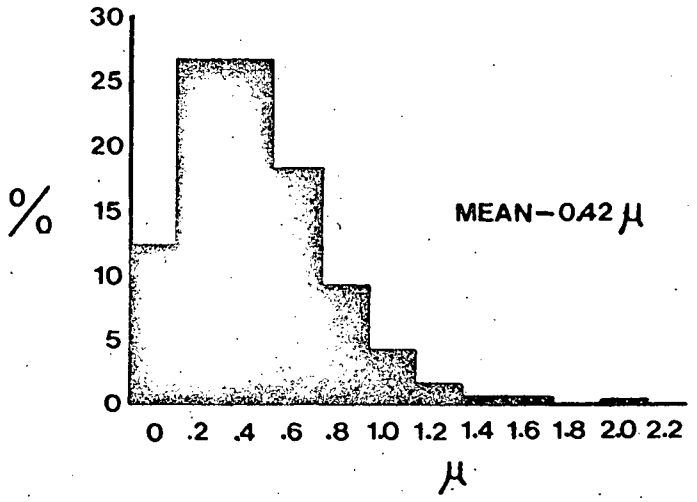
μ

34 B

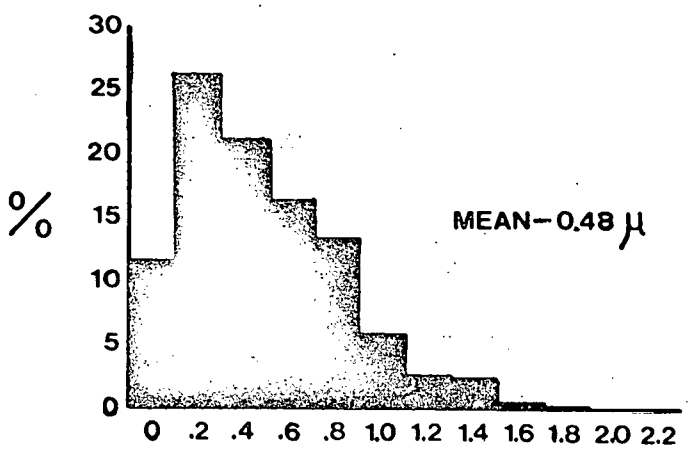

25
20
15
10
5
0
%
MEAN – 0.44 μ
0 .2 .4 .6 .8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8 3.0
CHANGE IN INTER–GRANULE DISTANCE (μ)

34C

Figure 35. Frequency distributions of changes in inter-granule distance in A) control and B) colcemid-treated, fertilized eggs prior to division. Both eggs were obtained from the same female.



35 A



35 B

CHANGE IN INTER–GRANULE DISTANCE (μ)

within clear areas appear to be the same as those in other regions of the cortex of eggs at the same stage or in the cortex of fertilized eggs prior to cleavage.

DISCUSSION

1. Involvement of microtubules and microfilaments in echinochrome granule movements.

a. Microtubules

Microtubules which display an orientation parallel with that of granule movement have been found in several types of cells (Reviewed by Rebhun, 1972). Treatment with colchicine or other agents thought to disrupt microtubules inhibits movement of some of these granules, including vitally-stained granules in eggs of the surf clam, Spisula and the sea urchin, Lytechinus (Rebhun, 1972), pigment granules in erythrophores of the squirrel fish, Homocentrus and melanophores of the killifish, Fundulus (Junqueira and Porter, 1969), and various particles in cultured mammalian cells (Freed and Lebowitz, 1970). However, colchicine has no effect upon saltation of echinochrome granules in unfertilized Arbacia eggs or upon their migration to the cortex at fertilization (Rebhun, 1967). On the other hand, colchicine does inhibit the aster-associated saltatory movements of those echinochrome granules which do not migrate to the cortex (Rebhun, 1973). This suggests that microtubules are required for some, but not all, movements of pigment granules in Arbacia eggs.

The present study has demonstrated that prefertilization saltation of echinochrome pigment granules and their migration to the cortex at fertilization are not inhibited by concentrations of col-

cemid (2.7×10^{-5} - 2.7×10^{-3}M) or vinblastine (10^{-5} - 10^{-4}M) which are sufficient to block pronuclear fusion and mitosis. (Treatment with 10^{-2}M colcemid did inhibit these pigment granule movements; however, this concentration produced toxic effects resulting in aggregation, swelling and lysis of pigment granules.) In addition, migration of pigment granules out of the presumptive micromere region, which begins during the 4-cell stage in normal embryos, also occurred in non-dividing eggs which had been treated with these agents.

Colcemid (N-desacetyl-N-methyl colchicine) is less toxic and more effective in disrupting the mitotic spindle than colchicine at the same concentrations (Kobayashi and Nakamura, 1957a; 1957b). The molecular mechanism of action of colcemid is generally assumed to be the same as that of colchicine, i.e., binding to microtubule subunit protein and thus disrupting or preventing assembly of microtubules (Taylor, 1965; Borisy and Taylor, 1967). Vinblastine inhibits mitotic events by inducing the aggregation of microtubule protein into crystalline arrays and has been reported to bind to a different site than colchicine (Weisenberg and Timasheff, 1970; Olmsted et al., 1970). The occurrence of normal pigment granule movements not only in colchicine (Rebhun, 1967), but also in both colcemid and vinblastine strengthens the hypothesis that microtubules are not required for these movements.

Microtubules have not been found in electron micrographs of unfertilized Arbacia eggs, even though other cytoplasmic structures (mitochondria, Golgi, annulate lamellae) appeared normal (see Jubin-

ville et al., 1967; Anderson, 1968; 1970). Spindle microtubules are well-preserved in dividing eggs (Schroeder, 1972), as are those in the general cytoplasm of later developmental stages (Gibbins et al., 1969; Tilney and Gibbins, 1969a; 1969b; Tilney and Goddard, 1970). Thus, failure to observe microtubules in unfertilized eggs suggests the absence of these structures unless there are gross differences in the rate and degree of penetration of fixative into unfertilized and fertilized eggs.

In summary, it appears that microtubules are not required for saltatory movements of echinochrome granules in unfertilized eggs, for their migration to the cortex at fertilization, or for their movement out of the presumptive micromere region. The apparent dependence of aster-associated saltations on the presence of microtubules (Rebhun, 1973) suggests that different cytoplasmic elements are involved in echinochrome granule movements in different regions and/or stages of development.

b. Microfilaments

Cytoplasmic filaments ranging from 40 - 80Å in diameter have been observed in a number of cells which display motile or contractile behavior. It has been suggested that these microfilaments may be involved in cytoplasmic streaming in plant cells (O'Brien and Thimann, 1966; Nagai and Rebhun, 1966), and in amoeboid movement (Bhisey and Freed, 1971), membrane ruffling (Goldman and Follett, 1969) and intracellular particle movements (Buckley and Porter, 1967) in cultured mammalian cells. A band of microfilaments has been

found just under the plasma membrane in the furrow region of many dividing cells and is thought to be involved in cytokinesis (see Schroeder, 1970; 1972). Several embryonic processes, including echinoderm gastrulation (Tilney and Gibbins, 1969b), amphibian neuralation (Baker and Schroeder, 1967) and ascidian tail resorption (Cloney, 1966; 1969) appear to involve bundles of microfilaments.

Several observations suggest that these microfilaments consist of actin-like proteins. Heavy meromyosin (HMM) binds to microfilaments in glycerinated preparations of a variety of non-muscle cells, forming arrowhead configurations similar to those formed in combination with muscle actin (Ishikawa et al., 1969; Perry et al., 1971). Actin-like proteins have been isolated from Acanthamoeba (Pollard et al., 1970), from plasmodia of the acellular slime mold Physarum (Hatano and Oosawa, 1966) and from unfertilized eggs of the sea urchins Hemicentrotus and Pseudocentrotus (Miki-Noumura and Oosawa, 1969). The amino acid composition and other properties of these isolated "actins" resemble those of muscle actin. Actins from Physarum (Nachmias et al., 1970) and Acanthamoeba (Pollard et al., 1970) also bind HMM in vitro. Thus, the resemblance of microfilaments to actin supports the hypothesis that these structures are involved in contractile processes and motility.

Hypotheses of the mechanism of action of CB.--Cytochalasin B (CB), a metabolite of the mold Helminthosporium dematioideum, was shown by Carter (1967) to inhibit movement and cytokinesis of cultured fibroblasts. Schroeder (1969) observed that cytokinesis in

Arbacia eggs was inhibited by CB and that the band of microfilaments in the furrow region disappeared at the same time. Subsequently, the disappearance or disorganization of microfilament bundles was observed in a variety of systems in which cytokinesis, cell movement or morphogenetic processes had been inhibited by CB. The latter included invertebrate gastrulation, ascidian tail resorption, formation of tubular glands in chick oviducts, branching of mouse salivary gland epithelium and axon elongation (see review by Wessells et al., 1971). On the basis of these observations, Wessells and his colleagues suggested that the effect of CB on these processes was primarily due to selective destruction of 40 - 80Å microfilaments.

Since these microfilaments may contain actin-like proteins, several investigations have been concerned with the possible interaction of CB with actin from muscle. Spudich and Lin (1972) found that the viscosity of actomyosin preparations from rabbit skeletal muscle and the activity of the acto-HMM ATPase were decreased in the presence of CB. These authors suggested that CB competes with myosin for binding to actin filaments. However, the CB concentrations used in these experiments were at least ten times those normally used to produce inhibition of microfilament-related processes. In addition, CB did not prevent the in vitro transformation of isolated G-actin to F-actin, nor did it interfere with the HMM binding to, or release from, isolated F-actin filaments (Forer et al., 1972). Thus, isolated actin filaments can retain several structural and functional properties in concentrations of CB which inhibit motility and morphogene-

sis in non-muscle cells. Actin within muscle cells also appears to be resistant to CB. Myoblasts cultured in CB were able to produce actin and myosin and contract spontaneously (Sanger et al., 1971). Spontaneous contraction was also observed in CB-treated cultures of cardiac and smooth muscle cells isolated from chick embryos (Sanger and Holtzer, 1972). However, CB reversibly inhibited beating in hearts of 13 - 14-day chick embryos. Myofibrillar organization was also disrupted in CB-treated hearts (Manasek et al., 1972). The difference between the responses to CB of cardiac muscle cells in culture and in situ might reflect an effect on functional interactions between cells, rather than on properties of individual cells. A difference in permeability to CB might also explain this discrepancy. In any event, CB does not always appear to exert an effect on the structure on the structure or function of actin filaments in vitro or in vivo.

In certain non-muscle cells, microfilaments were not disrupted by CB-treatments which inhibited movement or morphogenetic processes. Microfilaments were still present in cells of the alga, Nitella after cytoplasmic streaming had been inhibited by CB (Rebhun, 1972). Leukocytes in which spontaneous motility was inhibited by CB also showed no changes in the arrangement of microfilaments (Becker et al., 1972). The response of BHK fibroblasts to CB was variable: in some cells microfilaments disappeared, in others, they remained in the normal pattern, and in a third group, the microfilament bundles under the cell membrane disappeared while other microfilaments were found

with thicker filaments in "muscle-like" configurations (Goldman, 1972). Chang liver cells, in which endocytosis had been inhibited by CB still contained microfilaments, but their distribution within the cells had been altered (Wagner et al., 1971). Cessation of axon elongation and growth cone activity in cultured neurons treated with CB was also accompanied by a redistribution, but not a disappearance of microfilaments (Yamada et al., 1970). The band of microfilaments in the furrow region of Xenopus eggs remained after CB-induced forrow regression (Bluemink, 1971a; 1971b). However, the diameter of these filaments was 80 - 100Å rather than 40 - 80Å, which might explain their insensitivity to CB. Thus, in the presence of CB, microfilaments may retain their structural integrity and organization, be redistributed within the cell, or be completely disrupted. However, the continued presence of microfilaments in some CB-treated cells cannot be regarded as proof that these structures are able to function in CB.

Observations of cleavage inhibition by CB in a number of cells led to an alternative hypothesis that the primary effect of CB is on membrane structures. Membrane effects could indirectly cause changes in associated microfilaments in some cells (Bluemink, 1971a; 1971b). Only in HeLa cells and Arbacia eggs has complete prevention of furrowing been observed (Schroeder, 1970; 1972). Furrows were nearly completed and then regressed in some other cultured mammalian cells (Carter, 1967; Krishan and Ray-Chaudhuri, 1969; Krishan, 1971) and in Xenopus eggs (Bluemink, 1971a; 1971b; Hammer

et al., 1971) treated with CB. Carter (1967) suggested that the apparent increase in adhesion to the glass substrate might explain the inability of mouse fibroblasts to complete the furrowing process in CB. In addition to the initial furrow formation, growth of new membrane is required for cell division in Xenopus eggs. Since furrowing began normally in CB-treated Xenopus eggs, Bluemink (1971a; 1971b) has suggested that CB inhibits membrane growth and/or junction formation between blastomeres, rather than the activity of the equatorial band of microfilaments. Hammer and her colleagues (1971) have proposed a similar mechanism for cleavage inhibition by CB in Xenopus eggs. If the equatorial band of microfilaments does provide the impetus for furrowing (see Section 2), the above observations suggest that CB does not interfere with the functioning of these microfilaments in several types of cells. The initial furrowing in CB-treated cells might have resulted from a difference in the permeability of the cell membrane to CB, rather than from a specific effect of CB on membrane functions.

Inhibition of transport of specific substances into cells provides further support for the hypothesis that CB affects membrane properties. In cultured mammalian cells treated with CB, uptake of glucose and glucosamine (Cohn et al., 1972; Estensen and Plagemann, 1972; Kletzien et al., 1972; Zigmond and Hirsch, 1972) and uridine and thymidine (Plagemann and Estensen, 1972) was inhibited, while other substances were transported at the normal rates. This inhibition of transport appears to display a degree of cell specificity,

since the uptake of RNA precursors into chick embryonic cells was normal in the presence of CB (Maslow and Mayhew, 1972).

When cells from different embryonic tissues are combined in culture, they sort out, forming histotypic aggregates. This process was inhibited by CB in some combinations of cells (Maslow and Mayhew, 1972; Steinberg and Wiseman, 1972; Sanger and Holtzer, 1972), but continued normally in others (Armstrong and Parenti, 1972). Failure of cells to sort out was not merely a consequence of inhibition of movement by CB, since the initial aggregation was normal. Thus, CB apparently interferes with the specificity of recognition, attraction and/or adhesion between cells of a particular tissue. These processes appear to be functions of the cell membrane or of extracellular surface coats.

Phagocytosis and pinocytosis are also inhibited by CB in some cells. CB inhibited phagocytosis of bacteria by leukocytes and macrophages (Allison et al., 1971; Davis et al., 1971; Malawista et al., 1971) and pinocytosis of sucrose by Chang liver cells (Wagner et al., 1971), while uptake of colloidal gold or ferritin occurred normally in macrophages (Wills et al., 1972). Cell membranes are involved in these processes. However, a cortical layer of microfilaments which can bind HMM has been observed in normal mouse macrophages (Allison et al., 1971). It seems quite possible that the inhibition of phagocytosis and pinocytosis by CB was the result of disruption of these microfilaments.

In summary, inhibition of movement, morphogenesis and other processes by CB involves physiological effects on components of the cell surface, namely membranes and underlying microfilaments. The variation in CB-sensitivity of similar processes in different cells may reflect intercellular differences in the composition of membranes and microfilaments. Alternatively, the degree of involvement of the CB-sensitive structure(s) or event(s) in a given process may vary between different types of cells. The primary site and mechanism of action of CB remain uncertain. In many cells, the structural integrity of arrays of microfilaments was disrupted by CB; however, specific binding of CB to possibly homologous actin filaments in muscle has not been demonstrated. Since both membranes and microfilaments appear to be affected, CB may be interfering with interactions within a complex of cell surface structures which are involved in movement and morphogenesis as well as other processes.

Effects of CB on granule movements.--Treatment with CB inhibits saltatory movements and cytoplasmic streaming in algal cells (Wessells et al., 1971; Rebhun, 1972; Williamson, 1972), movement of melanin granules within melanocytes of frog skin (McGuire and Moellmann, 1972; McGuire et al., 1972) and saltatory movement of echinochrome granules in unfertilized Arbacia eggs (Van Wie, 1969; Belanger and Rustad, 1972; Rebhun, 1972). In melanocyte processes, microfilaments disappeared when granule movement was inhibited (McGuire and Moellman, 1972; McGuire et al., 1972).

In the experiments described herein, CB inhibited certain movements of echinochrome granules, allowed others to continue normally and even appeared to induce granule movement at one stage. This variability in response to CB reinforces the hypothesis that echinochrome granule movements do not occur by a single mechanism, but rather are governed by structures or processes in particular regions of the egg during different stages. Thus, conclusions regarding the mechanisms of these movements must take into account the location of the moving granules and the concurrent developmental events, as well as the sensitivity of the movements to CB and colcemid. This information is summarized in Table 2.

Saltation of echinochrome granules in unfertilized Arbacia eggs is random in direction (Parpart, 1964) and appears to represent independent granule motion rather than a response to cytoplasmic streaming. Migration of echinochrome granules to the cortex at fertilization occurs by a similar process in which most, but not all, saltatory movements are oriented toward the cortex. Both of these "independent" granule movements occurred in the presence of colcemid, but were inhibited by CB. If a cytoplasmic structural element is required for these movements, the preceding observations suggest that microfilaments rather than microtubules may be involved.

Pigment granules associated with the asters of the mitotic apparatus display saltatory movements along the astral rays. However, these movements continue in CB, but not in colchicine (Rebhun, 1973), suggesting involvement of astral microtubules, but not microfilaments.

TABLE 2

ECHINOCHROME GRANULE MOVEMENTS
IN COLCEMID AND CYTOCHALASIN B

Movement	Location	Associated Processes	Sensitivity to: CB	Colcemid
Prefertilization saltation	Cytoplasm		Blocks	No effect
Migration to cortex	Cytoplasm to cortex	Fertilization	Blocks	No effect
Aster-associated saltation	Cytoplasm	Mitosis	No effect*	Blocks*
Aggregation into clusters	Cortex	Pre-cleavage	Induces	Does not induce
Equatorial pigment accumulation	Cortex	Cleavage	Enhances	Blocks
Clear area formation	Cortex		Blocks**	No effect

* From Rebhun, 1973. Colchicine rather than colcemid was used in this experiment.

** In colcemid-treated, undivided eggs.

During cleavage in normal Arbacia eggs, pigment granules embedded in the cortex tend to accumulate in the furrow region and become less concentrated at the poles. This has been regarded as a reflection of cortical changes involved in furrowing rather than as independent granule movement (see Section 2). In CB-treated eggs which did not form furrows, the accumulation of pigment granules was much more dramatic than normal, resulting in an equatorial band of closely packed granules. Thus, cortical changes involved in migration of pigment granules to the equator during cleavage are not blocked by CB.

The transient aggregation of cortical pigment granules into clusters, which was often induced when eggs were placed in CB at metaphase, was never seen in normal or colcemid-treated eggs. This might reflect an effect of CB on the cortex or plasma membrane, but could also be a result of enhanced affinity or adhesion between pigment granules.

Movement of individual granules comprising clusters and equatorial bands was also observed. This provides a further demonstration that CB does not prevent movement of echinochrome granules within the cortex of fertilized Arbacia eggs. These movements might have been similar to the "slow shifting" observed in the cortex of normal and colcemid-treated fertilized eggs. Alternatively, they might have reflected changes or movements of the cortex or liquification of its "gel" structure. Since granules did not migrate by Brownian movement from the cortical layer into the general cytoplasm in these eggs, it

seems unlikely that the cortical gel was completely disrupted by CB treatment.

Movements of pigment granules leading to formation of a clear area in the cortex of the presumptive micromere region were not inhibited by colcemid. When CB was added to colcemid-treated eggs as clear areas were beginning to form, the movement of individual pigment granules within this region was inhibited. The formation of a clear area may be the result of "independent" granule movement with a preferential orientation or of changes or movements of the "cortical gel" (see Section 4 for a discussion of these possibilities). The sensitivity of pigment granule movements within the presumptive micromere region to CB suggests that they are more similar to "independent" granule movements, such as migration to the cortex, than to the cortical changes involved in cleavage which result in movement of pigment granules. Alternatively, the process of clear area formation might involve both independent and cortical movements. In either case, the inhibition of pigment granule movement within the presumptive micromere region by CB suggests that microfilaments may be involved.

Thus, the variation in sensitivity of different pigment granule movements to CB appears to reflect the location and general characteristics of the movements. Movements which were inhibited by CB seem to involve independent granule movement, with or without a preferential orientation. Granule movements observed in CB either occur in the cortex and appear to reflect movements of changes of this

layer as a whole, or are specifically associated with the microtubules of the mitotic apparatus.

The inhibition of independent granule movement by CB has been regarded as support for the hypothesis that microfilaments are required for these movements. Although microfilaments have not been found in electron micrographs of unfertilized Arbacia eggs, they are known to occur in the cortical region of fertilized sea urchin eggs during certain stages. In Arbacia, microfilaments have been observed in a band (0.2u thick and 8.0u wide, Schroeder, 1972) just under the plasma membrane in the furrow region during cleavage. The microvilli of fertilized Strongylocentrotus eggs contain cores of microfilaments, which extend down into the cortical layer (Harris, 1968). Very few microfilaments have been observed in the microvilli of fertilized Arbacia eggs (Schroeder, 1972). However, they are present in the microvilli of ectodermal cells of gastrulae, pseudopodial processes of of primary mesenchyme cells (Gibbins et al., 1969; Tilney and Gibbins, 1969a), and filopodia of secondary mesenchyme cells, which are involved in gastrulation (Tilney and Gibbins, 1969b).

The disappearance of the band of microfilaments in the furrow region in CB (Schroeder, 1972) demonstrates that one of the effects of this drug on Arbacia eggs is the disruption of microfilaments. Inhibition of gastrulation by CB in Strongylocentrotus and Lytechinus embryos (Wessells et al., 1971) is also thought to involve an effect on microfilaments. Thus, disruption of microfilaments could explain the present observations of inhibition of pigment

granule movement by CB. However, both prefertilization saltation and migration of pigment granules to the cortex occur at stages in which microfilaments have been rarely, if ever, observed. This might reflect increased lability of microfilaments at these stages or they might be present as a fine network, which would be more difficult to identify than discrete bundles of microfilaments.

Alternatively, CB might be causing a general effect on the structural and/or functional properties of the cortex and cell surface. This hypothesis is supported by observations of changes in the appearance of the egg surface in the presence of CB. The hyaline layer, which includes the surface microvilli, often was hypertrophied and granular in appearance after prolonged CB treatment. The surface of the egg below this layer often became wrinkled or bumpy in CB (hyaline layer hypertrophy and surface wrinkling appeared to be separate phenomena, since each was observed alone as well as in combination with the other). Although these changes might represent general physiological effects, or specific effects on the plasma membrane, they could also be explained by disruption of microfilaments, possibly in the microvilli. The elevation of normal-appearing fertilization membranes in unfertilized eggs exposed to CB involves changes in both the cortex and plasma membrane. However, the specific structures on which CB acts to produce this effect remain uncertain. Lysis of the majority of pigment granules in the cortex of fertilized eggs was sometimes observed in the presence of CB and could represent an effect on the membranes surrounding pigment granules. Since both

parthenogenetic activation and pigment granule lysis can be induced by a variety of experimental treatments (see Harvey, 1956), their occurrence in CB may reflect either a non-specific or specific effect. In some eggs, pigment granules were extruded out into the hyaline layer after prolonged CB treatment. This seemed to parallel the extrusion of nuclei observed by Carter (1967) in cultured cells. The mechanism for these effects is unknown.

Thus, CB causes several effects on the cell surface, in addition to inhibiting certain pigment granule movements. Although the cell surface changes might involve effects on membranes or other structures within this region, many of these phenomena could also be explained by direct or indirect effects on microfilaments. If prefertilization saltation of echinochrome granules, their migration to the cortex and movements during clear area formation do in fact depend on cytoplasmic structural elements, inhibition of these movements by CB suggests that microfilaments are involved.

2. Pigment granule movements in eggs exposed to CB before cytokinesis

Many attempts to understand the mechanism of cytokinesis have been concerned with the site of origin of the "active force" causing the formation of a furrow. In the "expanding membrane" hypothesis, Mitchison (1952) and Swann(1952) proposed that the polar cortex expands, pushing the furrow in passively. Alternatively, Marsland and Landau (1954) suggested the "contractile ring" hypothesis involving active contraction of a band of cortical gel at the equator to form

the furrow, and thus induce passive stretching of the polar cortex. The "astral relaxation" hypothesis of Wolpert (1960) embodies features of each of the preceding hypotheses, but reduces both polar expansion and equatorial contraction to passive processes. Wolpert proposed that material originating in the asters preferentially relaxes tension at the poles, thus allowing contraction at the equator.

In a wide variety of cells, including Arbacia eggs, a bundle of microfilaments (the "contractile ring") has been identified beneath the plasma membrane of the furrow region and is thought to correspond to the cortical gel (see Schroeder, 1970; 1972). These microfilaments disappear or become disoriented when cytokinesis is inhibited by CB in HeLa cells and Arbacia eggs (Schroeder, 1970; 1972). This has been regarded as evidence that furrowing results from equatorial contraction, involving "contractile ring" microfilaments (Schroeder, 1970; 1972) (for alternative interpretations of the mechanism by which CB inhibits cleavage in other cells, see Section 1b).

In Arbacia eggs, Dan (1951; 1954) and Scott (1960) have demonstrated that pigment granules accumulate in the furrow region during cleavage and become less concentrated in the polar regions. These movements are thought to reflect the equatorial contraction and polar expansion of the cortex and are consistent with all of the above hypotheses.

In the present investigation, it was found that the accumulation of pigment granules in the equatorial region occurred in the

presence of CB, even though no furrow formed (see Figure 14). As pigment granules migrated toward the equator, blebs of the hyaline layer formed above the concentrated band of pigment (see Figure 15). The accumulation of pigment granules appears to correspond to that observed during normal cleavage; the hyaline layer blebs probably represent material that would have been distributed along the sides of the furrow.

From observations of the movements of pigment granules during furrowing, Scott (1960) has inferred that cortical contraction in the furrow region consists of two components; longitudinal contraction (perpendicular to the plane of the furrow) and latitudinal contraction (parallel to the furrow circumference). A plausible explanation for the accumulation of pigment granules at the equator in CB is that latitudinal contraction was inhibited by CB-treatment, while longitudinal contraction continued. Longitudinal contraction would then be independent of latitudinal contraction, which might involve "contractile ring" microfilaments. If pigment accumulation and hyaline layer blebbing also reflect polar expansion, this expansion neither induced (as predicted by the "expanding membrane" hypothesis) nor resulted from (as in the "contractile ring" hypothesis) formation of a furrow in these CB-treated, non-dividing eggs. Polar expansion coupled with longitudinal equatorial contraction may be events which are independent of, and equally as "active" as furrowing.

Thus, certain phenomena associated with normal cleavage, including accumulation of pigment and hyaline layer material at the

equator can occur in the absence of furrowing. Normal changes in cortical birefringence and rigidity have also been observed in cells which did not divide (Monroy and Montalenti, 1947; Swann and Mitchison, 1953). However, in such eggs cleavage did not occur because the mitotic spindle had been disrupted by colchicine treatment. In this connection, it may be important to note that no concentrated bands of pigment or hyaline layer blebs were ever seen in colcemid-treated, non-dividing eggs during the course of the present experiments. Thus, these phenomena may represent activities occurring during cleavage which depend on the presence of a spindle or asters.

In eggs of the clam, Spisula, treated with CB, cortical events associated with the cleavages forming polar bodies also occur in the absence of furrowing (Longo, 1972). In untreated eggs, the spindle migrates to the cortex, cortical granules are moved out of this region, a cytoplasmic protrusion forms and is separated from the egg by cleavage. When eggs were treated with CB, all of the above events occurred normally with the exception of cleavage. The cytoplasmic protrusion was resorbed and appeared again at the time of formation of the second polar body in CB-treated eggs. The movement of cortical granules and appearance of cytoplasmic protrusions may be related to the presence and movement of the spindle. The preceding observations, as well as those of the present study demonstrate that cleavage may involve a group of independent events, some of which can be separated by CB-treatment.

3. Differentiation of the presumptive micromere cortex in the absence of mitosis and cleavage

During the 4-cell stage of normal Arbacia embryos, pigment granules begin to migrate out of the region which will become the micromeres of the 16-cell stage, leaving a clear area on each blastomere (Morgan, 1893). The present study has demonstrated that this pigment migration takes place at the normal time in eggs in which mitosis and cleavage were prevented by a variety of experimental treatments. Clear areas were formed in eggs treated with colcemid or vinblastine, both of which inhibit mitosis by interfering with microtubule function. Colcemid can either prevent pronuclear migration and subsequent fusion (Zimmerman and Zimmerman, 1967) or block mitosis at metaphase (Sauaia and Mazia, 1961), depending on the time of application. Vinblastine blocks the same stages. In eggs treated with these agents soon after fertilization, a mitotic apparatus was never formed and thus could not have influenced the migration of pigment out of the presumptive micromere region. Clear areas also formed in undivided eggs which had been exposed to puromycin, which blocks mitosis in the early prophase "streak" stage (Hultin, 1961). Protein synthesis is known to be substantially reduced in puromycin-treated eggs (Hultin, 1961; Black et al., 1967), but the mitotic inhibition may not be a result of a deficiency in essential proteins (Rebhun et al., 1969; Rebhun and Monroy, 1970). The formation of clear areas was also observed in γ-irradiated, undivided eggs. Gamma-irradiation

of unfertilized eggs (Henshaw and Francis, 1936) or newly-fertilized eggs (Henshaw and Cohen, 1940) causes a dose-dependent delay in mitosis. This delay is primarily expressed during prophase stages (Henshaw, 1940). As yet, the specific mechanism of the antimitotic action of Y-irradiation is unknown (see review by Rustad, 1971). Clear areas were also observed in non-dividing eggs which had been parthenogenetically activated by CB treatment and subsequently washed. In eggs which had been incubated between 4° and 8°C, clear areas formed in the absence of mitosis or cleavage, but at a much slower rate. Thus, the migration of pigment granules out of the presumptive micromere region occurred in both activated eggs and fertilized eggs in which mitosis and cleavage were blocked at different stages (before pronuclear fusion, during prophase or at metaphase).

The migration of pigment granules out of the presumptive micromere region is the earliest visible sign of the differentiation of micromeres. Previous observations of micromere formation at the normal time in eggs in which mitosis was delayed suggested that the time of formation of micromeres is controlled by a cytoplasmic "clock", which is independent of the number of mitoses which have occurred (Driesch, 1906; Painter, 1915; Harvey, 1956; Rustad, 1960; Ikeda, 1965). Ikeda (1965) proposed that this "clock" might involve rhythmic fluctuations in the sulfhydryl content of a KCl-soluble protein isolated from sea urchin eggs, which occur during each division cycle. Sakai (1968) suggested that these fluctuations might be the result of exchange of sulfhydryl groups between KCl-soluble protein

in the cortex and a water soluble protein in the cytoplasm, thereby increasing the contractility of the cortex at the time of cleavage. If mitosis was temporarily inhibited by exposure to UV-radiation, dinitrophenol or nitroquinoline oxide during one or more division cycles, the sulfhydryl cycle continued. In these eggs, micromeres were formed at the fourth peak of the sulfhydryl cycle, regardless of the number of cells which were produced at that time (Dan and Ikeda, 1971). When the sulfhydryl cycle (and cleavage) was temporarily inhibited by 0.6% ether in sea water, nuclear division continued normally. However, when cleavage was allowed to resume in these eggs, no micromeres were formed at the division which produced 16 cells (Dan and Ikeda, 1971). This further supports the hypothesis that the sulfhydryl cycle may be the cytoplasmic "clock" responsible for micromere formation.

It has not been determined whether the sulfhydryl cycle occurs preferentially in the cortex or the general cytoplasm or represents an interaction between the two (the KCl-soluble protein can be isolated from both the cortex and the cytoplasm). Cyclic changes in birefringence and rigidity associated with cleavage are known to occur in the cortex and continue in non-dividing eggs treated with colchicine (Monroy and Montalenti, 1947; Swann and Mitchison, 1953). However, the relationship of these cortical events to micromere formation has not been investigated. The present study demonstrates that a cortical event which is specifically involved in the differentiation of micromeres occurs in the complete absence of mitosis and

cleavage.

Several other examples of "differentiation without cleavage" have been reported. Lillie (1902) observed that Chaetopterus eggs placed in sea water containing KCl developed functional cilia and underwent other cytoplasmic changes resulting in a structure resembling the normal trochophore larva. These events were observed in eggs which did not cleave; in some eggs mitosis occurred, but in others a single nuclear mass was present throughout. Harvey (1956) has reported the formation of cytoplasmic protrusions resembling cilia in "blastulae" from anucleate Arbacia half-eggs which had been parthenogenetically activated. Although these "parthenogenetic merogones" contained no nuclei, cleavage did occur during their development. In eggs of Rana pipiens, which were induced to mature in vitro by progesterone treatment, a series of morphogenetic movements resembling normal gastrulation occurred after the completion of maturation (Smith and Ecker, 1970). No cleavage was observed in these eggs and the initial events of "pseudogastrulation" could occur in eggs from which the germinal vesicle (nucleus) had been removed prior to progesterone treatment. Thus, in a number of eggs, including those of Arbacia, certain events involved in differentiation appear to be controlled by cytoplasmic processes which are independent of both the mitotic cycle and cleavage.

4. Characterization of pigment granule movements during clear area formation

Analysis of the movement of pigment granules out of the presumptive micromere region of colcemid-treated eggs has been made in order to answer the following questions: (1) Are pigment granule movements within the presumptive micromere region preferentially oriented toward its perimeter or are they random in direction until granules become trapped at the periphery? (2) Do the rates of granule movement within the presumptive micromere region differ from those in other regions of the cortex of eggs at the same stage or in the cortex of fertilized eggs prior to division?

This analysis has revealed that the majority of pigment granules within the presumptive micromere region move predominantly toward its perimeter during formation of a clear area. Movements of individual granules in another region of the cortex of eggs at the same stage and in the cortex of fertilized eggs prior to division show no dominant orientation. The rates of movement of these pigment granules are not substantially different from those of granules within clear areas. In addition, treatment of eggs with hypo- or hypertonic sea water revealed no preferential weakness of the cell surface which might correspond to liquification of the "cortical gel", allowing increased freedom of granule movement in the presumptive micromere region. These observations demonstrate that formation of a clear area results from preferential orientation of pigment granule movements toward the perimeter of the region and does not require increased rates of granule movement.

Oriented movement of pigment granules might reflect movement of the cortical gel as a sheet from the center to the periphery of the region, carrying pigment granules with it. Alternatively, pigment granules might move independently within the cortical layer in response to some other orienting process. Any complete explanation of the orientation of pigment granule movements within the presumptive micromere region must take into account all of the observations listed in Table 3.

Movement of a small number of granules toward the center of the presumptive micromere region, erratic rather than straight granule paths, and independent movement of initially adjacent granules would not occur if moving sheets of cortical gel were carrying pigment granules toward the perimeter of the region. High concentrations of pigment granules would also be unlikely to remain in the center of some clear areas (see Figures 8, 11 and 12) as a result of such a process, unless sheet movement began at some distance from the center of the region. Thus, the observations reported here suggest movement of individual granules rather than of the cortical layer as a whole.

Several types of processes could result in the observed granule movements. According to Morgan and Spooner's (1909) account of clear area formation, "the red granules . . . move off to the sides as though material from below was being carried to the surface." Thus, one possible explanation might be preferential assembly of new cortex in the presumptive micromere region, forcing most pigment

TABLE 3

OBSERVATIONS OF PIGMENT GRANULE MOVEMENT
DURING CLEAR AREA FORMATION

1. Pigment granule movements are preferentially oriented toward the perimeter of the presumptive micromere region.
2. A small number of granules move with the opposite orientation.
3. High concentrations of pigment granules remain in the center of some clear areas (see Figures 8, 11 and 12).
4. Granules initially adjacent to each other move independently.
5. Moving granules follow tortuous, rather than straight, paths.

granules to move to the perimeter. Localized insertion of cortical material between pigment granules could explain the independent movement of adjacent granules, their erratic courses and the failure of all granules to leave the region.

McClendon (1910) suggested that the migration of pigment granules to the rim of the presumptive micromere region was analogous to similar granule movements in the cortex of other eggs during polar body formation (see also Longo, 1972), and to the accumulation of pigment granules in the furrow of cleaving Arbacia eggs. The resemblance between the concentrated ring of pigment granules at the perimeter of many clear areas in undivided eggs (see Figures 8 and 29) and the band of pigment granules at the equator of eggs in which cleavage was inhibited by CB (see Figure 14) concurs with this analogy. The pigment granule movements involved in furrowing appear to be a result of equatorial contraction of the cortex accompanied by expansion or stretching at the poles (see Section 2). Localized expansion of the cell surface could account for all movements of pigment granules observed during clear area formation. In fact, expansion of the egg surface to form pigment-free evaginations was observed after prolonged incubation of fertilized eggs at low temperatures. When these evaginations were reabsorbed (at room temperature), the region remained free of pigment. Unpigmented evaginations also formed when eggs were treated with hypotonic sea water or when part of the egg surface was extruded through a broken fertilization membrane. Thus, a pigment-free region can appear as the result of an

expansion process, but does not necessarily do so in the presumptive micromere region of normal or non-dividing eggs.

Individual pigment granules might also travel through oriented channels in the cortical cytoplasm. However, this would not seem to explain the tortuous nature of granule paths, including movement toward the center of the region for short distances. Failure to observe two granules following the same path is also inconsistent with this hypothesis.

Finally, pigment granule movements might also be associated with cytoplasmic structural elements such as microfilaments or microtubules. The inhibition of pigment granule movement within the clear area by CB, but not by colcemid suggests that microfilaments rather than microtubules could be involved in these movements (see Section 1).

In summary, the characteristics of pigment granule movement during clear area formation are inconsistent with movement of a sheet of cortical gel and strongly suggest independent movement of each pigment granule within the cortex. This movement might occur as a result of localized insertion of new cortex, expansion of the cell surface, or movement of granules through cytoplasmic channels or in association with oriented microfilaments.

5. Conclusion

Movements of echinochrome granules in *Arbacia* embryos reflect

cytoplasmic and cortical events involved in fertilization, mitosis, cleavage and the differentiation of micromeres. Characteristics of granule movement (rates, orientation and sensitivity to inhibitors) vary with the location of the granules and the stage of development of the embryo, suggesting that more than one process can result in granule movement. Observations made in the course of this study are consistent with the hypothesis that prefertilization saltation, migration to the cortex and movement of pigment granules out of the presumptive micromere region involve microfilaments, but not microtubules.

The accumulation of pigment granules at the equator of cleaving eggs appears to be the result of cortical processes involved in furrowing. Appearance of a concentrated band of pigment granules at the equator of CB-treated eggs which never formed furrows demonstrates that cortical events leading to pigment accumulation are independent of furrowing.

Migration of pigment granules out of the presumptive micromere region to form a clear area is the earliest visible sign of micromere differentiation. Occurrence of this migration in non-dividing eggs which had been blocked before pronuclear fusion, during prophase or at metaphase demonstrates the independence of this cortical differentiation from mitosis and cleavage.

Formation of a clear area in the presumptive micromere region involves oriented movement of individual pigment granules at rates

no greater than those occurring in other regions of the cortex at the same stage or in the cortex of fertilized eggs prior to division.

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