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AN ANALYSIS OF GASTRULATION

IN *LOLIGO PEALEI*

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN ZOOLOGY

AUGUST 1977

By

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Abstract

Time-lapse cinemicrography, light microscopy, and electron microscopy were used to study the cellular events and processes of gastrulation in Loligo pealei. Early cleavage (cleavages 1 through 7) are reviewed and later divisions (8 through 11), which occur coincidentally with the onset of gastrulation, are described. Cleavage of the blastodisc results in a single-layered blastoderm. After eighth cleavage a specific population of blastomeres spreads vegetally over presumptive middle-layer cells (= mesendodermal layer of older literature) by the active extension of lamellipodia and filopodia. The process of overlapping is essentially epibolic. After the presumptive middle-layer cells are overlapped they cleave (ninth cleavage) perpendicular to the axis of overlap. The processes of overlapping and cell division together produce a ring of cells between the blastoderm and the yolk cytoplasmic layer.

After segregation of the middle layer the yolk syncytium is formed by the vegetal movements of blastocone nuclei and retraction of the furrows of the blastocones. Furrow retraction is a result of the dissociation of the contractile bands of microfilaments from the bases of furrows and a combined folding and vesiculation of the plasma membranes of the furrows. After their initial

vegetal movements, the yolk syncytial nuclei divide twice by mitosis with the formation of a circumferential, transitory furrow. The yolk syncytial nuclei then come to lie beneath the two-layered blastoderm by means of nuclear movements and epibolic spreading of the blastoderm.

The ultrastructural features of cellular extensions of overlapping cells in Loligo are similar to those of other cells during locomotion. Lamellipodia and filopodia extend from leading edges of overlapping cells and make 20 nm appositional contacts with underlying cells. Overlapping cell processes frequently contain arrays of oriented microfilaments, but few microtubules. Progressive extension and retraction of these cell processes results in the overlapping cells being positioned above the presumptive middle-layer cells.

The significance of cell movement relative to that of cell division in cephalopod gastrulation was assessed using the inhibitors colchicine and cytochalasin B. Cell movement of overlapping and yolk syncytium formation are inhibited by application of 0.2 μg/ml cytochalasin B, but not by 10^{-4}M colchicine. In relation to gastrulation, cytochalasin B inhibits the formation of a two-layered blastoderm whereas colchicine does not.

Descriptive and experimental results are discussed in relation to concepts of cephalopod gastrulation and current

concepts of mechanisms of cell movement. A generalized description of cephalopod gastrulation is presented and discussed with regard to recent literature on cephalopod development.

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1. INTRODUCTION

Gastrulation is of fundamental importance in the embryogenesis of all Metazoa. Since the pioneering work of Holtfreter (1939, 1943, 1944), it has become increasingly clear that gastrulation involves cellular shape change and translocation (see Balinsky, 1970; Johnson, 1974; Berrill and Karp, 1976; Trinkaus, 1976). For example, cellular shape changes have been implicated as a driving force in amphibian gastrulation (Baker, 1965) and in vegetal plate formation in echinoderms (Dan and Okazuki, 1956; Dan 1960; Gustafson, 1963; Gibbins, et al., 1969). Formation of pseudopodia and ruffled membranes by mesodermal cells of amphibians indicate that active migration of individual cells is also involved (Nakatsuji, 1974; Johnson, 1976). The appearance of chorda mesodermal cells with scanning electron microscopy indicates that these cells translocate during gastrulation by means of lamellipodia and filopodia (Monroy, et al., 1976). Active cell movements have been observed during the formation of the archenteron and mesenchyme in echinoderms (Gustafson and Wolpert, 1967; Gustafson and Toneby, 1971) and during epiboly in teleosts (Trinkaus, 1973; Wourms, 1972). Avian gastrulation also appears to involve translocations by various cells (Trelsted, et al., 1967; Hay, 1968; Nicolet, 1971). Although there is

now considerable information concerning the processes of gastrulation, as Trinkaus (1976) has recently stated, there is still a great deal to be learned.

The concept of cephalopod gastrulation has a history paralleling the development of the general concept of gastrulation. Many of the earliest observations of cephalopod development dealt almost exclusively with external morphology (e.g. Kolliker, 1844; Lankester, 1875; Grenacher, 1874; Brooks, 1880). Writers who examined and described gastrulation may be categorized according to their explanation of the supposed mechanisms of gastrulation. Regarding the segregation of the middle (= mesendodermal) layer, early studies fall into three groups. The majority of writers described the formation of the middle layer in cephalopods as a process of mitotic delamination comparable to what was then thought to be the mechanism of presumptive mesoderm formation in birds, teleosts and mammals (Metschnikoff, 1867; Ussow, 1875; Bruce, 1886, Watase, 1891; Teichmann, 1903). Horizontal divisions occurring in either a restricted area (Watase, 1891) or throughout the single-layered blastoderm (Metschnikoff, 1867; Ussow, 1874, 1875, 1881) were thought to give rise to a second layer of cells between the yolk and the blastoderm, thereby producing a "Gastrula" stage embryo. The most descriptive writer, Ussow (1875) gave the following account:

> ". . . the cells, which are continually undergoing further division in a longitudinal direction, begin also to divide gradually in a transverse direction, the division commencing at the lower periphery and advancing towards the center. . . . As the result of this transverse division a second germ-lamella is produced, at first only in the median ring and in the segment part." (Ussow, 1875, p. 109).

Disputing the idea of delamination, Korschelt (1892; and in Korschelt and Heider, 1900, 1936) reported that the middle layer was established by a modified form of invagination which he termed "immigration." He thought that cells near the edge of the blastoderm migrated into the subblastodermal area. Korschelt proposed that ". . . the edge of the germ-disc corresponds to the blastopore which is filled by the large yolk-plug" (Korschelt and Heider, 1900, p. 279). This homologization of the blastodermal perimeter with the blastopore was tenuous, admittedly having been based principally on the proximity of the stomodeal invagination to the perimeter in Grenacher's (1874) Oegopsid embryo. Korschelt's opinions concerning this homology and Naef's (1928) later extensions of the idea were discussed by Sacarrão (1949, 1952 a, b, c, 1953).

Leading a third group of writers, Bobretzky (1877) observed development in Loligo vulgaris and described the middle layer as formed by means of a folding under at the edge of the blastoderm (Umschlagsrand). This view of middle layer formation was further espoused by Vialleton

(1888) and Faussek (1896, 1900). Naef (1928), in his extensive monograph on the Cephalopoda, embraced this theory to derive a scheme for the evolution of the cephalopod embryo. Naef proposed a hypothetical form having an amount of yolk and form of development intermediate between Prosobranchs and Cephalopods. His interpretation, however, ignores obvious differences in the origin and ultimate fate of the germinal layers in these two groups (for a detailed discussion of Naef's views, see Sacarrão, 1953; Arnold, 1971). Other attempts were made to find an intermediate form of development among extant molluscan species. Conklin (1907) found that the origin and fate of the germ layers in the large (1.7 mm diameter), yolky eggs of the whelk Busycon corresponded precisely with those of other prosobranchs. He found that the same was true for Crepidula which also has a large (400 um), yolky egg (Conklin, 1897). Naef's theories have gained little support and it is generally accepted that the cephalopod form of development is unique among the molluscs (Raven, 1958; Arnold, 1971).

Sacarrão (1953) discussed at length problems of segregation and fates of germ layers in cephalopods. His interpretations have gained widest acceptance in recent literature (Arnold, 1965, 1971; Arnold, et al., 1974; Fields, 1965; Raven, 1958; Meister, 1972; De Leo, 1972; Fuchs, 1973; Fioroni, 1974; Fioroni and Meister, 1974). Sacarrão reaffirmed the idea that the middle layer constituted a

complex endomesoderm which arose by means of a mitotic delamination of the originally single-layered blastoderm. However, he observed in only one instance a mitotic figure which would give rise to a cell in the middle region. This provoked the remark that "...la delamination du blastodisque est tres probablement un acte unique."[1] (Sacarrão, 1953 p. 34). Though he favored the idea of mitotic delamination, Sacarrão was aware of the limitation and dangers of interpretation solely from fixed materials. Thus he wrote the following:

> "... on ne peut pas rejecter à priori l'idée d'une migration de quelques elements vers la profondeur. La position oblique d'une ou autre cellule de la marge du blastoderm, comme qu'enchevauchée par l'èlement voisin, fait penser à cette possibilité...."[2] (Sacarrão, 1953 p. 34).

Sacarrão's allusion to the possibility of the movement of cells during germ layer segregation has been ignored by most subsequent investigators of cephalopod development.

Aside from Sacarrão's (1953) observation of a single mitotic figure, little evidence for delamination has been presented. Arnold (1971) presented evidence for delamination in the form of light micrographs showing a mitotic figure

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[1]["... the delamination of the blastodisc is very probably a unique occurrence."]

[2]["... one cannot reject a priori the idea of a migration of some elements toward the interior. The oblique position of one or another cell at the margin of the blastoderm, such as that overlapped by the neighboring element, poses that possibility."]

oriented so that it might give rise to a middle layer of cells. Both Sacarrão's and Arnold's evidence are, however, open to a different interpretation.

Recently, Fioroni (1974) reiterated Korschelt's opinion that middle-layer formation takes place by means of immigration of cells from the periphery of the blastoderm. Fioroni's opinion, like that of Korschelt, was based upon study of sections of paraffin-embedded embryos observed with light microscopy. Neither investigator presented evidence, other than rather interpretive drawings of what he observed. It seems unreasonable to expect to be able to interpret the dynamic phenomena of gastrulation solely by observing sections of fixed embryos. Fioroni concluded by refuting Naef's theory of invagination at an enlarged blastopore and proposing a mechanism of middle-layer formation involving unverified cell movements.

Another aspect of the problem of cephalopod gastrulation is the establishment of the yolk syncytium (= yolk epithelium, yolk membrane or periblast of older literature) (see Arnold, 1971; Arnold and Williams-Arnolds, 1976). Various conflicting descriptions are found in the early literature. Lankester (1875) felt that the yolk syncytium nuclei were derived from the yolk mass as was, at that time, thought of the so-called "yolk nuclei" in arthropods and vertebrates. Ussow (1875) did not recognize the syncytial nature of the yolk syncytial layer and termed it the

"intestino-fibrous layer." He described the "cells" forming this layer as derived by delamination from the middle layer. Ussow felt that the middle layer ultimately produced the muscular envelope of the intestinal tract and ink sac, and the walls of the circulatory system. Vialleton (1888) coined the name "yolk epithelium" to describe the yolk syncytium although he seems to have recognized its syncytial quality. Vialleton described the "yolk epithelium" as forming from the marginal elements of the blastoderm which he named blastocones. The blastocones, he saw, underwent an "essential alteration." Their cellular nature was lost giving rise to a "plasmodium" that moved by "ingression" under the middle layer to the animal apex of the egg as well as down around the yolk mass. Vialleton considered the "yolk epithelium" to be purely endodermal in nature.

Watase (1891) first recognized the role of the yolk syncytium as a transitory digestive organ. He felt that it functioned in the digestion of the yolk during embryogenesis, and that it produced no adult tissue. Korschelt (1900) used Vialleton's term "yolk epithelium" to describe a layer he saw as consisting of very flattened cells. He did not discuss its origin, although he recognized the yolk syncytial layer's role in embryogenesis. Teichmann (1903) thought, wrongly, that the middle layer and yolk

syncytium together constituted the definitive endoderm. He also thought that mesoderm arose from infoldings of the ectoderm. Teichmann did not discuss the formation of the yolk syncytium. The role of the yolk syncytium was later clarified by the investigations of Portmann and Bidder (1928) and of Boletzky (1967, 1975). These authors showed conclusively that the yolk syncytium functioned as a digestive organ and that it was not incorporated into the definitive endoderm which, as Kolliker (1844) had earlier recognized, developed from a small plaque of middle-layer cells. Sacarrão (1953) agreed with Vialleton's description of the mode of formation and syncytial character of the yolk syncytium. Sacarrão (1953) considered that the endoderm in cephalopods was divided into two ontogenetically and functionally distinct tissues: the yolk syncytium was derived from the blastocones and had a transitory role, digesting yolk during development; the definitive endoderm of the adult was derived from the complex endomesodermal layer.

More recently Fuchs (1973) and Fioroni (1974) have confirmed, in various octopods, the fact that the yolk syncytium nuclei arise from the blastocones. However, neither of these writers has addressed questions of the cellular basis of yolk syncytium formation. Both report that, in Eledone, nuclei and cytoplasm of the yolk

syncytium lying under the center of the blastoderm are derived by detachment and incorporation of cells from that area of the blastoderm. Fuchs (1973) summarizes his findings as follows:

> "Im Innern der Keimscheibe. Die Bildung des Syncytium vollzieht sich in diesem Fall am Detachierungsort, direkt unter dem Blastoderm. Vielleicht erhellt daraus, warum nach gewissen älteren Autoren (Ussow 1881; Korschelt 1892) das Dotterepithel aus dem Entomesoderm hervorgeht. Freilich wollen wir damit nicht sagen, daß Entomesodermzellen bei der Bildung des Dotter-epithels mitwirken. Vielmehr differenziert sich das Dotterepithel direkt aus den Blastomeren Gleichzeitig oder sogar noch vor den Entomeso-dermzellen."[1] (Fuchs, 1973 p. 37)

This manner of yolk syncytium development is found in no other cephalopod species. Furthermore, it conflicts with the theory of an organogenetic inductive function of the yolk syncytium (Arnold and Williams-Arnold, 1974, 1976) and with the conservative nature of the cleavage patterns of most cephalopods. Even with these more recent studies knowledge of the mechanism of yolk syncytium nucleation is little advanced beyond Vialleton's (1888) description.

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[1] ["In the interior of the germinal disc. The formation of the syncytium occurs in this case at the point of attachment directly beneath the blastoderm. Maybe this clarifies the fact that according to some previous authors (Ussow, 1881; Korschelt, 1892) the yolk epithelium develops from endomesoderm. Certainly we do not say with this that the endomesodermal cells have a part in the formation of the yolk epithelium. Rather the yolk epithelium develops directly from the blastomeres after or even before the formation of the endomesodermal cells."]

The investigations reported here provide a description of gastrulation in Loligo pealei. Observations made using techniques of light microscopy, electron microscopy and time-lapse cinemicrography are employed to describe: 1) the later phases of cleavage in order to clarify the ontogenetic history of the germ layers, particularly the middle-layer cells; 2) the events of middle-layer segregation; and 3) the events and mechanism of yolk syncytium formation. Observational data are used to present a generalized description of cephalopod gastrulation and are discussed in relation to recent literature on cephalopod development.

Results of experiments with the inhibitors colchicine and cytochalasin B further describe and differentiate the morphogenetic mechanisms involved in segregation of the middle layer and formation of the yolk syncytium. Data from these experiments are compared with observational data on the normal development of Loligo pealei. Observational and experimental data are discussed in relation to cephalopod gastrulation and to problems of morphogenetic cell movement. A testable model is presented as a possible explanation of the mechanism of epibolic cell movement in Loligo.

2. MATERIALS AND METHODS

2.1. Acquisition and Maintenance of Experimental Animals

Adult Loligo pealei were obtained from the Supply Department of the Marine Biological Laboratory at Woods Hole, Massachusetts. These animals were maintained for short periods (3 to 6 days) in fiberglass tanks (0.50 m X 0.75 m X 1.10 m) supplied with fresh, running sea water (ca. 0.35 m in depth) at a temperature of 15° to 21°C. No attempts were made to feed adults to extend their maintenance capacity (see Summers and McMahon, 1970, 1974; Summers, et al., 1974).

Fertilized eggs of L. pealei were obtained by artificially inducing egg laying (Arnold, 1962). Embryos were separated from their surrounding jelly layers using fine forceps. Dejellied embryos were placed in Syracuse watchglasses with fresh sea water for observation. Those to be maintained for longer periods were placed in Stender dishes (62 mm X 36 mm) containing sea water (15 to 20 ml) at room temperature (15° to 21°C). Sea water was changed at least three times daily. A maximum of twelve embryos was maintained in any dish.

Adult Euprymna scolopes were captured and maintained as previously reported (Arnold, et al., 1972). There is no known stimulus for egg laying behavior in this species. To obtain eggs from E. scolopes a mature adult of each sex

was placed in a common tank. Copulation occurred only at night; usually after 2200 hours. The duration of copulation varied from 20 minutes to over 60 minutes. The females usually laid eggs within one week after copulation and then died within the following two weeks. E. scolopes embryos were observed and maintained as described for L. pealei.

2.2. Methods of Light Microscopy, Transmission Electron Microscopy, and Scanning Electron Microscopy

For the study of early cleavage stages and cell lineage, embryos were fixed according to the method of Timmermans, Geilenkirchen, and Verdonk (1970). Whole embryos were fixed in a mixture of formalin, ethanol and acetic acid (FAA), treated with 95% ethanol for 24 hours, and stained by a modified Feulgen stain (Pearse, 1968 p. 648). Stained embryos were dehydrated, cleared in xylene and mounted, whole or with the vegetal half removed, on standard microscope slides. Some FAA-Feulgen treated and Bouin's fixed embryos were embedded in Epon (Luft, 1961) and sectioned at 1 μm for observation with the light microscope. Whole-mounted embryos were observed with a compound microscope and camera lucida drawings made of the various stages.

Embryos were fixed for transmission electron microscopy using several methods. The procedure giving the best and

most consistent results was that of Palade (1952). This was a single fixation procedure using 1% osmium tetroxide in a veronal acetate buffer at pH 6.8. Embryos were fixed for ten to twenty minutes and rinsed in several changes of 50% ethanol. Dehydration continued through a graded ethanol series into propylene oxide. Dehydrated embryos were embedded in Epon (Luft, 1961). Sections 60 nm to 90 nm in thickness, as judged by their interference colors, were taken with a diamond knife using a Reichert OM-U2 Ultramicrotome. Sections were picked up on copper mesh grids and stained with saturated uranyl acetate in 50% methanol (Stempak and Ward, 1964) and lead citrate (Venable and Coggeshall, 1965). Stained sections were observed using a Philips EM 201 electron microscope.

Thick sections (1 to 1.5 μm) of osmium fixed, Epon embedded embryos were made with glass knives for observation with the light microscope. Thick sections were mounted on standard glass slides and stained with either Richardson's methylene blue-azure II mixture (Richardson, et al. 1960) or a sequential methylene blue, azure II, and basic fuchsin series as described by Humphry and Pittman (1974).

Other fixation procedures used for electron microscopy were as follows:

1. Embryos were prefixed in 2.5% glutaraldehyde buffered to pH 7.2 with 0.2 M Millonig's phosphate, adjusted

to 960 milliosmoles with NaCl. Prefixation was followed by three buffer rinses. Post fixation was accomplished in 1% osmium tetroxide in Millonig's phosphate buffer at pH 7.2 for 15 to 20 minutes (Cloney and Florey, 1968).

2. Embryos were prefixed for 1 hour in 2% glutaraldehyde in 0.1 M cacodylate buffered sea water at pH 7.2, which was adjusted to 1.5% sucrose. Prefixation was followed by two 15 minute rinses in 0.1 M cacodylate in sea water plus 5% sucrose. Post fixation was accomplished in 2% osmium tetroxide in distilled water followed by two 15 minute rinses in 0.1 M cacodylate in distilled water.

3. Embryos were prefixed for 1/2 to 1 hour in 2.6% glutaraldehyde in 0.1 M collidine in sea water at pH 7.4 followed by a 30 minute rinse in filtered sea water. Embryos were post fixed in 1% osmium tetroxide in collidine buffered sea water at pH 7.4, rinsed in filtered sea water and dehydrated (Arnold, personal communication).

Embryos prepared for scanning electron microscopy were fixed as for transmission electron microscopy, dehydrated in acetone and dried in a Sorvall Critical Point Drying System (Du Pont-Sorvall Inc., Newtown, CT) using Freon 113. Dried specimens were coated with carbon and gold and observed using a JOELCO Scanning Electron Microscope.

2.3. Methods for Time-Lapse Cinemicrography

Time-lapse sequences of germ layer segregation in L. pealei were made using a Sage 500 Cinephotomicrographic Apparatus (Sage Instruments, White Plains, NY) in conjunction with a Zeiss RA microscope (Carl Zeiss, West Germany) fitted with Nomarski interference-contrast optics. The microscope's built-in illuminator or alternatively a fiber-optics source (Bausch and Lomb, Rochester, NY) was used as a light source. Use of the latter obviated the need of a beam interruptor to avoid heating the embryos. Exposures of 0.25 seconds were made at a rate of 8 or 12 frames per minute on Kodak Ektachrome EF Film (7242) (Eastman Kodak, Rochester, NY).

For filming, embryos of an appropriate stage were separated from their surrounding jelly layers, installed in a perfusion chamber as illustrated in Fig. 1 and placed on the microscope stage. Embryos from the same egg string were maintained as controls. A flow of fresh sea water through the apparatus was maintained at a rate of 10 to 12 drops per minute (ca. 0.5 ml/min.) throughout filming.

Time-lapse films were analyzed in the following manner. Single frames were projected onto translucent paper and the outlines of individual cells traced. Tracings of successive frames were superimposed to determine changes in the morphology of the leading edge and extent of cell movement. Cell movement was measured on tracings,

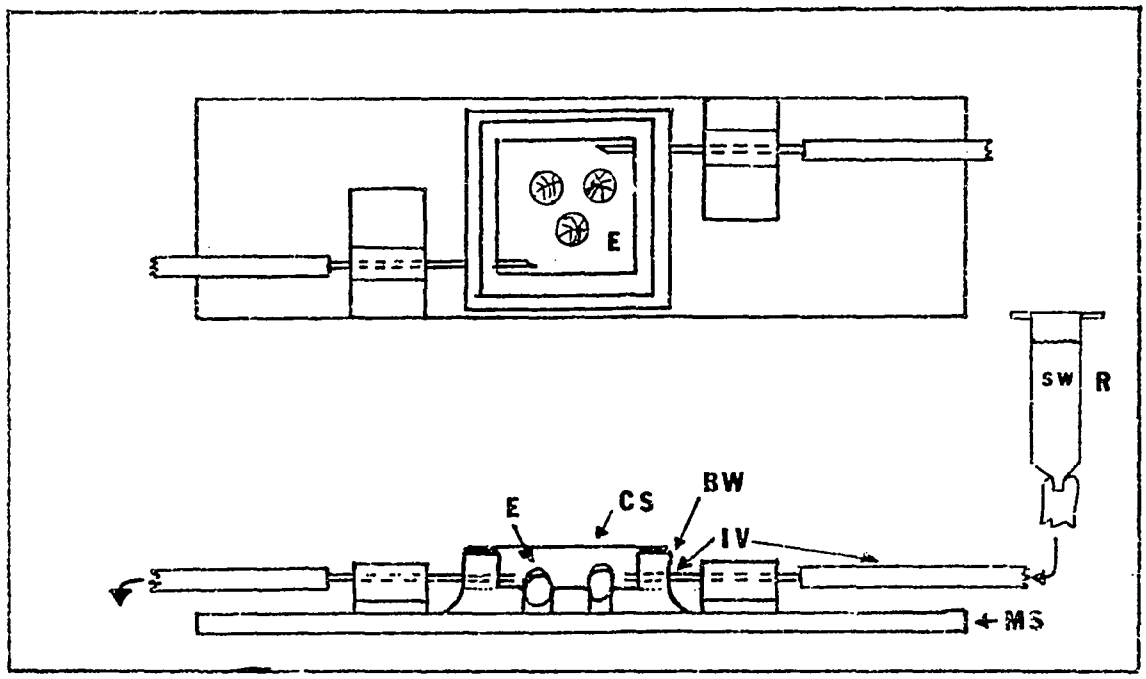


Fig. 1. Perfusion Apparatus for Time-Lapse Filming

The perfusion apparatus illustrated above was constructed with the following materials. A square (25 mm) piece of Dental Bite-Wax (BW)(Hygienic Dental Mfg. C., Akron Ohio) was affixed to a standard microscope slide (MS) with moderate warming. Intravenous needle assemblies (IV) ("Small Vein Kit," Becton Dickenson and Co., Parsippany, N.Y.) were placed at opposite corners of the wax piece and secured to the slide with tape. Another 25 mm square piece of wax having an 18 mm square hole was placed atop the first and secured around its edges by melting the wax with a hot needle. Holes (1.0 mm in diameter) were bored into the lower wax piece to receive the embryos (E). A standard 22mm square #1 coverslip (CS) was secured to the wax with petroleum jelly. Fresh sea water (SW) was allowed to flow through the apparatus at a rate of about 0.5 to 1.0 milliliters per minute from the attached reservoir (R). Plans for the construction of this apparatus were provided by Dr. J. M. Arnold.

projected images, and prints of rephotographed frames of 16mm films. Cell movement was determined by measuring the distance between the position of a cell's leading edge in any frame and its position at an arbitrary starting point (time = 0). Time = 0 was chosen as the frame in which minimal overlap was exhibited by those cells which were to overlap the ring of presumptive middle-layer cells after eighth cleavage. Measurements were made at three sites along the leading edge of each cell. The average of these measurements was taken as the distance moved by each cell over the interval between measured frames. Frames were chosen such that the interval between frames was approximately 10 minutes. Rates of movement were determined by dividing measurements of the distance moved by the time interval over which the movement occurred. Time intervals between measurements were determined by multiplying the film exposure interval (i.e. at 8 frames/minute the exposure interval was 7.5 seconds) by the number of frames plus one between measured frames. Suitable sequences were rephotographed on 35mm Kodak Plus X film (Plates V through XII).

2.4. Methods of Experimental Inhibition of Cell Movement and Division

Colchicine and cytochalasin B were used in attempts to specifically inhibit mitosis and cell motility during

early phases of germ layer segregation in Loligo pealei. In each experiment, control and experimental embryos were taken from a single egg string. Each string contains about 180 eggs all of which have been fertilized simultaneously and develop synchronously. Each experimental and control group consisted of 10 or 15 embryos. At least four experimental replications were made for any single time/concentration combination. Experimental results were discarded if more than ten percent of the controls showed aberrant development. This situation occurred only with "end of the season" animals.

Cytochalasin B solutions were made up according to the method of Carter (1967 a). Cytochalasin B was dissolved in dimethyl sulfoxide at a concentration of 1 mg/ml and stored at -4° to -20° C until used. Serial dilutions of the stock solution were made with fresh, filtered sea water to concentrations of 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0μgm/ml. Control embryos were maintained in sea water alone and in appropriate concentrations of dimethyl sulfoxide in sea water corresponding. Embryos at specific stages were treated with each of the above concentrations of cytochalasin B for periods of 5, 10, 30, 60 minutes and continuous immersion.

A few embryos (4) were treated by regional application of cytochalasin B using the method of Arnold and Williams-Arnold (1976). Restricted regions of the blastoderm were

treated by applying (for 10 min.) a small glass probe (ca. 75 m to 100 m at the tip) which had been coated with agar, dried, and soaked in 2.0 µg/ml cytochalasin B.

Colchicine was dissolved in sea water to a concentration of 10^{-4}M. Initially, embryos of various stages were treated with this solution for periods ranging from five minutes to one hour. Because the block of mitosis produced by colchicine at this concentration is irreversible in Loligo, variation of treatment time was discontinued in favor of a single ten or fifteen minute treatment followed by at least three five minute rinses in fresh sea water.

After treatment, the embryos were maintained for periods of three to five days. Daily observations were made and any changes in the morphology of the treated embryos over the maintenance period were recorded. Control and treated embryos were fixed for either light or electron microscopy at the end of the experimental period.

2.5. Terminology of Cephalopod Development Used in This Study

Except as noted below the terminology for cephalopod development used herein will be that of Arnold (1971) and Arnold and Williams-Arnold (1976). A review of the literature on cephalopod development reveals some confusion regarding certain terms descriptive of the structure of the embryo. This has led to considerable misinterpretation by some

workers. The problems are primarily semantic, but nonetheless real. The following are often the source of misconception and are defined as they are used here:

Yolk Cytoplasmic Layer. This is the uncleaved portion original egg cytoplasm before it is invaded by yolk syncytial nuclei. This portion of the embryo is usually lumped with the yolk syncytium. I feel, however, that it must be renamed because of functional and morphological differences and to lend clarity to descriptive analysis.

Yolk Syncytium. The yolk syncytium is that portion of the yolk cytoplasmic layer which has become nucleated and thus constitutes a true syncytium. This structure is, on the average, more than twice as thick as the yolk cytoplasmic layer, and is associated with active yolk digestion. This term corresponds to the terms yolk epithelium and periblast of the literature.

Yolk Syncytium Nuclei. The nuclei of the yolk syncytium. These are larger than the nuclei of the blastomeres, are greatly flattened when they lie beneath the blastoderm, have dispersed chromatin, and are more basophilic than blastoderm nuclei. This corresponds to the term yolk epithelium nuclei of the literature.

Blastomere. As in the usual sense, this term is used to describe the cells resulting from the complete cleavage of the egg cytoplasm. In cephalopods the blastomeres are distinguished from the blastocones by not being continuous

with the yolk cytoplasmic layer. This term has caused confusion by being used in the literature to describe all the products of cleavage.

Blastocones. These are the products of the partial cleavage of the blastodisc which are defined by the meridional and undercutting furrows and which remain continuous with the yolk cytoplasmic layer. Because they remain throughout their history continuous with the yolk cytoplasmic layer, they cannot, by any rigid definition, be called cells. The term "blastocone cell" of the literature is therefore discarded in favor of the simpler blastocone.

3. RESULTS

3.1. Cleavage Pattern in Loligo pealei

The cleavage pattern in Loligo was followed using living and FAA-Feulgen treated embryos and time-lapse cinemicrography. The principal questions of interest with regard to cleavage are: 1) What is the lineage of the cells from which the middle layer arises? 2) Are the middle-layer cells products of a delaminating cleavage, blastomeres which migrate inward from the periphery of the blastoderm or are they derived in some other manner? 3) What is the lineage of the yolk syncytium nuclei? Information relating to each of these questions is presented below.

3.1.1. Establishment of the Blastoderm by Cleavage

The early cleavage pattern of Loligo pealei has previously been described to seventh cleavage (Watase, 1891; Arnold, 1971). Little has been said, however, about the later cleavages just prior to and through germ layer segregation. The following description of cleavage includes a brief redescription of the egg and first seven cleavages in order to lend clarity and continuity to descriptions of the later cleavages.

The egg of Loligo is ovate with one end more pointed than the other, much like a hen's egg. Prior to fertilization the egg cytoplasm exists as a thin layer surrounding the mass of yolk (see Arnold, 1971). Fertilization initiates a streaming of the cytoplasm toward the pointed (animal) end of the egg. There, part of the cytoplasm forms a thickened cap, the blastodisc. At this stage the uncleaved egg possesses a symmetry which is maintained throughout development, and corresponds to the axes of the adult animal (Fig. 2; Watase, 1891; Arnold, 1971). The animal-vegetal plane of the egg corresponds to the caudo-cephalic plane of the adult. The egg is bisected by the anterior-posterior plane, but asymmetrical around the dorsal-ventral axis. Viewed from the side, the anterior portion of the egg is more convex and thus more massive than the posterior. In addition, the place of the zygotic

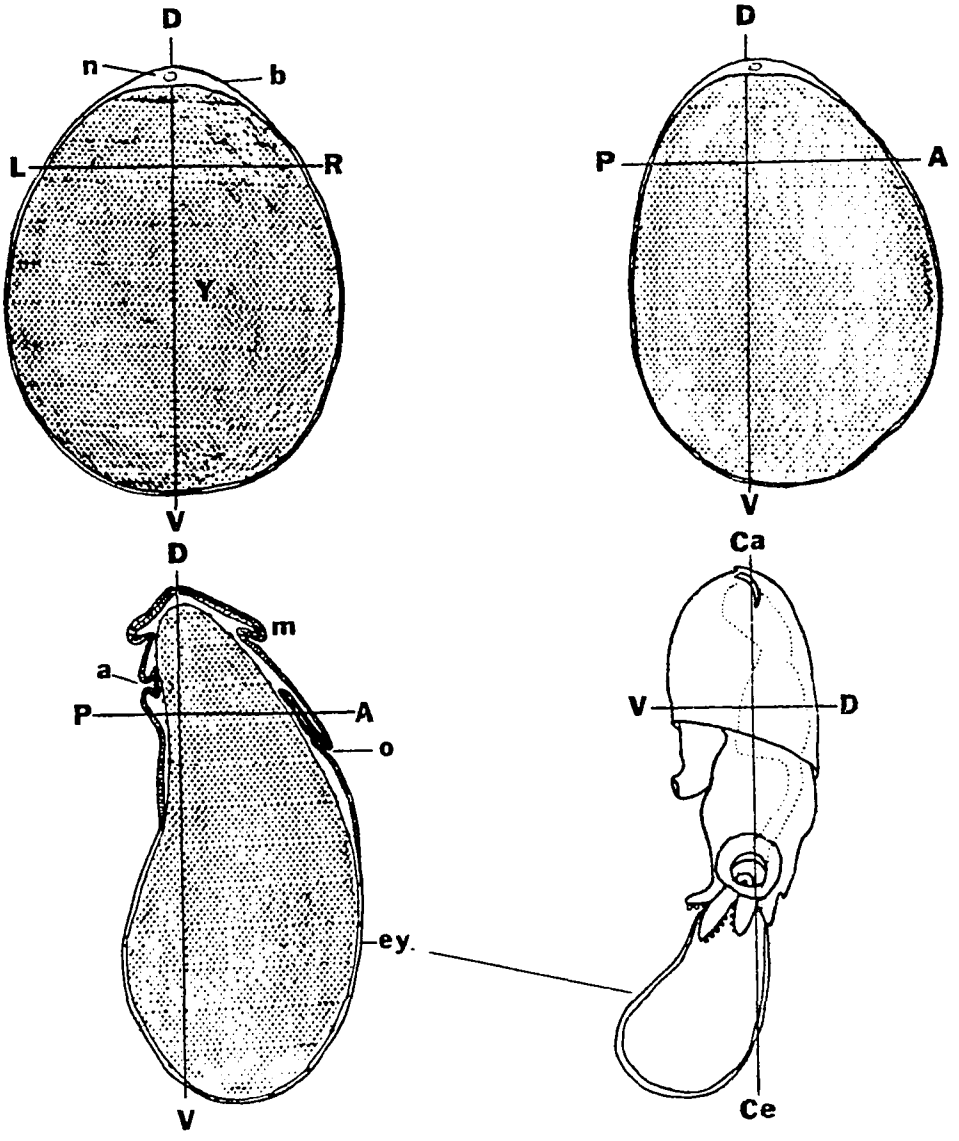


Fig. 2. Symmetry of the egg and embryo of Loligo pealei.

a. An optical section of an uncleaved egg. The optical axis coincides with the antero-posterior plane bisecting the egg. b. Right profile of the egg illustrating the asymmetry around the dorso-ventral axis. The anterior part is larger and more convex. The nucleus is posterior of center in the blastodisc. c. A sagital section at ca. Stage 20. Flexure of the body corresponds to the asymmetry of the egg. d. An embryo at ca. Stage 27 showing the future axes of symmetry of the adult. D, dorsal; V, ventral; A, anterior; P, posterior; b, blastodisc; Ca, caudal; Ce, cephalic; n, nucleus; a, anus; m, mantle; mo, mouth; Y, yolk; eys, external yolk sac; iys, internal yolk sac (modified from Watase, 1891).

nucleus is asymmetric, being disposed to the posterior of a transverse line bisecting the blastodisc (Fig. 2). This anterior-posterior asymmetry persists throughout embryogenesis and may be used to orient within the blastoderm during cleavage and gastrulation stages (Arnold, 1971).

First cleavage (Stage 4 of Arnold, 1965) separates the future right and left halves of the embryo along its anteroposterior plane (Arnold, 1965). The furrows of the first three cleavages cut through the cytoplasm of the blastodisc, but the cytoplasm remains continuous at the periphery of the resultant "cells." A second furrow divides the blastodisc somewhat symmetrically into an anterior and posterior portion. The posterior portion is the smaller due to the above-mentioned acentric displacement of the zygotic nucleus.

The third cleavage (Stage 6) furrow is asymmetric in the right and left halves. In the posterior half the third furrow forms at right angles to the second furrow, but occurs at an angle of 30° to the perpendicular anterior of the second furrow (PLATE I, Fig. 1). Thus the daughter "cells" adjacent to the first furrow in the anterior half are larger than those in the posterior half. It has been suggested that this asymmetry is a remnant of ancestral Spiralian relationships (Arnold and Williams-Arnold, 1976).

At third cleavage a mitotic asynchrony becomes more evident. The posterior daughter "cells" lag somewhat

PLATE I

Early Cleavage: Cleavages 3, 4, 5, and 6

Figs. 1 to 4 are camera lucida drawings of FAA-Feulgen treated embryos of Loligo pealei. Numerals indicate the cleavage number of the corresponding furrow. Dotted lines indicate the positions of undercutting furrow bases. The figures are oriented with the anterior-posterior axes of the embryos vertical. Nuclei are drawn as they appear with Feulgen stain. Mitotic phases appear as follows: ○ Interphase, ◎ Early Prophase, ✱ Late Prophase, ‖ Metaphase, ‖ Anaphase, and ⊂⊃ Telophase. Bars = 0.1 mm (ca. 75 X).

Fig. 1. Third cleavage (Stage 6). The third cleavage furrows are unequal in the posterior half and symmetrical in the right and left halves. Note the mitotic phase lag of the posterior-medial bastocones.

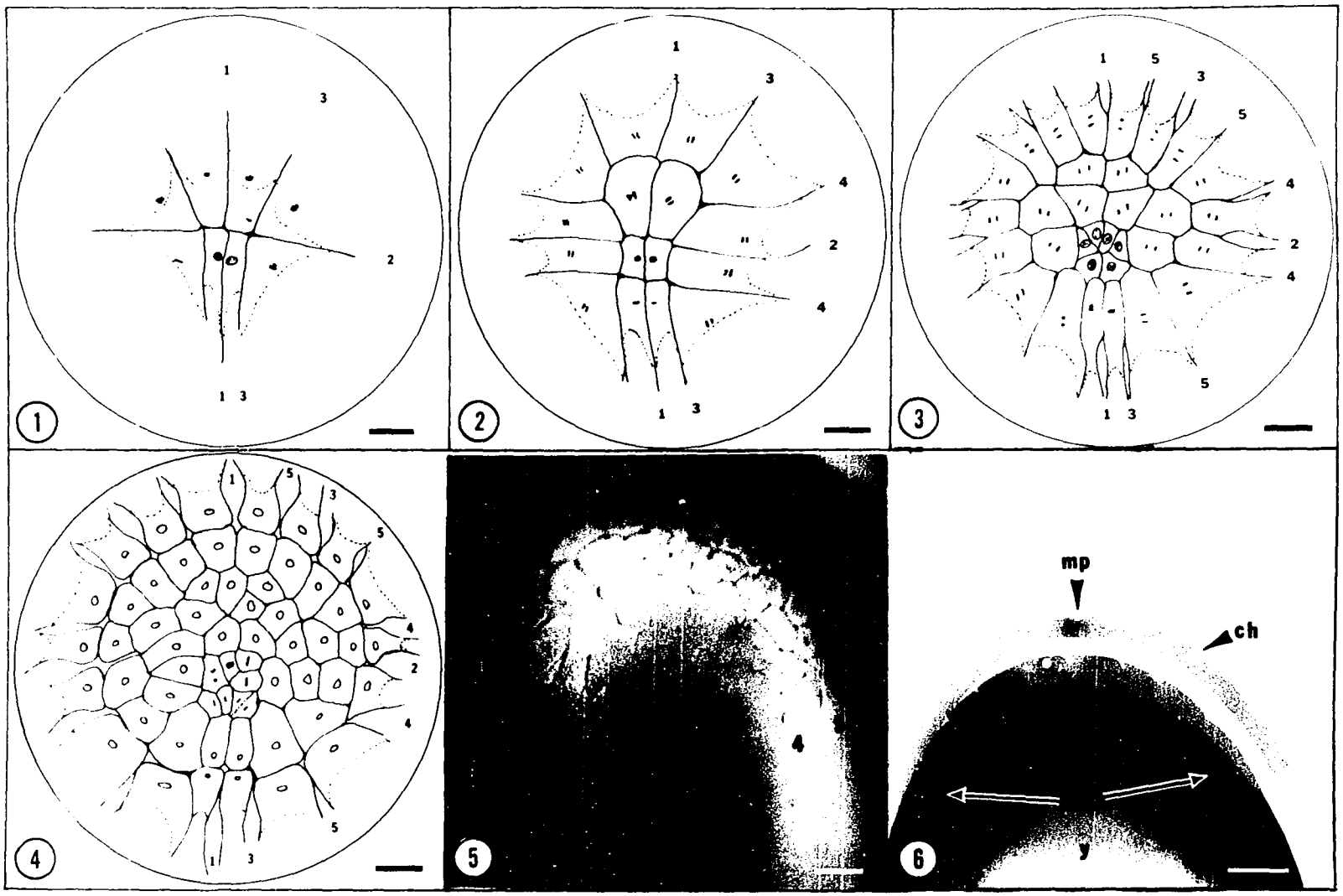
Fig. 2. Fourth cleavage (Stage 7). Fourth cleavage is roughly parallel to the second furrow and forms the first four blastomeres. The degree of mitotic phase lag has increased.

Fig. 3. Fifth cleavage (Stage 8). Cleavage planes are radial except in the blastocones adjacent to the second furrow and in the posterior-medial blastocones.

Fig. 4. Sixth cleavage (Stage 9). All blastocones and the large blastomeres abutting the second furrow in Stage 8 have cleaved trans-radially. The orientation of cleavage in central blastomeres varies.

Fig. 5. Sixth cleavage embryo of Loligo pealei. View is of the right posterior quadrant. Numerals indicate furrows as above. Bar = 0.1 mm (ca. 75 X).

Fig. 6. Section through a sixth cleavage embryo illustrating the single-layered aspect of the blastoderm at this stage. The section plane is parallel and just posterior to the second furrow. The blastocones (bc), chorion (ch), yolk (y) and micropyle (mp) are indicated. Bar = 0.1 mm (ca. 100 X).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behind the anterior "cells" as regards their mitotic phase. The period of the mitotic cycle at this stage is 50 to 60 minutes at 21°C. After second cleavage, undercutting furrows begin to separate the cleavage elements from the underlying yolk (see Arnold and Williams-Arnold, 1976).

Fourth cleavage (Stage 7) produces the first four complete blastomeres. These are surrounded by twelve radially arranged elements, the blastocones (Vialleton, 1888) (PLATE I, Fig. 2). The blastocones are confluent with the yolk cytoplasmic layer and remain so through ninth cleavage. The mitotic phase lag of the posterior nuclei is more evident at fourth cleavage. The nuclei of the blastomeres and blastocones adjacent the first furrow lag behind the remaining synchronously dividing nuclei (PLATE I, Figs. 2 and 3). Nuclei of the smallest blastomeres and of the blastocones which lie along the posterior midline are in an earlier phase of mitosis than the remaining nuclei. Thus the asynchrony is symmetrical in the right and left halves of the embryo. The mitotic phase difference of the blastomeres and blastocones lying along the posterior midline is very apparent through tenth cleavage. After tenth cleavage the numbers of cells and the presence of the middle layer prevent a clear determination of the pattern.

The pattern of cleavages five through nine are difficult to describe simply. For convenience and descriptive continuity, the terms chosen here to describe furrow

orientation are the following:

1). Radial furrow. With reference to the circular geometry of the blastodisc and blastoderm, this is a furrow which lies roughly along a radius of the blastodisc or blastoderm.

2). Trans-radial furrow. Again with reference to blastoderm geometry, a furrow which lies at right angles (or approximately right angles) to a radius of the blastoderm. These terms are in no way meant to imply a relation to an overall pattern of cleavage, vide radial cleavage versus spiral cleavage.

Furrows of the fifth cleavage (Stage 8) which divide the anterolateral and posterolateral blastocones of the fourth cleavage embryo are oriented radially (PLATE I. Fig. 3). The remaining furrows of the fifth cleavage are trans-radial. The fifth cleavage blastoderm thus consists of six small cells at its center and eight large cells anteriorly and laterally. This blastoderm is surrounded by eighteen blastocones (PLATE I, Fig. 3).

Sixth cleavage (Stage 9) is trans-radial in all blastocones and large blastomeres (compare PLATE I, Figs. 3 and 4). Sixth cleavage establishes two concentric rings of blastomeres surrounding eight small central blastomeres. The blastomeres together constitute the blastoderm which is surrounded by eighteen blastocones. Plate I, Fig. 5 is a photograph of the right rear quadrant of an embryo

having completed sixth cleavage. Furrow orientation can easily be discerned. In cross section the blastoderm is seen as a single layer of blastomeres boardered by blastocones (PLATE I, Fig. 6).

Furrows of the seventh cleavage (Stage 9) are radial in all blastocones posterior to the second furrow except the postero-medial two in which they are trans-radial (PLATE II, Fig. 1). Anteriorly, seventh cleavage divides the two blastocones boardered by furrows three and five at sixth cleavage by radial furrows. The remaining blastocones divide trans-radially.

Seventh cleavage is also trans-radial in most divisions of outer ring blastomeres. Blastomeres adjacent to the second furrow, both anteriorly and posteriorly, however, are divided radially. The central blastomeres formed by sixth cleavage are divided trans-radially by seventh cleavage. The cleavage pattern is consistent to this point, but may show some variation within the central group of blastomeres. Completion of seventh cleavage yields a blastoderm consisting of about 104 cells arrayed in approximately concentric rings. The blastoderm is surrounded by blastocones now numbering 24 (or occasionally 26 if the lateral most blastocones in the posterior portion divide radially at sixth cleavage). Plate II, Fig. 2 illustrates an embryo of L. pealei at seventh cleavage.

PLATE II

Late Cleavage: Cleavages 7 and 8

Fig. 1. Seventh cleavage (Stage 9); see Plate I legend. Blastomeres of the sixth cleavage outer ring are, in general, cleaved trans-radially. The large posterior blastocones and the two anterior blastocones boardered by furrows 3 and 5 at sixth cleavage are cleaved radially. The mitotic phase lag can be seen in the blastomeres along the posterior midline. Bar = 0.1 mm (ca. 75 X).

Fig. 2. Embryo of Loligo pealei at Seventh cleavage. This is the same embryo as Plate I, Fig. 5. Bar = 0.1 (ca. 75 X).

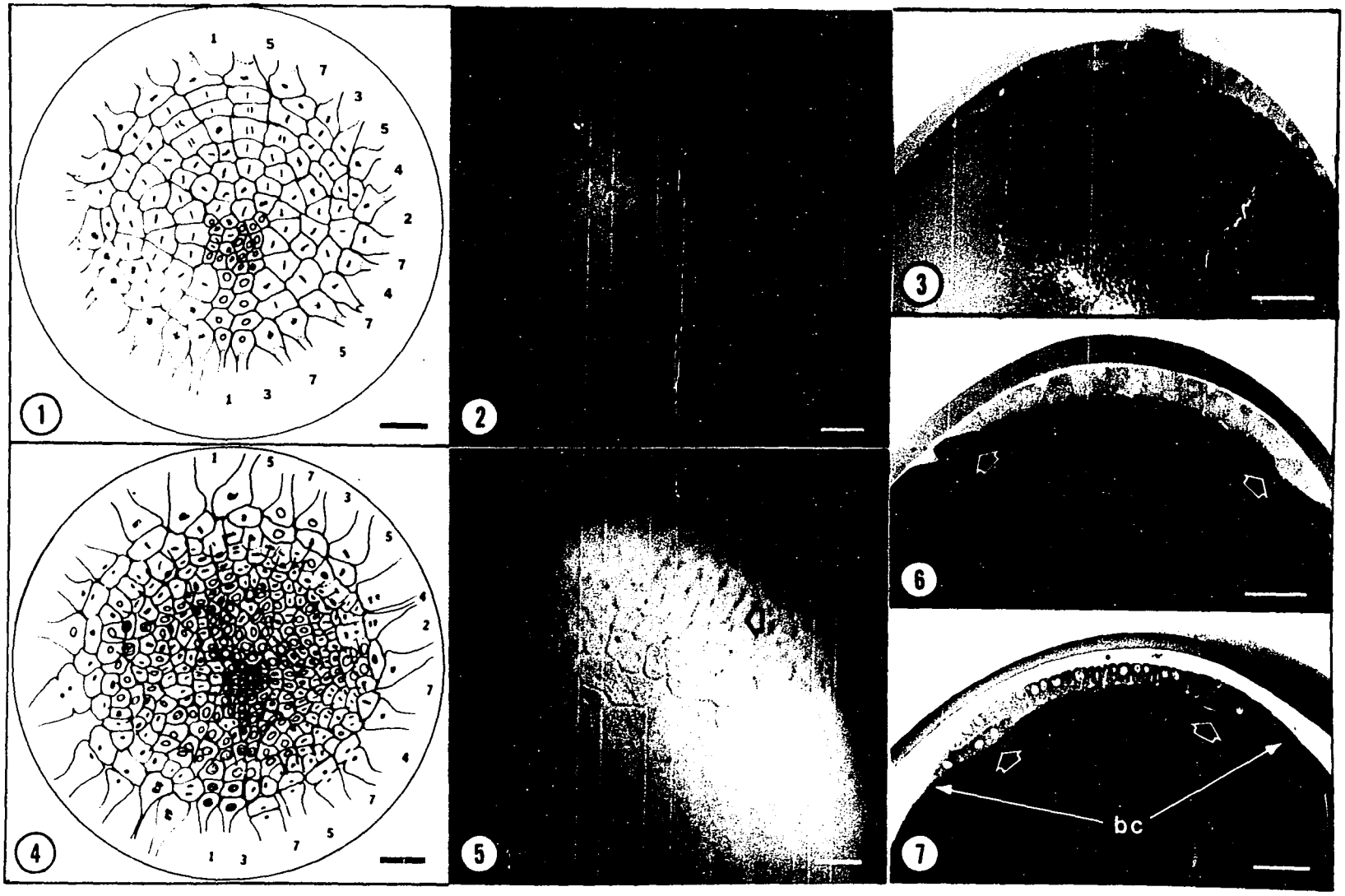
Fig. 3. Section of a seventh cleavage embryo. The blastoderm remains as a single layer of blastomeres surrounded by blastocones. Bar = 0.1 mm (ca. 100 X).

Fig. 4. Eighth cleavage (Stage 9). Cleavage planes are radial in most blastomeres and trans-radial in all blastocones. Blastomeres now number about 230 and blastocones 24 or 26. Overlapping of the presumptive middle-layer cells begins at this stage. The mitotic phase lag of the posterior-medial cells is still very evident. Bar = 0.1 mm (ca. 75 X).

Fig. 5. Embryo of Loligo pealei at eighth cleavage. This is the embryo of Fig. 5 above. The arrow indicates the area of visible overlap. Bar = 0.1 mm (ca. 75 X).

Fig. 6. Section of an eighth cleavage embryo. Overlapping is moderately advanced causing a depression of the yolk (open arrows). Bar = 0.1 mm (ca. 100 X).

Fig. 7. Section of a ninth cleavage (Stage 10) embryo. A ring of middle-layer cells is produced below the blastoderm (open arrows). The blastocones (bc) are still present. Bar = 0.1 mm (ca. 100 X).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In cross section the blastoderm is still seen as a single layer of now smaller blastomeres surrounded by peripherally attached blastocones (PLATE II, Fig. 6).

Furrows of the eighth cleavage are oriented radially in most blastomeres excepting some 24 large blastomeres which lie adjacent to the second furrow (PLATE II, Fig. 4). These 24 cells are all daughters of the large blastomeres and blastocones which abut the second furrow at fifth and sixth cleavages. Orientation of the eighth cleavage furrows in the central blastomeres appears to be random. The number of blastocones remains at 24 or 26 as they are cleaved trans-radially (compare PLATE II, Figs. 1 and 4). Eighth cleavage completes the cleavage phase of development. The ninth cell divisions are, however, termed ninth cleavage for the sake of continuity and simplicity.

3.1.2. Post Cleavage Division and the First Phase of Gastrulation

After eighth cleavage is completed the third ring of blastomeres centripetal from the margin of the blastoderm is overlapped by the centrifugal extensions of cells of the fourth ring (see PLATE II, Figs. 4, 5, and 6). The ring of overlapped cells constitutes a population of cells which will give rise to the middle layer (presumptive middle-layer cells) (PLATE II, Fig. 6).

At ninth cleavage the blastomeres of the two most peripheral rings are divided radially, whereas in the centripedally adjacent ring of presumptive middle-layer cells the furrows are oriented trans-radially. The blastomeres of the fourth ring in from the periphery of the blastoderm (overlapping cells) also divide trans-radially. This trans-radial division of the presumptive middle-layer cells in conjunction with their overlapping by the centripetally adjacent cells is one of the two basic morphogenetic processes of germ layer segregation in Loligo. Interference with either of these processes, as described later, produces an abnormal segregation of the middle-layer.

The orientation of division in the central blastomeres is less consistent at ninth cleavage. Division has also become increasingly asynchronous. The blastoderms of FAA-Feulgen treated embryos at ninth cleavage display a symmetrical pattern of asynchrony. Symmetrically arrayed bands of cells in the right and left halves of the blastodermere seen to be in a phase of mitosis which is later with respect to the cells anterior to the bands and earlier with respect to those posterior of the band. Thus an anterior-posterior gradient of division rate is established which persists through early phases of gastrulation. With time-lapse cinemicrography the pattern of asynchrony is visible as waves of division which originate along the anterior midline and then sweep toward the posterior midline.

Ninth cleavage divides the blastocones in a trans-radial direction (PLATE III, Fig. 1). The number of blastocones thus remains at 24 or 26. The nuclei of the blastocones produced by ninth cleavage are later incorporated into the yolk cytoplasmic layer to form the yolk syncytium as described below. The blastomeres produced by this cleavage of the blastocones form a population of cells which will produce the leading edge of the blastoderm during later spreading phases (Stages 12 through 18 of Arnold, 1965a).

During the two subsequent divisions, the number of cells, asynchrony of division, and the presence of the middle-layer prohibit the accurate observation of all divisions. I have, therefore, termed the first of these two divisions tenth nuclear division. That is, the mitotic division of the blastocone nuclei produced by the ninth cleavage. This terminology is in no way meant to imply that the cells of the blastoderm no longer undergo cytokinesis. It is meant only to be descriptive of the most obvious, important, and easily followed events subsequent to the completion of cleavage.

At the time of the tenth nuclear division these nuclei constitute the yolk syncytium (= yolk epithelium) and are thus termed the yolk syncytium nuclei (PLATE III, Fig. 3). During mitotic division of these nuclei a trans-radially oriented furrow is formed between each pair of daughter nuclei. These furrows appear to form a single continuous

PLATE III

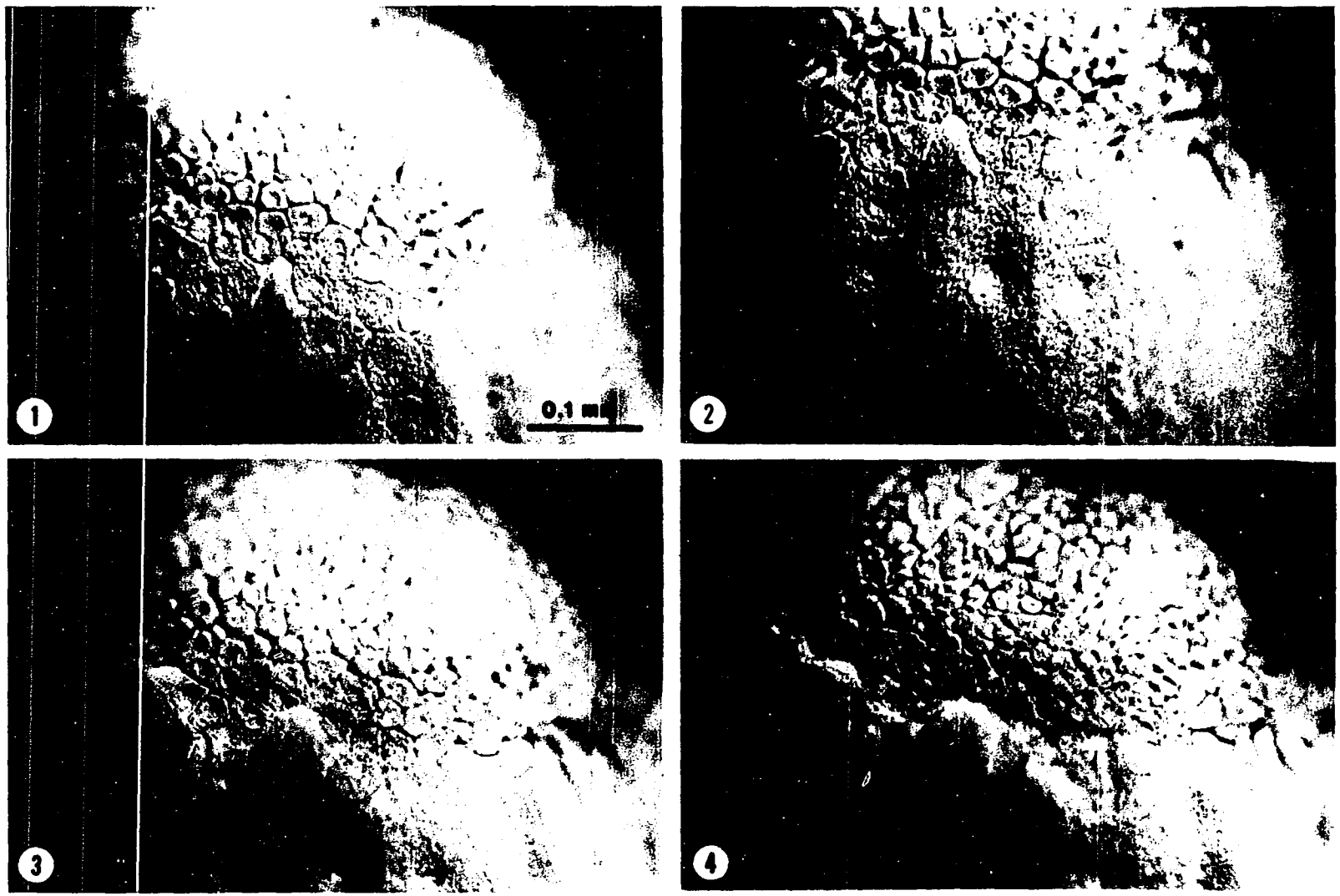
Ninth Cleavage and Tenth Nuclear Division

Fig. 1. Ninth cleavage (Early Stage 10). The blastocones have undergone ninth cleavage and are delineated by the meridional and undercutting furrows. In this embryo the furrows have already begun to retract. At the perimeter of the blastoderm the middle-layer cells are visible beneath the outer layer of cells. Elapse time = 0.00. 250 X.

Fig. 2. Ninth cleavage (Early Stage 10). The meridional and undercutting furrows of blastocones have almost disappeared as retraction continues. The framing of this micrograph has shifted somewhat vegetally. Elapse time = 0:17. 250X.

Fig. 3. Ninth cleavage (Early Stage 10). The blastocone nuclei have moved vegetally into the yolk cytoplasmic layer to produce the yolk syncytium. Note that the blastomeres produced by ninth cleavage of the blastocones have moved toward the margin of the blastoderm. A lenticulate mass of cytoplasm surrounds each yolk syncytium nucleus. The ring of middle-layer cells produces a slight depression in the underlying yolk at the edge of the blastoderm. Elapse time = 1.16. 250 X.

Fig. 4. Tenth nuclear division (Mid-Stage 10). The yolk syncytium nuclei are in prophase of the tenth nuclear division. The peripheral blastomeres have moved farther toward the edge of the blastoderm. The yolk depression is more prominent at this time. Elapse time = 2:05. 250 X.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circumferential ring around the embryo (PLATE IV, Fig. 1).

The circumferential furrow is transitory, existing for about one hour. Contraction of the filamentous band at the base of the furrow produces a depression in the surface of the embryo, but it does not cut through the syncytial cytoplasm to the yolk. Later the furrow relaxes and disappears (PLATE IV, Fig. 2). Thus although cleavage furrows are formed during this division, a syncytium remains. The maintenance of the syncytial character of this layer is of critical importance for the later movements of the yolk syncytium nuclei discussed below.

During tenth nuclear division there is an increase in the number of middle-layer cells. This increase causes a deepening of the depression in the yolk near the edge of the blastoderm (PLATE III, Fig. 4). As the number of middle-layer cells increases, their absence in the area under the center of the blastoderm produces a "central yolk papilla" (see Arnold, 1971). The middle layer is eventually completed by continued division of the middle-layer cells. The blastomeres produced by ninth cleavage of the blastocones begin to spread around the perimeter of the blastoderm (compare for example PLATE III, Figs. 3 and 4; PLATE IV, Figs. 1 to 4).

Eleventh nuclear division is similarly designated by the second mitotic division of the yolk syncytium nuclei. Prior to eleventh nuclear division the proximal ring

PLATE IV

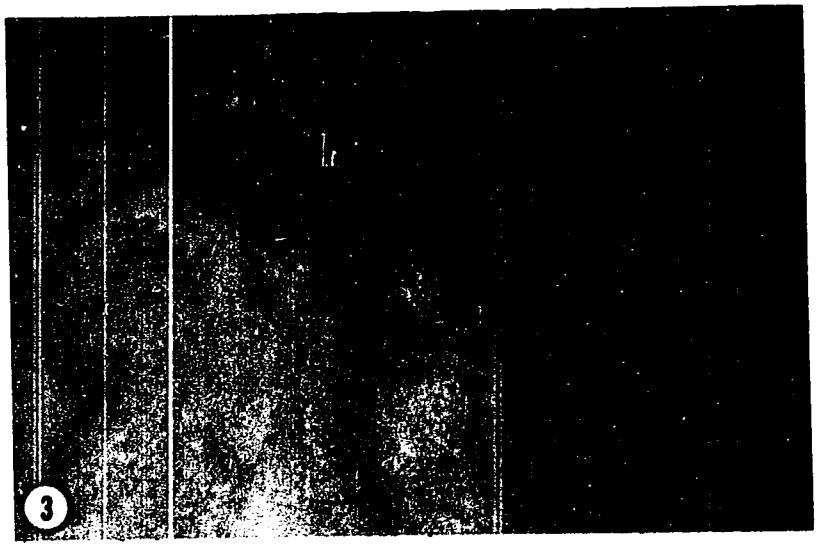
Tenth Nuclear Division

Fig. 1. Tenth nuclear division (Mid-Stage 10). This and the following figures are a continuation of the series of figures in Plate III. The yolk syncytium nuclei have completed mitosis of tenth division and a circumferential, transitory furrow has formed (arrow). The peripheral blastomeres have assumed a rounded outline and are moving toward the margin of the blastoderm. Elapse time = 2:29. 250 X.

Fig. 2. Tenth nuclear division (Mid-Stage 10). At this time the transitory furrow has disappeared (arrow) and the yolk syncytium nuclei have moved farther apart. The nuclei proximal to the blastoderm have moved toward the animal pole and the distal nuclei have moved vegetally. Elapse time = 3:49. 250 X.

Fig. 3. Tenth nuclear division (Mid-Stage 10). The yolk syncytium nuclei have moved farther apart and assumed a more uniform distribution prior to eleventh nuclear division. The peripheral blastomeres have moved farther onto the margin of the blastomere and are spreading laterally around its perimeter. Elapse time = 5:02. 250 X.

Fig. 4. Tenth nuclear division (Mid-Stage 10). Yolk syncytium nuclei proximal to the blastoderm have moved to the margin of the blastoderm just prior to their being overlaid by it. The distal yolk syncytium nuclei are positioned for eleventh nuclear division. The peripheral blastomeres exhibit signs of the onset of epiboly (compare with Fig. 3 in regard to the leading edges of these peripheral cells). The peripheral blastomeres have begun to flatten and extend lamellipodia. Elapse time = 6:30. 250 X.



of yolk syncytium nuclei moves centripetally as the blastoderm begins epibolic spreading (PLATE IV, Figs. 1 to 4). These nuclei divide almost synchronously and a circumferential, transitory furrow is formed as in the tenth nuclear division.

FAA-Feulgen treated embryos of Stage 10 reveal patches of synchronously dividing cells within the blastoderm. Time-lapse observation shows that the divisions continue in waves passing around the blastoderm. Eleventh nuclear division signals the end of the first phase of gastrulation. The principal events of this first phase are: 1) The segregation of the middle layer, and 2) the establishment of the yolk syncytium. The second phase of gastrulation involves the expansion, growth and spreading of the two-layered blastoderm over the mass of yolk. A complete analysis of this second phase has not yet been made. However, some aspects of the ultrastructural morphology of the peripheral blastoderm cells during initial stages of epiboly are described below.

3.2. Cell Movements of Gastrulation in *Loligo pealei*

After most cells have completed eighth cleavage, a specific population of blastoderms begins to overlap a second, more marginal population by a centrifugal extension of lamellipodia-like protrusions. The population of overlapping cells consists of 36 to 40 blastomeres.

These are arranged in a ring located three blastomeres in from the perimeter of the blastoderm (PLATE II, Fig. 3 and PLATE VI, T=2:10). The presumptive middle-layer cells are segregated into the area between the blastoderm and the yolk by the processes of overlapping and ninth cleavage. Time-lapse cinemicrography reveals the dynamic character of the overlapping cell movements.

Plates V through XII are reproductions of sequences of frames from two time-lapse films of germ layer segregation in Loligo pealei. The pattern of late cleavage can be seen clearly. Concentric rings of cells are established by seventh cleavage (PLATES V and VI). After eighth cleavage centrifugally directed cell protrusions progressively overlap adjacent cells. The onset of overlapping can be seen in several cells (PLATE VI, T=2:10, T=2:20 and PLATE IX, T=0:25.00 and T=0:37.30). These cells are followed through successive stages (see Plate captions for details). The extension and flattening of the overlapping cells progresses during the interval between eighth and ninth cleavage until the presumptive middle-layer cells are one-fifth to one-quarter overlapped (see PLATE VII, T=2:30 and T=2:40; PLATE IX, T=0:37.30 to T=1:03.00). Just prior to ninth cleavage the overlapping cells round up and their leading edges appear less active (PLATE VII, T=2:50; PLATE IX, T=1:03.00). After ninth cleavage the activity of the leading edges increases and the cells spread

PLATE V

Cinemicrographic Series I.

Cleavages 5 and 6

Plates V through VIII present a series of frames from a time-lapse cinemicrographic film of middle layer segregation in Loligo pealei. The view of the embryo is of the left anterior quadrant of the blastoderm. Magnification is 320 X as indicated by the first frame. Elapse time designations are in hours and minutes.

T=0:00. Fifth cleavage (Stage 8). Cleavage of the blastocones is radial.

T=0:10. Fifth cleavage (Stage 8). The blastocone nuclei are entering prophase of the sixth cleavage division.

T=0:20. Fifth cleavage (Stage 8). Sixth cleavage mitosis.

T=0:30. Fifth cleavage (Stage 8). Sixth cleavage mitosis.

T=0:40. Sixth cleavage (Stage 9). Sixth cleavage of all blastocones is trans-radial.

T=0:50. Sixth cleavage (Stage 9). The nuclei of the blastomeres are entering prophase of the seventh cleavage division.

T=1:00. Sixth cleavage (Stage 9). The nuclei are in mitosis of seventh cleavage.

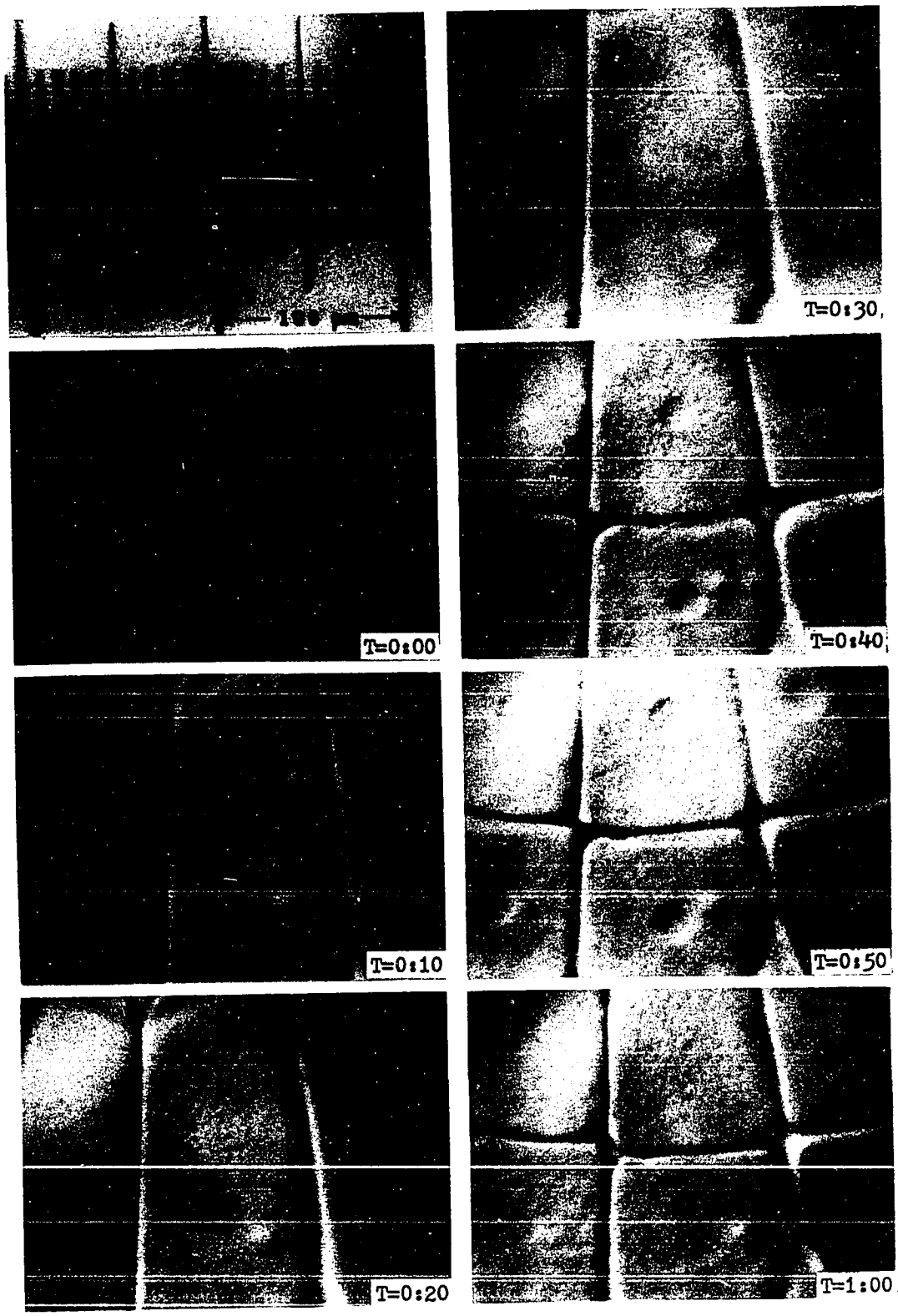

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PLATE VI

Cinemicrographic Series I.

Cleavages 7 and 8

T=1:10. Late sixth cleavage (Stage 9). The nuclei are in early anaphase of mitosis prior to seventh cleavage. The beginning formation of seventh cleavage furrows is visible as slight indentations along the radial furrows (arrows).

T=1:20. Seventh cleavage (Stage 9). Nuclei of the cells within the frame are in late telophase. Orientation of the cleavage furrows is trans-radial.

T=1:30. Seventh cleavage (Stage 9). The nuclei have reformed and moved to the centers of the cells.

T=1:40. Seventh cleavage (Stage 9). The cells are in the G1 phase of the cell cycle. Note that the nuclei have increased in size.

T=1:50. Seventh cleavage (Stage 9). Nuclei are in mitosis prior to eighth cleavage. Note the asynchrony of mitosis.

T=2:00. Eighth cleavage (Stage 9). The cells in the upper-middle and left of the frame are undergoing cytokinesis. Asynchrony of division is more evident in this frame.

T=2:10. Eighth cleavage (Stage 9). The first signs of overlapping of the presumptive middle-layer cells are visible (arrow). The blastocones are cleaved trans-radially by eighth cleavage.

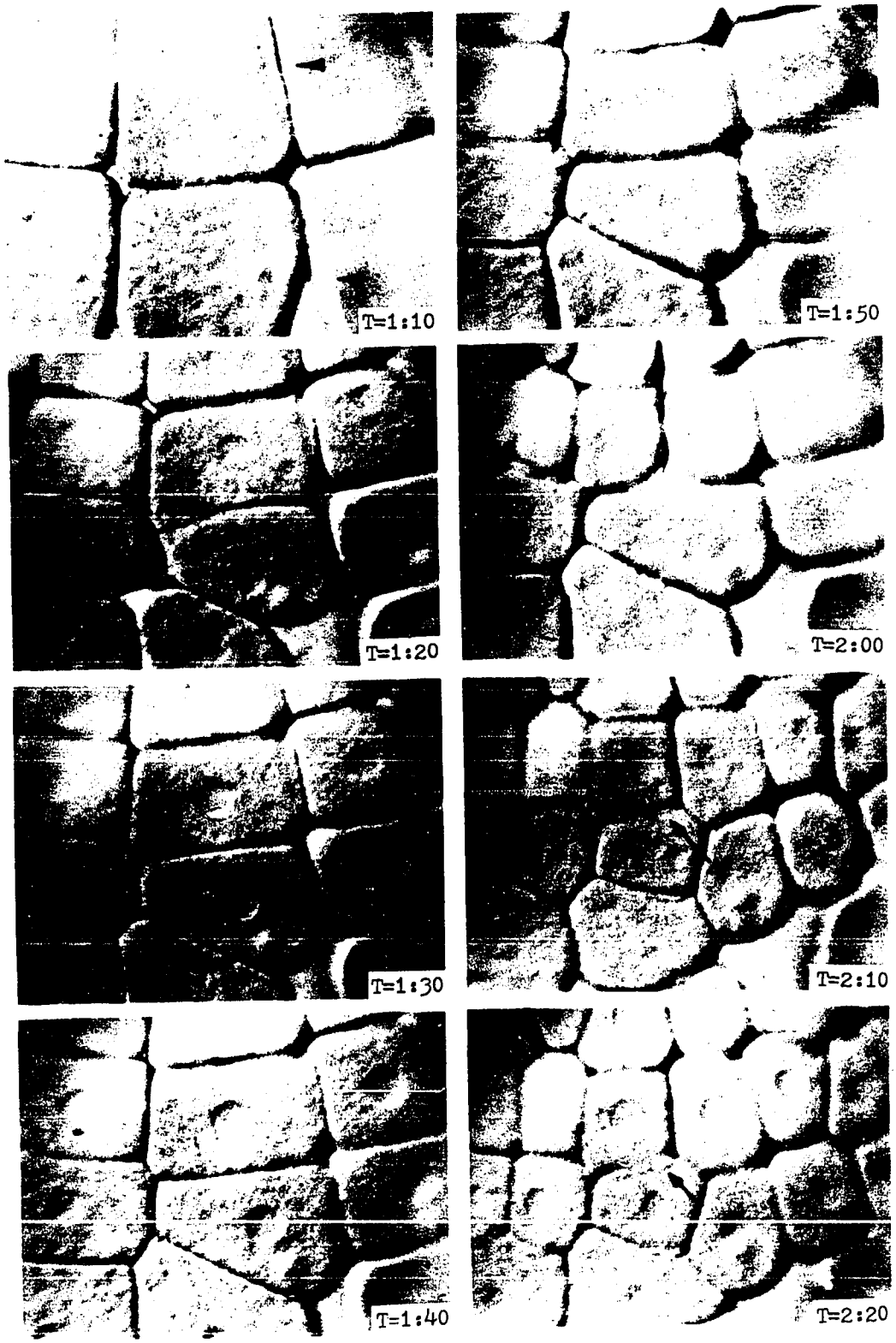
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PLATE VII

Cinemicrographic Series I.

Germ Layer Segregation

T=2:30. Late eighth cleavage (Stage 9). Overlapping is visible in all cells of the overlapping ring (arrows). Close scrutiny of this frame reveals that the leading edges are uneven and have numerous small pseudopodia.

T=2:40. Late eighth cleavage (Stage 9). Overlapping has increased, the cells are beginning to retract their leading edges (arrow), and are rounding up prior to ninth cleavage. These cells now overlap the presumptive middle-layer cells by more than 10 μm.

T=2:50. Early ninth cleavage (Stage 9-10). Two overlapping cells have undergone ninth cleavage (open arrow). The two vegetal-most daughter cells of the above cleavage are still somewhat rounded at their leading edges (closed arrow) as they begin to flatten and spread.

T=3:00. Ninth cleavage (Early Stage 10). Leading edges of the overlapping cells again show pseudopodial activity (arrows).

T=3:10. Ninth cleavage (Early Stage 10). Although this frame is somewhat out of focus, the arrow indicates the location of the leading edge of overlap. Some variation in the cleavage pattern is evident. For example, the presumptive middle-layer cell in the center of the field has cleaved radially whereas the others are cleaving trans-radially (open arrow).

T=3:20. Ninth cleavage (Early Stage 10). Presumptive middle-layer cells are one-fourth to one-half overlapped. The leading edge of overlap is indicated by the arrow.

T=3:30. Ninth cleavage (Early Stage 10). Presumptive middle-layer cells are now one-half to two-thirds overlapped (arrow). The leading edges of the overlapping cells show signs of intense activity.

T=3:40. Ninth cleavage (Stage 10). Presumptive middle-layer cells are now approximately three-fourths overlapped (arrow). Note the rounded aspect of the centripetal margins of the middle-layer cells. The appearance of the middle-layer cells contrasts sharply with that of the overlapping cells.

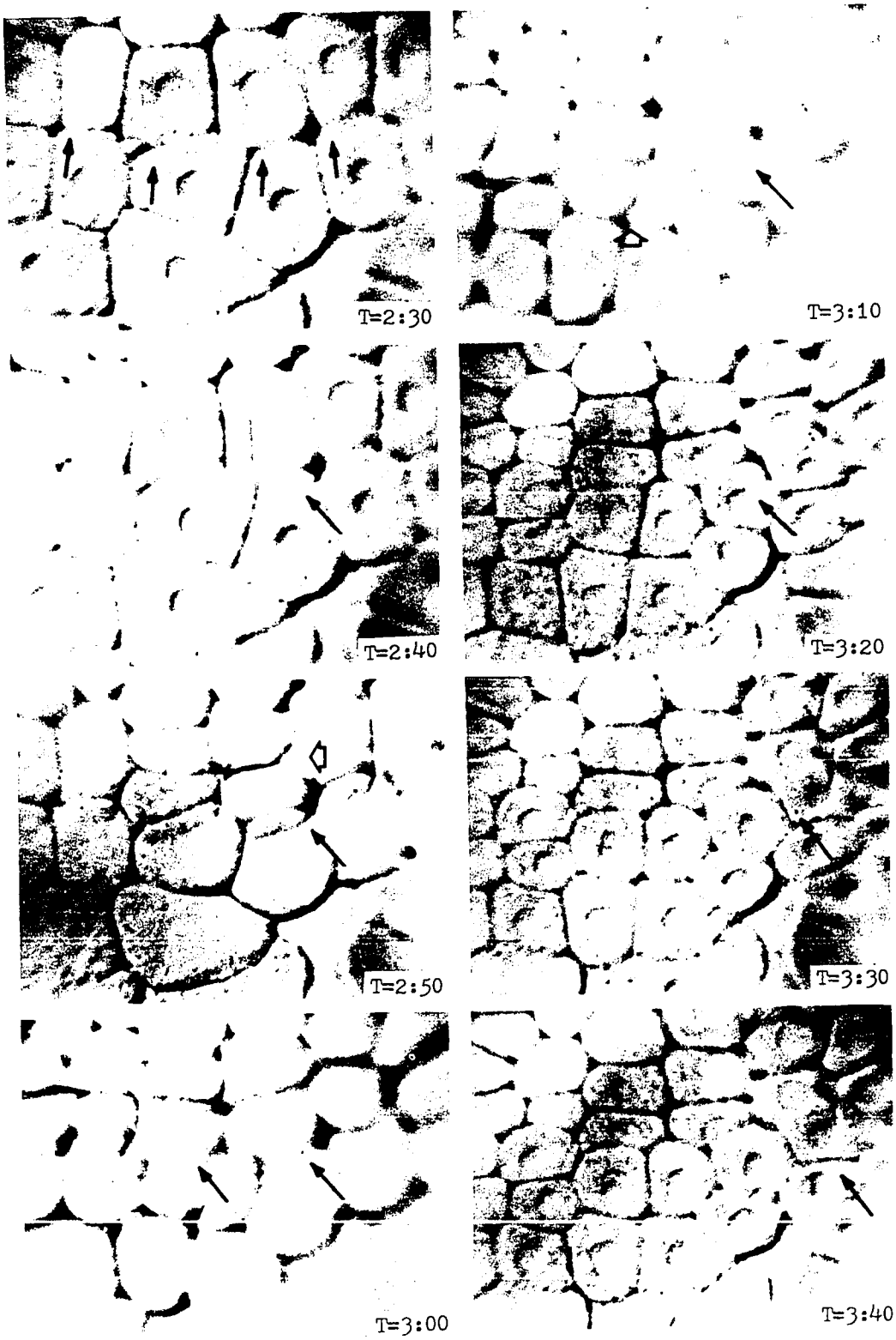
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PLATE VIII

Cinemicrographic Series I.

Germ Layer Segregation

T=3:50. Ninth Cleavage (Stage 10). Presumptive middle-layer cells are three-fourths-plus overlapped. Overlapping cells still display intense pseudopodial activity at their leading edges (arrow).

T=4:00. Ninth Cleavage (Stage 10). Overlapping is almost complete (arrow). Pseudopodial activity is evident (arrow). The light vertical line is artifact.

T=4:10. Tenth Division (Stage 10). Overlapping cells have completely overlapped their subjacent cells in most cases and are rounding up for cytokinesis. The two cells at the right of center of the frame are in early anaphase of mitosis. The two cells at center left (arrow) are in a slightly earlier phase.

T=4:20. Tenth Division (Stage 10). The cells at center right have cleaved and the remaining cells are rounding up for division.

T=4:30. Tenth Division (Stage 10). The cells in the center of the field have all cleaved. The middle-layer cells and blastocones are in mitosis prior to tenth cleavage.

T=4:40. Tenth Nuclear Division (Stage 10). Many blastomeres within the frame have cleaved. The blastocones and yolk syncytium nuclei are undergoing mitosis prior to tenth nuclear division. The yolk syncytium nuclei are outside the field of view.

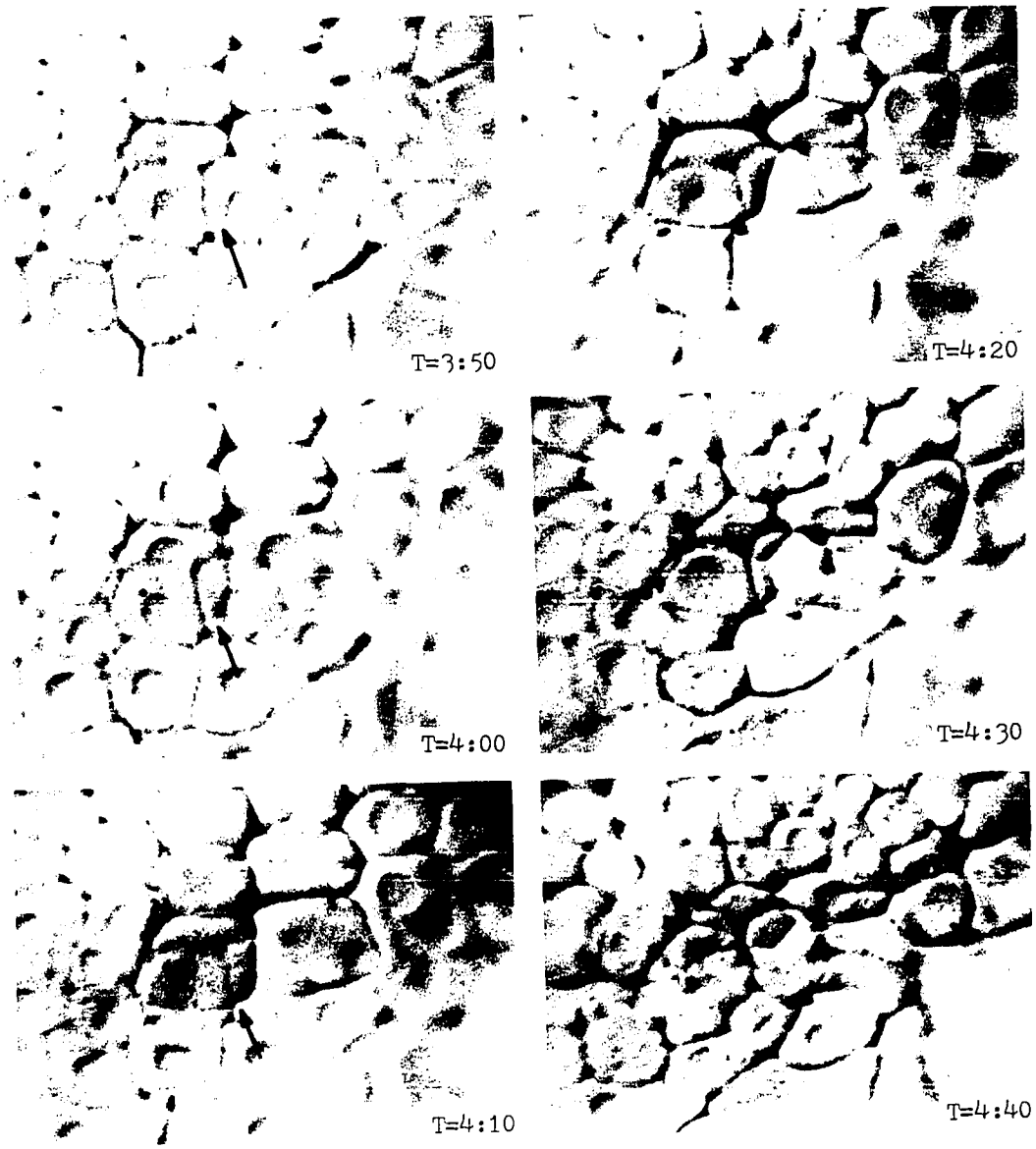

T=3:50
T=4:20
T=4:00
T=4:30
T=4:10
T=4:40

PLATE IX

Cinemicrographic Series II.

Cleavages 7 to 9 and Germ Layer Segregation

Plates IX through XII present a series of frames from a time-lapse cinemicrographic film of germ layer segregation and yolk syncytium formation in Loligo pealei. Magnification in this series is approximately 210 times normal. Elapse time designation is in hours, minutes and seconds.

T=0:00.00. Seventh Cleavage (Stage 9). Seventh cleavage is trans-radial in all blastomeres and blastocones within the frame.

T=0:12.30. Seventh Cleavage (Stage 9). All nuclei are in prophase of eighth cleavage mitosis.

T=0:25.00. Eighth Cleavage (Stage 9). All blastomeres have completed or are completing eighth cleavage. Some evidence of the initiation of overlapping can be seen in the form of a broad pseudopod extending over the subjacent presumptive middle-layer cell (arrow).

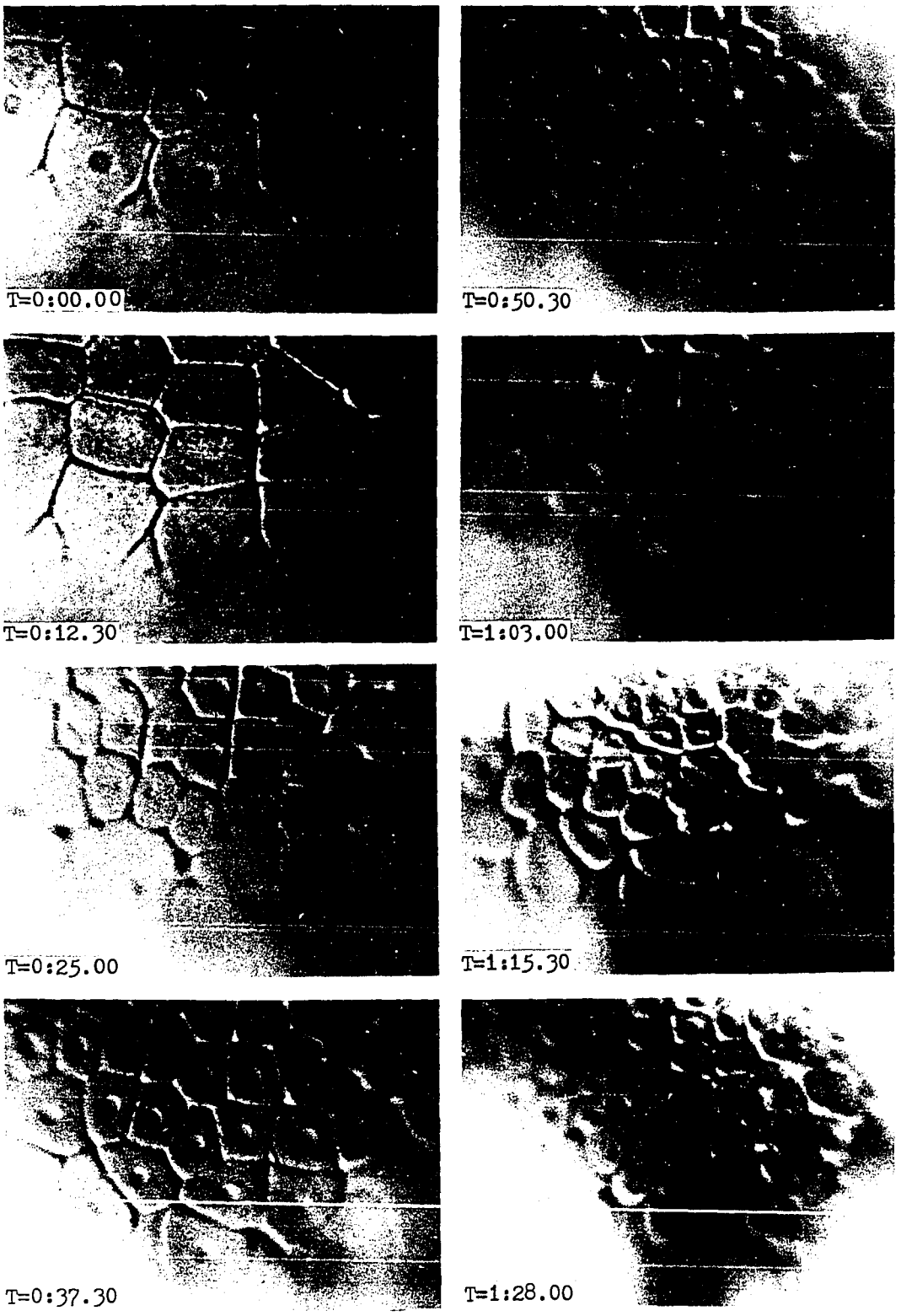
T=0:37.30. Eighth Cleavage (Stage 9). Most nuclei are in interphase of mitosis. The pseudopod of the previous frame has broadened and overlapping has begun in adjacent cells.

T=0:50.30. Eighth Cleavage (Stage 9-10). Overlapping has advanced and small pseudopodial processes can be seen at the margins of the leading edges (arrow).

T=1:03.00. Eighth Cleavage (Stage 9-10). The nuclei of the overlapping cells are entering prophase of the ninth cleavage division. The cells are rounded. The presumptive middle-layer cells appear to be about one-fourth overlapped.

T=1:15.30. Ninth Cleavage (Early Stage 10). Ninth cleavage has occurred in most blastomeres and blastocones. Cleavage in the ring of overlapping cells is indicated by the open arrow. Flattening and extension of overlapping processes is resumed (arrow).

T=1:28.00. Ninth Cleavage (Early Stage 10). Overlapping continues as the presumptive middle-layer cells cleave (arrow).


T=0:00.00
T=0:50.30
T=0:12.30
T=1:03.00
T=0:25.00
T=1:15.30
T=0:37.30
T=1:28.00

more rapidly over the underlying presumptive middle-layer cells (PLATE VII, T=3:00 to PLATE VIII, T=3:50; PLATE IX, T=1:15.30 to T=2:05.30). During spreading the leading margins of these flattened cellular protrusions send out smaller processes which extend and retract at a greater rate than the rate of advance of the cell margins (PLATE VII, T=3:00 and PLATE IX, T=1:15.30). The number of lamellipodia and filopodia each cell develops varies with time. These pseudopodial processes disappear completely just prior to and during cytokinesis. As cellular extensions make contact with underlying cells, they broaden and coalesce. This activity produces an overall vectorial flow of overlapping cells over the presumptive middle-layer cells.

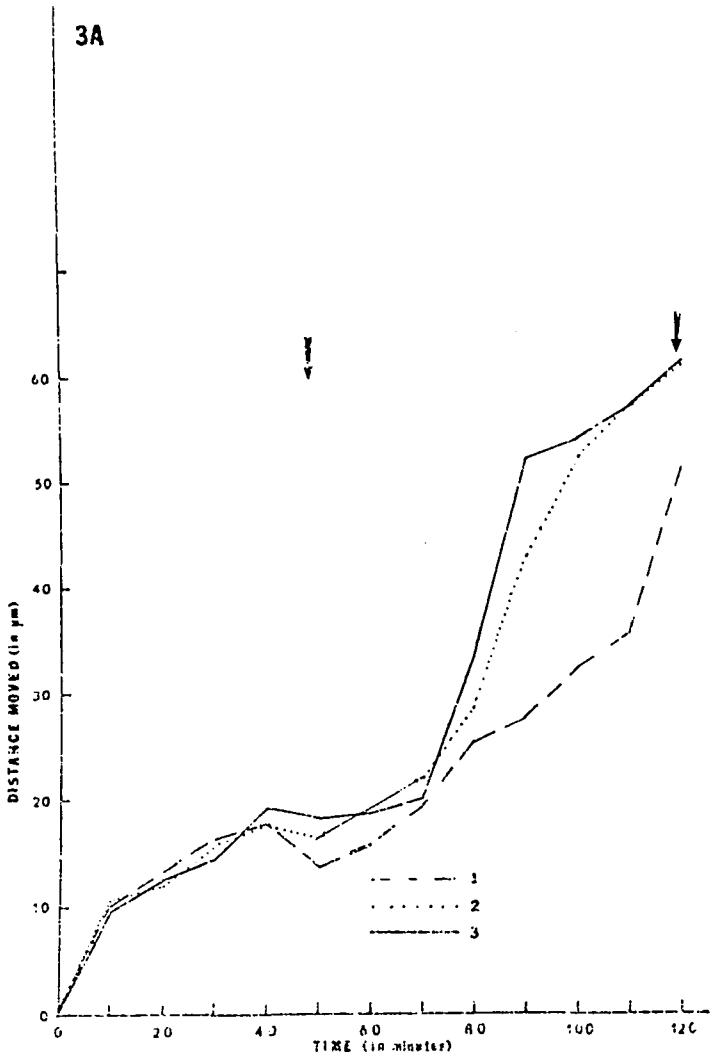
Overlapping accelerates subsequent to ninth cleavage (PLATE VII, T=3:00 to PLATE VIII, T=4:00 and PLATE IX, T=1:15.30 to PLATE X, T=2:18.00). The change in the rate of overlapping is further illustrated in Figs. 3 and 4. Measurements of the rate of overlapping show that the rate of oriented movement reaches a maximum of approximately 1.4 m per minute (Fig. 4A) between ninth cleavage and tenth nuclear division. Although the overlapping cells constitute an epithelial sheet, Figs. 3 and 4 illustrate that each cell moves somewhat independently of adjacent cells. Figs. 3B and 4B show that the rate of movement is not constant over the period observed and they illustrate the retraction of

Figure 3

Extent and Rate of Cell Movement During Overlapping I

Fig. 3-A. Distance moved by individual cells through time as measured on projected time-lapse cinemicrographic sequence. This graph illustrates the extent of movement of three cells between eighth and tenth cleavage. Distance moved was measured as the distance between the position of a cell's leading edge at time = 0 and its position at time = X. The time interval was determined by summation of the individual frame intervals between measured frames. The times of ninth and tenth cleavages are marked with arrows. Cell 2 could not be measured accurately at 60 minutes as indicated by the solid line.

Fig. 3-B. Rate of movement of the three cells of Fig. 3-A between eighth and tenth cleavage. Rate of movement (average rate between measured frames) was calculated by dividing the distance moved (Fig. 3-A) by the interval between measurements. Time was determined as above. Arrows indicate ninth and tenth cleavages. This figure illustrates that the rate of cell movement was not constant, cells did not all move at the same rate, and movement was interrupted by cleavage of the cells.


3A
DISTANCE MOVED (in cm)
0
10
20
30
40
50
60
0
20
40
60
80
100
120
TIME (in minutes)
1
2
3

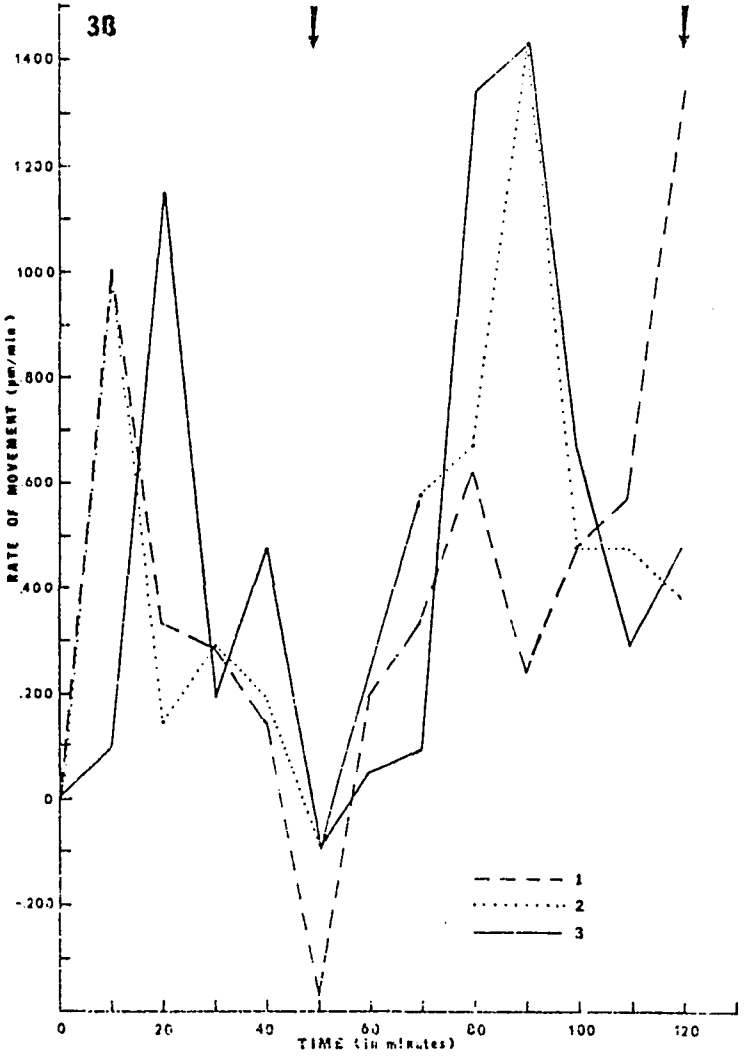
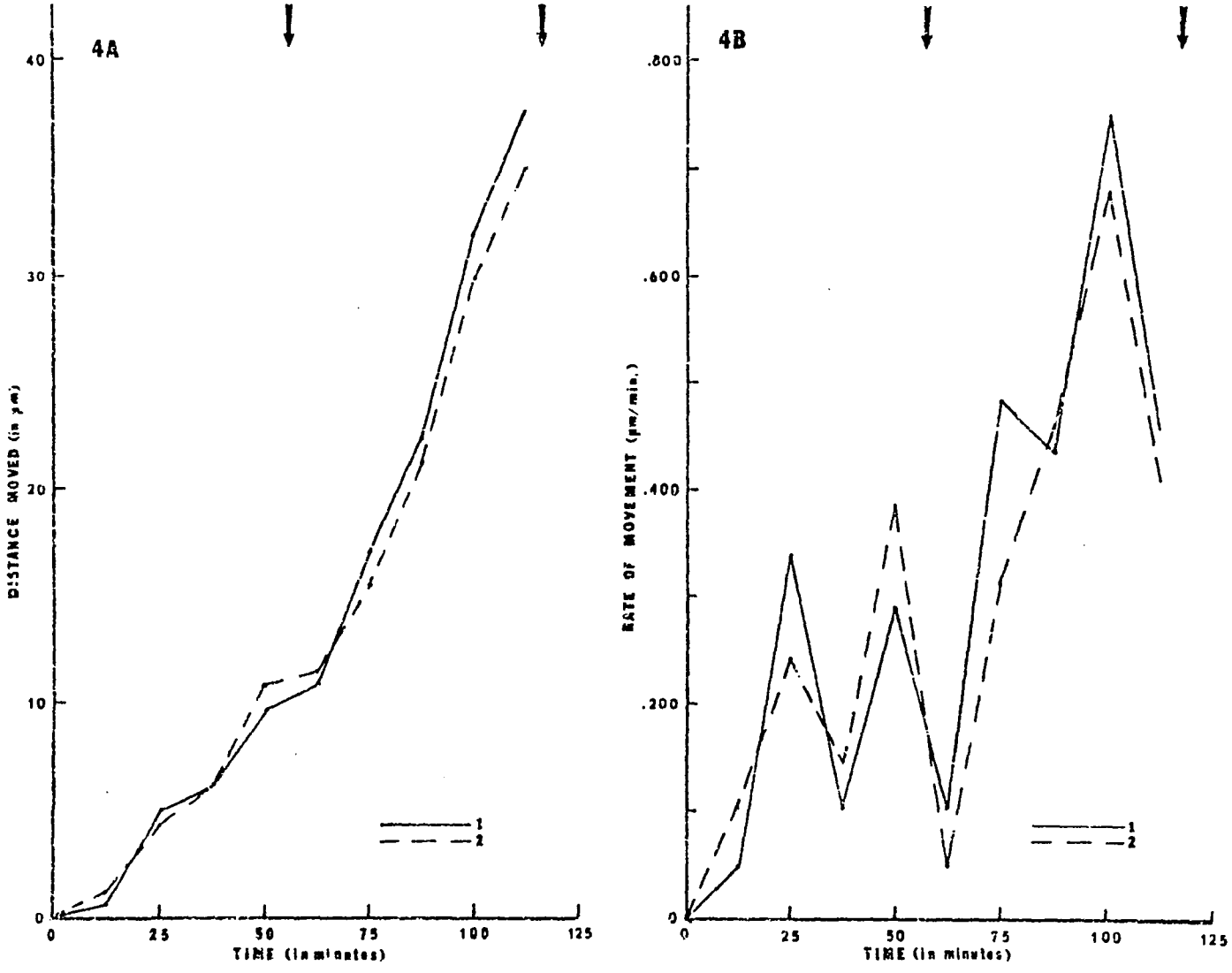

3B
RATE OF MOVEMENT (cm/min)
-200
0
200
400
600
800
1000
1200
1400
0
20
40
60
80
100
120
TIME (in minutes)
1
2
3

Figure 4

Extent and Rate of Cell Movement During Overlapping II

Fig. 4-A. Distance moved by individual cells through time as measured on projected time-lapse cinemicrographic sequences. This figure illustrates the extent of movement of two cells over the period between eighth and tenth cleavages. Distance moved was measured as the distance between the position of the cell's leading edge at time = 0 and its position at time = X. The time interval was determined by summation of the individual frame intervals between measured frames. The times of ninth and tenth cleavages are marked with arrows.

Fig. 4-B. Rate of movement of the two cells of Fig. 4-A between eighth and tenth cleavages. Rate of movement (average rate between measured frames) was calculated by dividing the distance moved (Fig. 4-A) by the period between measurements. Time was determined as above. Arrows indicate ninth and tenth cleavages. This figure illustrates as in Fig. 3-B that the rate of movement was not constant and that the rate of movement was reduced during cytokinesis.


4A
DISTANCE MOVED (in μm)
0
10
20
30
40
0
25
50
75
100
125
TIME (in minutes)
1
2
4B
RATE OF MOVEMENT (μm/min.)
0
.200
.400
.600
.800
0
25
50
75
100
125
TIME (in minutes)
1
2

rounding up of the leading edge prior to cleavage. During cytokinesis the rate of movement is reduced to near or below zero. Although the number of cells whose rates of movement were measured was necessarily limited by movements of the cells in and out of focus, Figs. 3 and 4 illustrate some behavioral characteristics of the overlapping cells. The significance of this behavior is discussed below.

3.3. Establishment of the Yolk Syncytium

3.3.1. Observation of Normal Development

The yolk syncytium is established by the incorporation into the yolk cytoplasmic layer of the nuclei of blastocones produced by ninth cleavage. Blastocones are, in essence, pockets within the continuous yolk cytoplasmic layer. They are formed by the membranes of meridional and undercutting furrows.

The sequence of events in the establishment of the yolk syncytium was followed by time-lapse cinemicrography as illustrated in PLATES X, XI, and XII. After ninth cleavage (Plate X, T=3:08.00) lateral and undercutting furrows of the blastocones retract toward the blastoderm and disappear (PLATE XI, T=3:20.30). The blastocone nuclei move vegetally (compare PLATE X, T=3:08.00 through PLATE XI, T=3:45.30). The vegetal movement of the nuclei and the retraction of the furrows leave the nuclei in a syncytial ring around the perimeter of the blastoderm. At this stage

PLATE X

Cinemicrographic Series II.

Ninth Cleavage

T=1:40.30. Ninth Cleavage (Early Stage 10). The centripetal ring of cells resulting from the ninth cleavage of the presumptive middle-layer cells is completely overlaid. The cells of this ring can be seen lying beneath the overlap cells (bracketed area). The active margin of the overlapping ring of cells is indicated (arrow).

T=1:53.00. Ninth Cleavage (Stage 10). Overlapping continues. The pesudopodial activity of the leading edge is visible (arrow).

T=2:05.00. Ninth Cleavage (Stage 10). The centrifugal ring of cells produced by the ninth cleavage of the presumptive middle-layer cells is now completely overlapped. The middle-layer ring of cells is now two cells wide and can be seen beneath the overlap cells. Active overlapping continues (arrow).

T=2:18.00. Ninth Cleavage (Stage 10). The cells in the center of the field are in mitosis and are rounding up for tenth cleavage which is trans-radial in these cells. Note the retracted leading edge (arrow).

T=2:30.30. Ninth Cleavage (Stage 10). The majority of the blastomeres appear to have undergone ninth cleavage. The blastocones have entered mitosis prior to ninth cleavage (broad arrow). The margin of overlap is indicated (arrow).

T=2:43.00. Ninth Cleavage (Stage 10). The blastocones are entering cytokinesis of ninth cleavage. Furrow orientation is trans-radial in this division of the blastocones. Note that the blastomeres produced by eighth cleavage of the blastocones are moving up onto the edge of the blastoderm. The arrow again indicates the margin of overlap.

T=2:55.30. Ninth Cleavage (Mid-Stage 10). Ninth cleavage is now complete within the field of view.

T=3:08.00. Ninth Cleavage (Mid-Stage 10). The daughter nuclei of blastocone ninth cleavage mitosis have moved apart (broad arrows) and the blastocone furrows have begun to retract. The middle layer is visible beneath the surface cells (bracket).

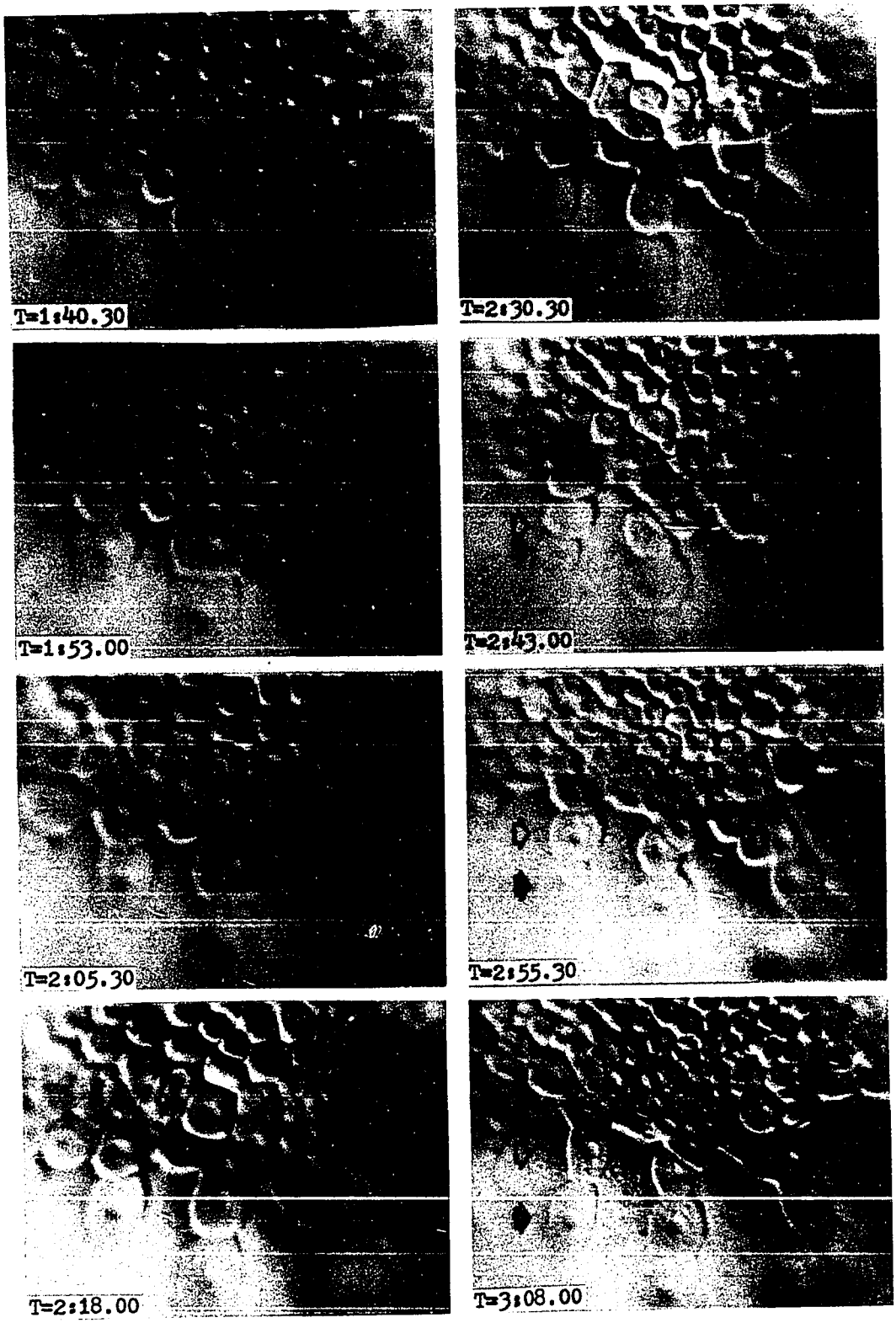

T=1:40.30
T=2:30.30
T=1:53.00
T=2:43.00
T=2:05.30
T=2:55.30
T=2:18.00
T=3:08.00

PLATE XI

Cinemicrographic Series II.

Ninth Cleavage and Establishment of the Yolk Syncytium

T=3:20.30. Ninth Cleavage (Mid-Stage 10). The blastocone furrows are retracting. The blastomeres of eighth cleavage of the blastocones have moved up onto the edge of the blastoderm (arrowheads). The middle layer is no longer clearly discernable.

T=3:33.00. Ninth Cleavage (Mid-Stage 10). The blastocone furrows are almost completely retracted and the blastocone nuclei have moved vegetally. These nuclei now constitute a syncytium and are termed the yolk syncytium nuclei. The blastocone ninth cleavage blastomeres are rounding up and moving toward the blastoderm. The blastocone eighth cleavage daughter blastomeres are indicated (arrowheads).

T=3:45.30. Ninth Cleavage (Mid-Stage 10). The blastocone ninth cleavage daughter blastomeres continue to move toward the blastoderm (open arrow). Blastocone eighth daughter blastomeres are indicated (arrowheads).

T=3:58.00. Ninth Cleavage (Mid-Stage 10). The blastocone ninth cleavage daughter blastomeres continue to move toward the blastoderm (open arrow). The yolk syncytium nuclei have not changed position (solid arrows).

T=4:10.30. Ninth Cleavage (Mid-Stage 10). The processes of the previous frames are continued (broad arrows). The yolk syncytium nuclei are entering prophase of Tenth Nuclear Division (small arrow). The black dot is an artifact.

T=4:23.00. Tenth Nuclear Division (Late Stage 10). The blastomeres followed above continue to move onto the blastoderm (open arrow and arrowhead). The yolk syncytium nuclei are in mitosis. The nucleus indicated by the broad, solid arrow is now in late prophase and the nucleus indicated by the small arrows is in late anaphase.

T=4:35.30. Tenth Nuclear Division (Late Stage 10). The blastomeres followed above continue to move onto the blastoderm (open arrow and arrowhead). The framing here has shifted vegetally and to the apparent right. The yolk syncytium nuclei continue mitosis (arrows).

T=4:48.00. Tenth Nuclear Division (Late Stage 10). The blastodermal cells are in various stages of cleavage. The blastomere indicated above continues moving onto the blastoderm (arrowhead).

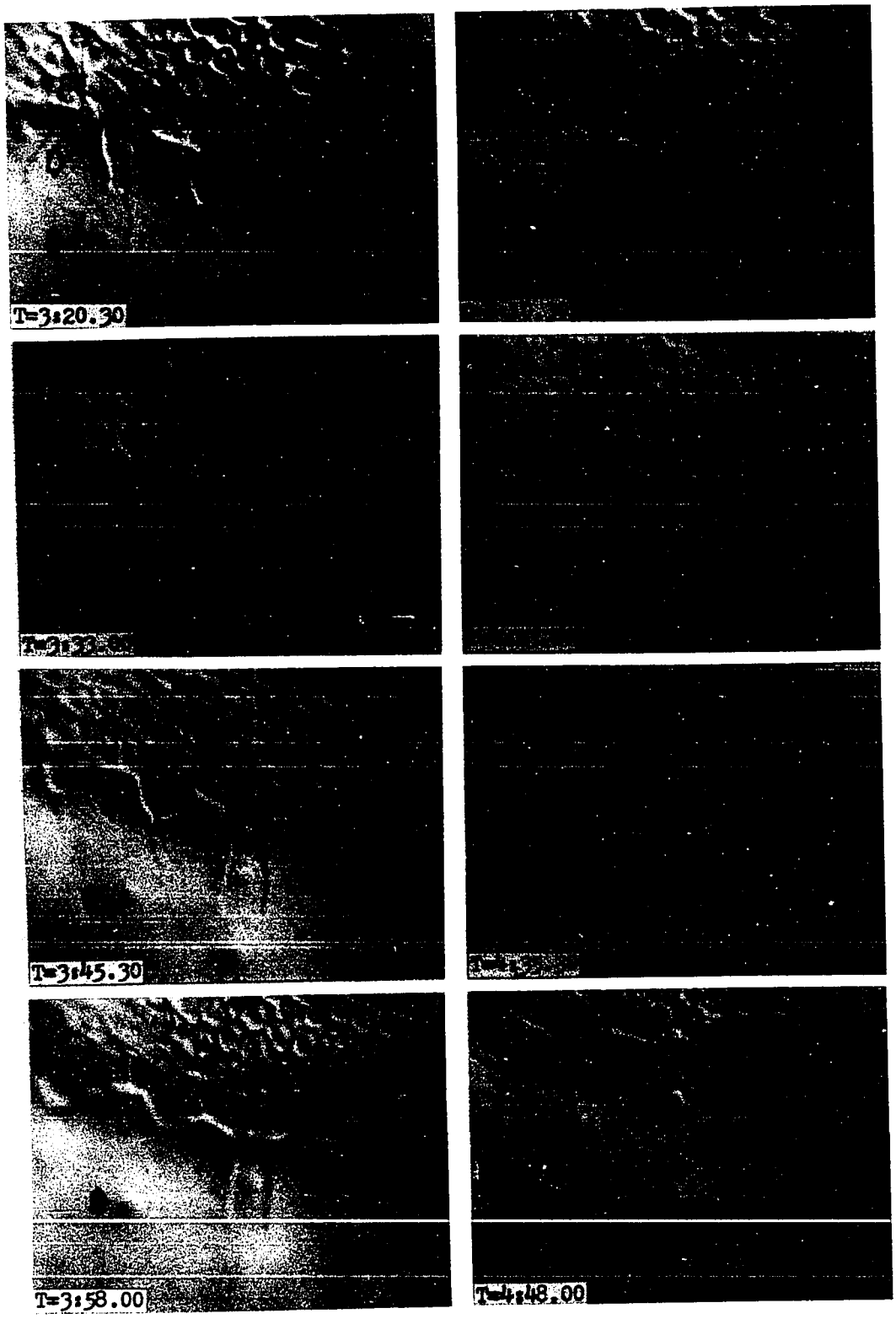

T=3:20.30
T=3:45.30
T=3:58.00
T=4:48.00

PLATE XII

Cinemicrographic Series II.

Establishment of the Yolk Syncytium

T=5:00.30. Tenth Nuclear Division (Late Stage 10). The proximal ring of yolk syncytium nuclei has moved a short distance toward the animal pole (arrow). The peripheral blastodermal cells are flattening in preparation for movements of epiboly.

T=5:13.00. Tenth Nuclear Division (Early Stage 11). Some blastodermal cells can be seen undergoing eleventh cleavage. The proximal ring of yolk syncytium nuclei is seen moving beneath the margin of the blastoderm by means of autonomous movement toward the animal pole and vegetal spreading of the blastoderm (arrow).

T=5:25.30. Tenth Nuclear Division (Early Stage 11). Epiboly is beginning and small pseudopodia are seen on the flattened margins of the peripheral cells of the blastoderm.

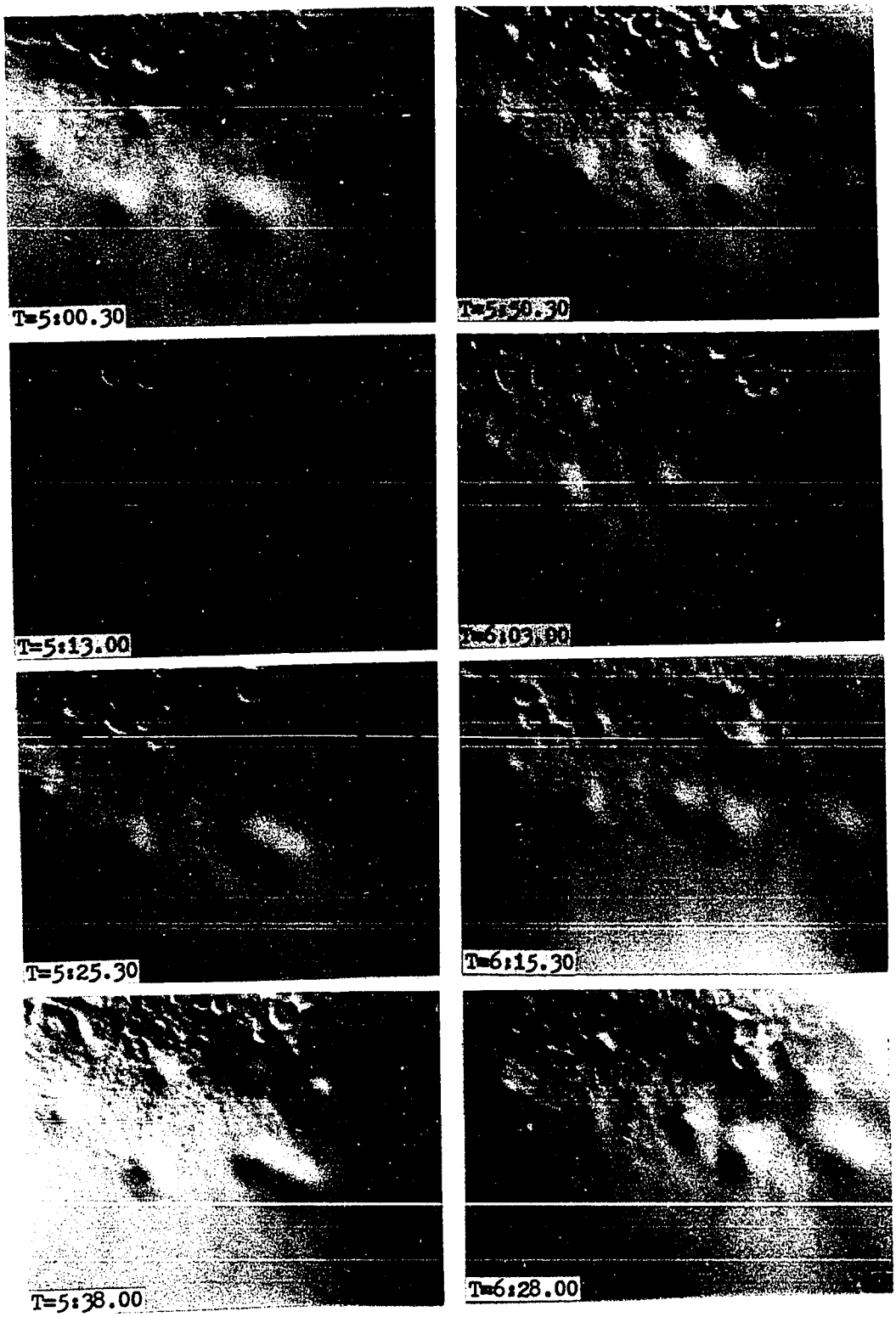
T=5:38.00. Tenth Nuclear Division (Early Stage 11). The proximal ring of yolk syncytium nuclei now lies beneath the expanding margin of the blastoderm (arrow).

T=5:50.30. Tenth Nuclear Division (Early Stage 11). The proximal yolk syncytium nuclei are completely overlaid by the blastoderm and continue their autonomous movement toward the animal pole (arrow).

T=6:03.00. Tenth Nuclear Division (Mid-Stage 11). The yolk syncytium nuclei followed previously are indicated lying beneath the blastoderm (arrow). The margin of the blastoderm consists of a single layer of flattened polygonal cells. This peripheral ring of cells remains as a single layer throughout epiboly and contributes to the formation of the external yolk sac. The distal yolk syncytium nuclei are entering prophase of eleventh nuclear division (arrowhead).

T=6:15.30. Tenth Nuclear Division (Mid-Stage 11). The yolk syncytium nuclei continue in mitosis. Epiboly continues.

T=6:28.00. Tenth Nuclear Division (Mid-Stage 11). Epibolic and nuclear movements continue. The distal yolk syncytium nucleus indicated (arrowhead) is in late anaphase. The proximal yolk syncytium nucleus indicated (arrow) is flattening and so appears to be getting larger. This stage completes the first phases of gastrulation. Gastrulation subsequently involves a continuation of epibolic spreading of the blastoderm and cytodifferentiation of the germ layers.


T=5:00.30
T=5:13.00
T=5:25.30
T=5:38.00
T=6:03.90
T=6:15.30
T=6:28.00

each nucleus is spheroidal and lies within a lenticulate portion of the common cytoplasm producing a slight depression in the underlying yolk.

During their vegetal movements the nuclei undergo a series of relatively rapid oscillations as seen by time-lapse cinemicrography. The nuclear membrane exhibits surface activity much like the surface activity of cultured cells during blebbing locomotion, and the entire nucleus moves back and forth. Similar oscillatory movements were observed in no other nuclei. The function of these movements is unknown. Measurements of the vegetal movement of fifteen blastocone nuclei were taken from photographic sequences of yolk syncytium formation. Over their approximately one hour existence, these nuclei moved vegetally on the average a distance of 37.5 μm.

The blastomeres produced by ninth cleavage of the blastocones round up and are shown by time-lapse to move up onto the perimeter of the blastoderm (see PLATE X, T=2:55.30 to PLATE XI, T=4:23.00). These blastomeres spread out around the perimeter of the blastoderm where they form the leading edge during the second phase of epibolic spreading of the blastoderm (PLATE XI, T=3:45.30 to T=4:23.00). These blastomeres establish a population of cells which will give rise to some of the extraembryonic tissues of the external yolk sac. Ninth cleavage has thus

established two unique populations of cells (i.e. the initial ring of middle-layer cells and marginal cells of the blastoderm) and a population of syncytial nuclei. The marginal cells of the blastoderm and the yolk syncytium are entirely extraembryonic.

Retraction of undercutting and meridional furrows of the blastocones involves a folding up of the furrows beginning at their bases and a possible vesiculation of the furrow membranes as revealed by electron microscopy. At ninth cleavage these furrows appear as long parallel membranes separated by approximately 10 nm and terminating in an expanded bulb-shaped structure typical of furrow bases in Loligo (PLATE XIII, Figs. 2 to 4: see also Arnold, 1971, 1974). Associated with the cytoplasmic face of the furrow base membrane is a band of 5 nm to 7 nm filaments (PLATE XIII, Fig. 4). The undercutting furrows extend through the syncytial cytoplasm for a distance of as much as 175 μm. During retraction the furrows become highly folded especially at the bases (PLATE XIV, Figs. 1 and 2). The microfilamentous band seen associated with normal furrows is noticeably absent in retracting furrows. This folded area of the base apparently moves toward the blastoderm as judged by the progressively shorter furrows seen in embryos fixed at progressively later stages. In later stages of retraction the furrows often appear as strings of membranous vesicles.

PLATE XIII

Undercutting Furrows of Blastocones

Fig. 1. Section of an osmiun tetroxide fixed, Epon embedded ninth cleavage (early Stage 10) Loligo pealei. This figure shows the organization of the edge of the blastoderm and blastocones early in ninth cleavage. Note the location of the blastocone (BC), middle-layer ring of cells (MLR) and the overlapping cell (OLC). The small, round, light areas beneath the cells are yolk platelets in various stages of digestion. 250 X.

Fig. 2. Electron micrograph of the area of the undercutting furrow (UCF) indicated by the bracket in Fig. 1. In cross section the furrow is seen as two parallel membranes separated by a space of approximately 100 Angstroms. At the furrow base the membranes diverge forming a bulb-like profile. Associated with the furrow base membrane is a band of microfilaments (MF and see Fig. 4). The platelet at the far right is in an early stage of digestion.

Fig. 3. This micrograph presents the base of the undercutting furrow in more detail (FB). The furrow membranes at this stage are parallel and exhibit little gross undulation or folding. Small undulations, however, produce apparent discontinuities in the membrane structure as the cross sectional-planes of the membranes tilt through the plane of section. The syncytial cytoplasm consists primarily of mitochondria, ribosomes and smooth endoplasmic reticulum. The fibrillar material (FM) between the chorion (CH) and the cytoplasmic layer lying above the yolk (Y) is the remnant of the fertilization membrane (see Arnold and Williams-Arnold, 1976). Yolk platelets are each surrounded by a unit membrane. Some evidence of membrane vesiculation is present near the furrow base. 22,200 X.

Fig. 4. Higher magnification micrograph of a furrow base illustrating the microfilamentous band (MF). The microfilamentous material associated with the furrow base is usually more abundant on the side of the furrow nearest the yolk. 50,000 X.

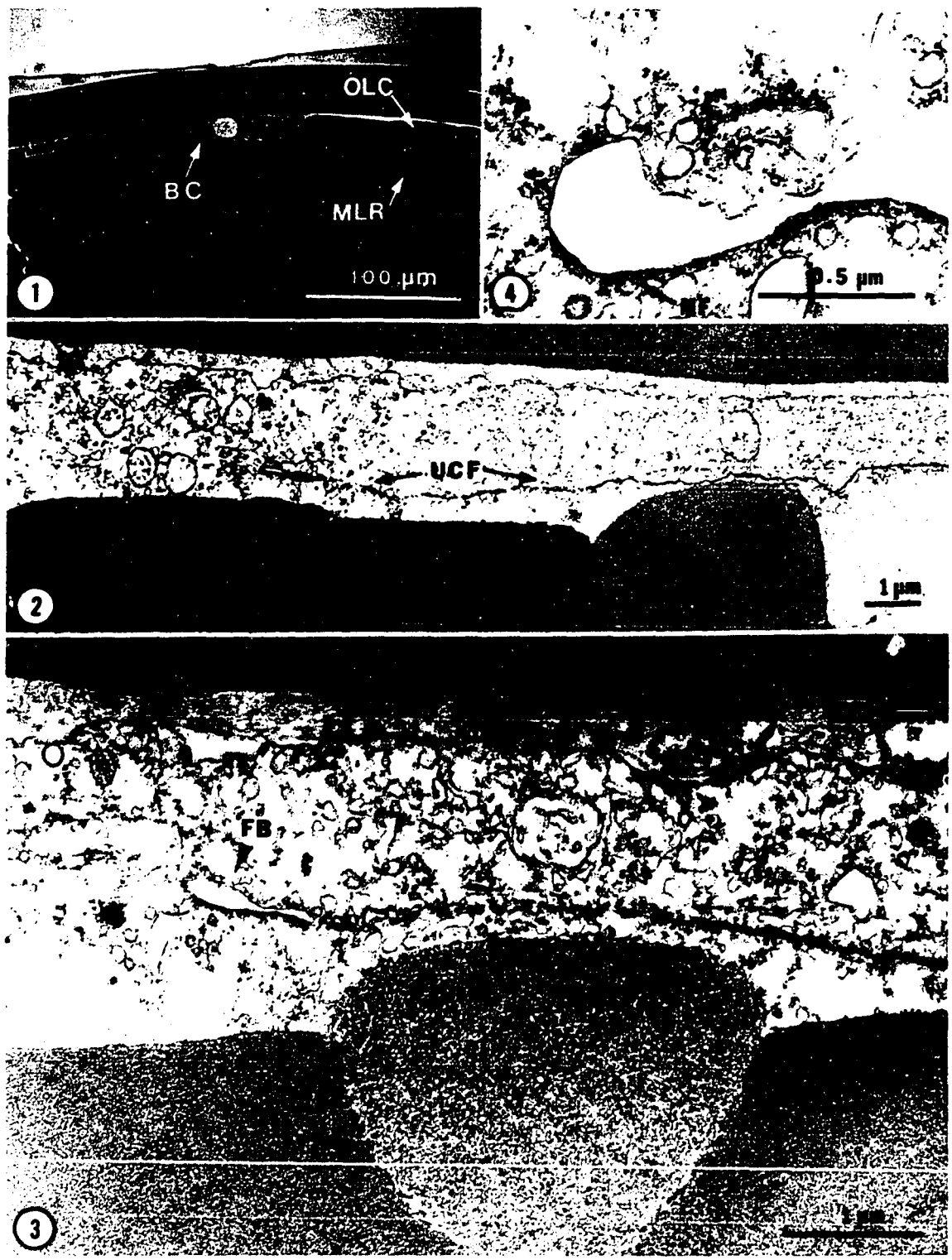

OLC
BC
MLR
100 µm
1
4
0.5 µm
UCF
2
1 µm
FB
3

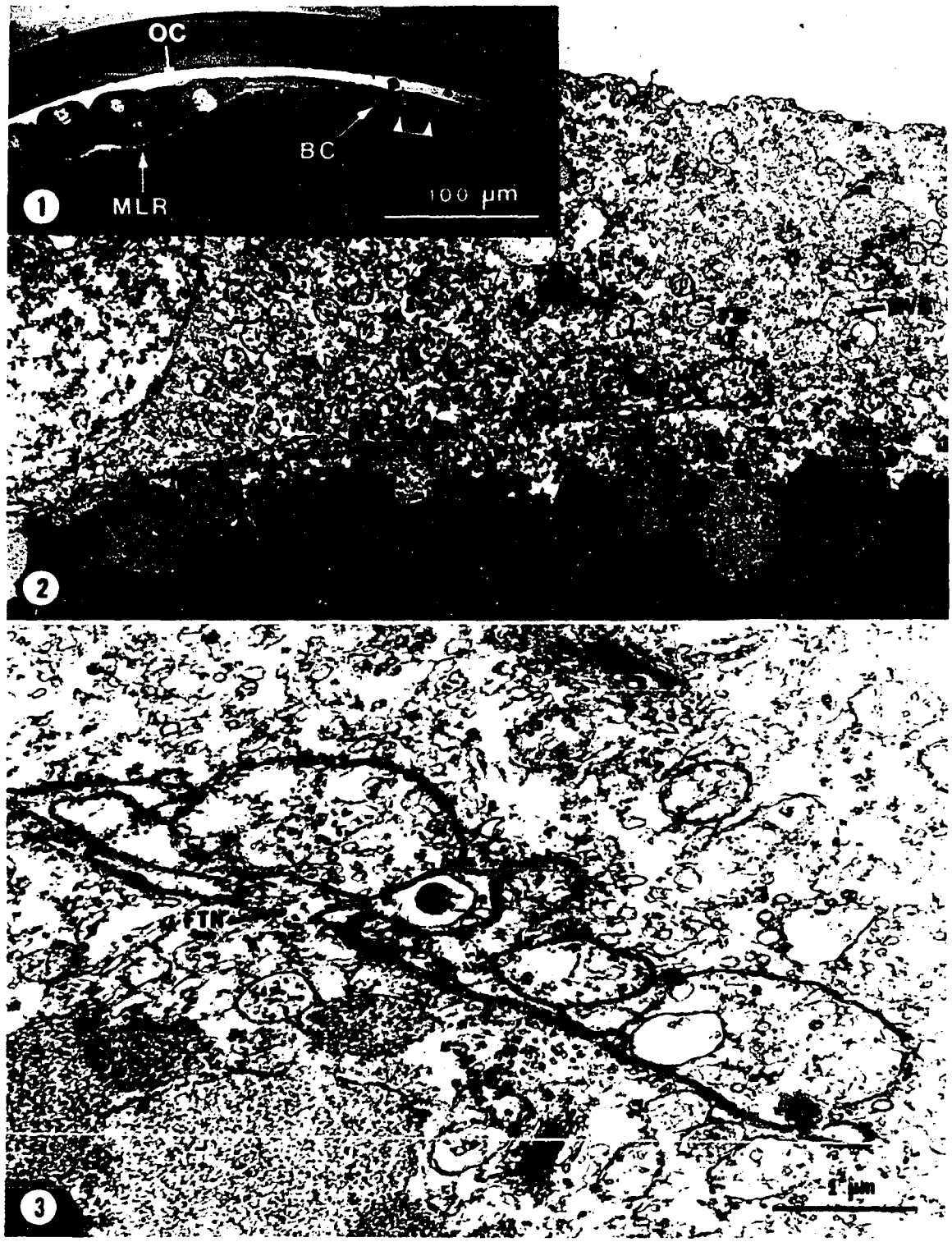
PLATE XIV

Furrow Retraction In A Blastocone

Fig. 1. Section of an osmium tetroxide fixed, Epon embedded, late ninth cleavage embryo of Loligo pealei. This shows the organization of the blastoderm, middle-layer ring (MLR), and blastocone (BC). The middle-layer ring (MLR) is two cells wide at this stage. The lateral edges of two overlapping cells (OC) lie above the middle-layer cells. The dark staining of the middle-layer cells is due to their relatively greater ribosome content. The undercutting furrow illustrated in Figs. 2 and 3 below is visible in the bracketed area of the blastocone. Compare the location of the furrow base in this figure with that of Plate XIII, Fig. 1. 250 X.

Fig. 2. Electron micrograph of a blastocone and its undercutting furrow during retraction. Note the highly folded appearance of the furrow at this early phase of retraction (UCF). The base of the furrow (FB) at this stage is much nearer the nucleus than in early stages. Multivesiculate bodies (MVB) and Golgi bodies (G) are common features of the cytoplasm in the area of the retracting furrow. 6,800 X.

Fig. 3. Higher magnification of a section through the retracting furrow base. The furrow appears to be folded back upon itself, there is a distinct lack of microfilamentous material, and membranous vesicles are common. Varying amounts of a fine tubular network (FTN) are present near the retracting base. 23,100 X.


OC
BC
MLR
100 µm
1
2
FTN
3

A number of cytoplasmic organelles not distributed throughout the cytoplasm are found associated with the undercutting furrow base during retraction. Multivesiculate bodies are commonly found in the area of the retracting furrow. In addition, there is a variable amount of a fine (ca. 25 nm diameter) network of tubular membranes associated with the retracting furrow base. Although Golgi bodies are commonly found throughout the cytoplasm, they appear to be always present near the retracting furrow base.

Microtubules are frequently seen within the cytoplasm immediately surrounding the yolk syncytium nuclei at the stage when these nuclei move vegetally, as well as when the nuclei are moving beneath the blastoderm. Microtubules are found lying close to the nuclear membranes and arrayed parallel to the plasma membrane of the yolk syncytium (PLATE XV, Figs. 2 and 3). No evidence of physical attachment of the microtubules and nuclear membrane was found (PLATE XV, Fig. 3).

3.3.2. Observations of Experimentally Altered Development

Experiments with the inhibitors colchicine and cytochalasin B indicate that microtubules seen associated with the yolk syncytium nuclei are not involved in nuclear migration in Loligo. Embryos which had completed ninth cleavage were treated with 10^{-4}M colchicine for 10 minutes, 30 minutes or continuously. Blastocone nuclei produced by

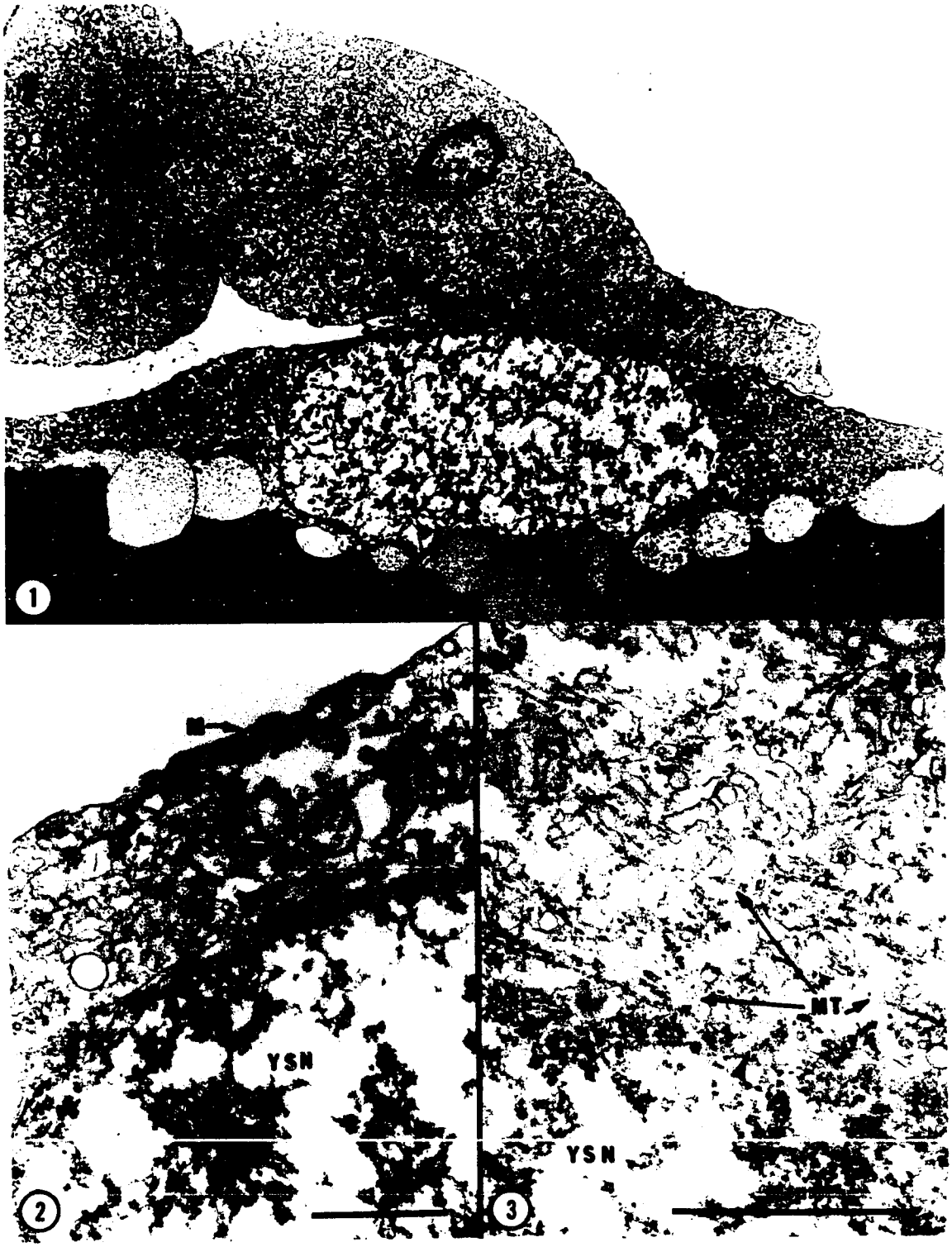
PLATE XV

Yolk Syncytium Nuclei and Microtubules

Fig. 1. Electron micrograph of the blastodermal margin at a stage between tenth nuclear division and eleventh nuclear division. Epibolic spreading is in its earliest phase as evidenced by the thick lamellipodium of the marginal cell. During this stage the yolk syncytium nuclei (YSN) move toward the animal pole. The yolk syncytium nuclei lie within a lenticulate portion of the syncytial cytoplasm which sits in a slight depression in the yolk. During phases of nuclear movement microtubules are commonly found arrayed near the nuclei (see Figs. 2 and 3). 3,900 X.

Fig. 2. Micrograph showing microtubules arrayed between the plasma membrane (M) of the yolk syncytium and the yolk syncytium nucleus (YSN). 22,100 X.

Fig. 3. Micrograph showing the close association of some microtubules (MT) and the nuclear membrane (arrowhead). No evidence was found of any physical contact between the microtubules and the nuclear membrane. 40,000 X.


1
M
YSN
2
MT
YSN
3

ninth cleavage division in treated embryos migrated out of the blastocones and continued vegetally at a rate comparable to the rate of advance of blastodermal spreading in controls (PLATE XXV, Fig. 6). Colchicine irreversibly blocked the maintenance of microtubules in embryos as judged by electron microscopy and totally inhibited mitosis. The results of colchicine treatment indicate that the migration of the yolk syncytium nuclei is independent of the spreading of the blastoderm.

In contrast with the results of colchicine treatment, nuclear migration was totally inhibited during treatment of embryos with 0.02 μgm/ml of cytochalasin B. Inhibition occurred only during treatment and migration resumed after removal of the cytochalasin. After removal of the cytochalasin from embryos treated for ten minutes at ninth cleavage the nuclei renewed their movements in both vegetal and animal directions. In cases where treatment extended through tenth nuclear division both daughter nuclei moved in a vegetal direction after removal of the cytochalasin. Prolonged treatment (30 minutes or longer at 0.02 μgm/ml) elicited a retraction of the leading edges of the peripheral blastoderm cells. After removal of the cytochalasin the proximal ring of yolk syncytium nuclei was out beyond the perimeter of the blastoderm, whereas in control embryos these nuclei lay just beneath the edge of the blastoderm. In such cases completion of gastrulation was totally

inhibited. The yolk syncytium nuclei moved vegetally while the blastodermal cells eventually formed a clump at the animal apex and the embryo died (PLATE XXIII, Figs. 3 and 4).

3.4. Observations with the Scanning Electron Microscope

3.4.1. Late Cleavage Stage Blastoderm and Blastocones

At eighth cleavage (Late Stage 9) the embryo of Loligo pealei consists of a single layered blastoderm comprised of ca. 230 blastomeres arranged in approximately concentric rings and surrounded by 24 or 26 blastocones (PLATE II, Figs. 4 and 5; PLATE XVI, Fig. 1). Plate XVI, Fig. 1 illustrates the overall appearance of the embryo at eighth cleavage. The remnants of the fertilization membrane (FM) are pulled back to reveal the blastoderm. The second complete ring of cells in from the margin of the blastoderm constitutes the population of presumptive middle-layer cells (PMC). The presumptive middle-layer cells in the embryo illustrated are partially overlapped by the centripetally adjacent ring of cells (OC). The free surfaces of the blastomeres possess numerous small blebs, filopodia (termed microspikes by Weiss, 1961) and intermediate sorts of protrusions. These small cellular protrusions are more numerous near the cells' apices. These protrusions extend out of the cells and into the

intrachorionic space or across the intercellular space where their tips make non-junctional contact with neighboring cells (PLATE XVI, Fig. 2; PLATE XVII, Figs. 1 and 2). The filopodia range in diameter from 0.2 μm to 0.4 μm and are thicker at the base tapering toward the tip. Their length ranges from less than 1.0 μm to over 10.0 μm.

Some blastomeres lack large numbers of filopodia, have a more rounded appearance, and do not closely abut adjacent cells apically. For example, in Plate XVI, Fig. 1, two radially arrayed bands of blastomeres exhibiting these characteristics can be seen. Rounding up and loss of surface activity are common phenomena associated with cell division (e.g. Revel, 1974). Furthermore, the number and distribution of cells having these characteristics is precisely that of mitotic cells observed in FAA-Feulgen treated embryos of the same stage. It is assumed, therefore, that the cells which show little surface activity have entered mitosis. The blastocones of the embryo in Plate XVI, Fig. 1 also have little surface activity and so are probably undergoing mitosis. FAA-Feulgen treated embryos of this stage often display mitotic figures in all but the posteromedial blastocones.

3.4.2. Overlapping Cells during Middle-Layer Segregation

The blastomeres, which with time-lapse cinemicrography were found to spread over the presumptive middle-layer cells,

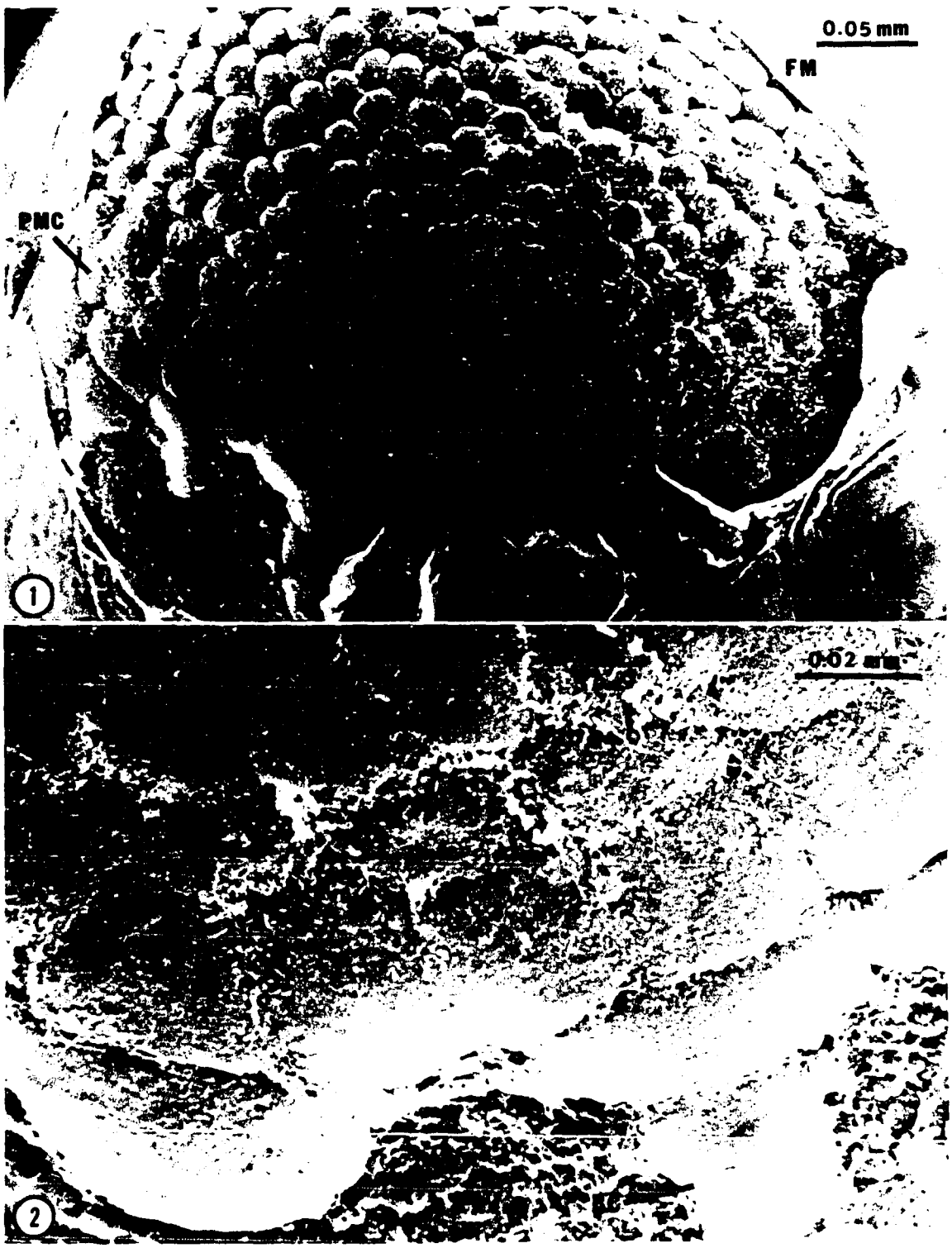
PLATE XVI

Scanning Electron Microscopy of an Eighth Cleavage Embryo

Fig. 1. Scanning electron micrograph of Loligo pealei at eighth cleavage. The embryo consists of a single layer of blastomeres and blastocones. The blastomeres are arranged in approximately concentric rings forming the blastoderm which is surrounded by the continuous blastocones (BC). The entire embryo is surrounded by a fibrous fertilization membrane (FM) which here has been fortuitously pulled back to reveal the blastoderm. The population of presumptive middle-layer cells (PMC) forms a ring of contiguous blastomeres as the third ring in from the perimeter of the blastoderm. The beginning of overlapping can be seen in the ring of cells centripetal from the presumptive middle-layer ring.

The blastomeres possess varying degrees of surface activity in the form of small cell protrusions. The relatively reduced surface activity and rounded appearance of the radially arrayed blastomeres at the top of the figure is indicative of their being in some phase of mitosis. 320 X.

Fig. 2. Scanning electron micrograph of the margin of the blastoderm of L. pealei at eighth cleavage. The overlap cells (OC) extend broad, flattened lamellipodia vegetally (arrow) over the presumptive middle-layer cells (PMC). Smaller lamellipodia and filopodia extend from the margins of most blastomeres and blastocones (BC). 985 X.


0.05 mm
FM
PMC
1
0.02 mm
2

with scanning electron microscopy display lamellipodial cell protrusions in addition to the various blebs and filopodia described above (PLATE XVII). The vegetally directed margins of these cells extend over the presumptive middle-layer cells as broad, tapering lamellipodia-like processes (PLATE XVII, Fig. 1, L). Their breadth varies with the size of the cell and ranges between 10 and 30 μm. These broad processes have narrower (ca. 1 to 5 μm) and thinner (ca. 0.1 to 0.5 μm) lamellipodia extending from their leading edges (PLATE XVII, Figs. 1 and 2, L and P). The smaller lamellipodia in turn extend long fine filopodia which again make contact at their tips with neighboring cells (PLATE XVII, Fig. 1, P). The lamellipodia of the overlapping cells, in general, extend from the cell in the direction of movement.

3.5. Observation with the Transmission Electron Microscope

3.5.1. Fine Structure of the Early Cleavage Blastoderm

As with embryos of other animal species, blastomeres of Loligo pealei characteristically lack the cytoplasmic specializations seen later in development and in adult tissues. The ground cytoplasm has a low electron opacity and contains few organelles (PLATE XVIII). The mitochondria consist of a round to irregularly rounded outer envelope surrounding a few tubular cristae. All of the endoplasmic

PLATE XVII

Scanning Electron Microscopy of Overlapping Cells

Fig. 1. Scanning electron micrograph of the blastoderm of Loligo pealei at eighth cleavage. This micrograph illustrates in more detail the relation of the overlap cells to the presumptive middle-layer cells. A broad, thick lamellipodium (L) is seen extending smaller lamellipodial processes (p) from its leading edge. These, in turn, possess smaller filopodial protrusions. In general these processes are extended in the direction of overlapping (arrow). Small blebs are also a common feature of the cells' surfaces at this stage. 4,500 X.

Fig. 2. Scanning electron micrograph of an eighth cleavage embryo illustrating cell surface activity. The broad lamellipodium (L, twoheaded arrow) is spreading in the direction of the arrow as it extends and contacts underlying cells via small lamellipodial (P) and filopodial (F) processes. Most blastomeres exhibit similar filopodial activity. An area of presumed junction formation (arrowhead) displays less surface activity than other areas of cell-cell apposition. 45,000 X.

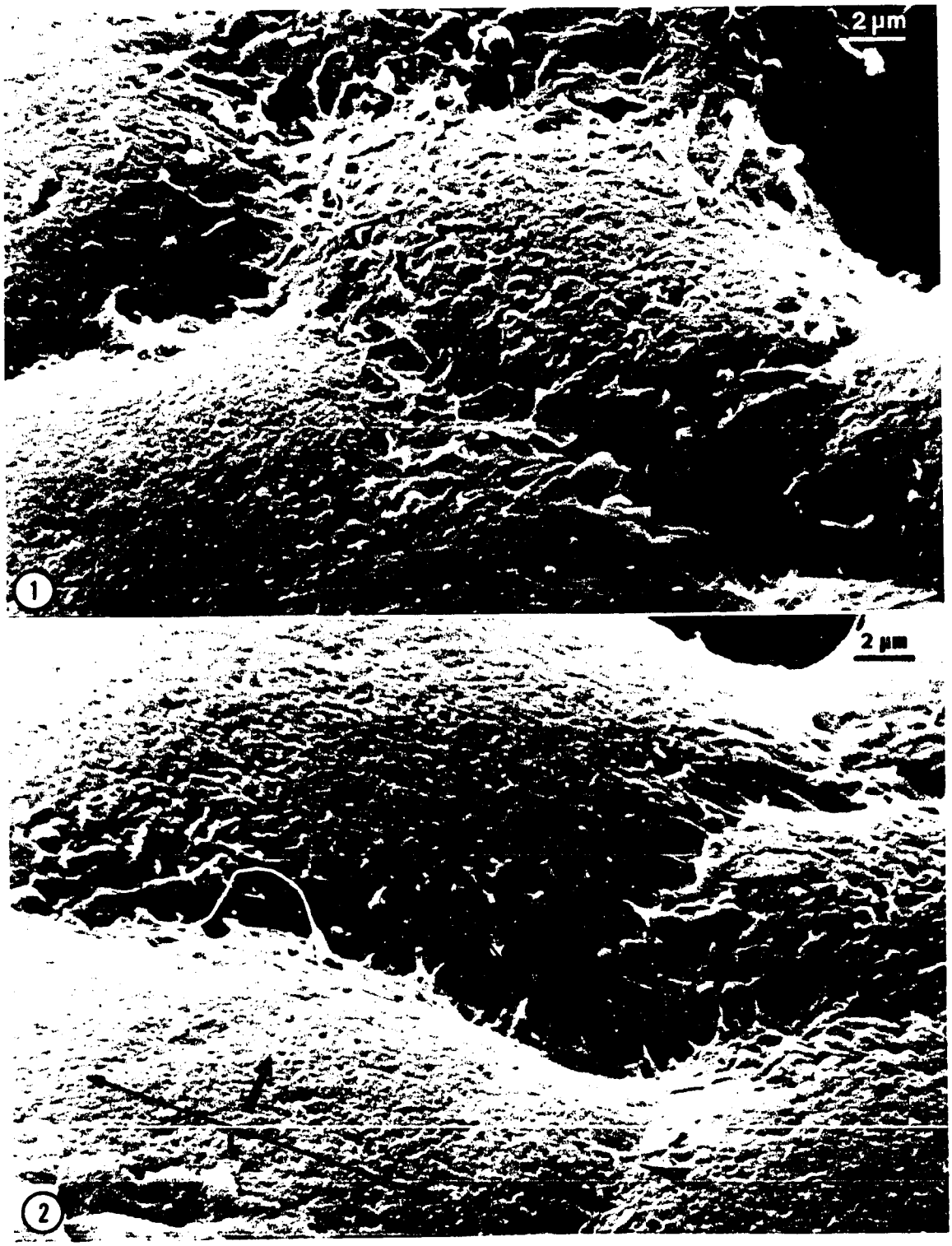

2 µm
1
2 µm
2

PLATE XVIII

Intercellular Junctions and Contacts

Fig. 1. Electron micrograph of an apical intercellular apposition of blastomeres in an eighth cleavage embryo. The apposition at its apex is 20 nm to 25 nm wide. No septa are seen in apical appositions prior to eighth cleavage. Note the microspike (filopodium) bridging the gap between cells. These filopodia make 20 nm non-junctional appositions with neighboring cells. These processes typically contain bundles of microfilaments. 13,000 X.

Fig. 2. Micrograph of an apical intercellular junction in a late ninth cleavage embryo. Within the 10 nm to 15 nm area of apposition 5 nm by 15 nm septa are appearing, but with a greater than 15 nm center to center distance. The distension of the membrane (between arrows) is due to fixative solution being hypo-osmotic (see Lord and Dibona, 1976). A Golgi body (G) is seen cut tangential to the forming face. Evidence of filopodial activity is seen at the apex of these cells. 25,600 X.

Fig. 3. Septate Junction. This micrograph illustrates the form of a typical septate junction below the apical desmosome in a Stage 11 embryo. The inter-membrane distance is constant at 15 nm in septate areas. The spot-like close appositions above and below the septate region are indicative of maculate desmosomes. However, these are not clear in this micrograph. 63,000 X.

Fig. 4. Intercellular interdigitation. Microvilli often extend from one cell into another. Interdigitations such as this are sometimes the remnant of cleavage furrow formation, but are often seen in other areas as well. The Golgi (G) at the right is cut somewhat tangential to the forming face. 20,000 X.

Fig. 5. Intercellular interdigitation similar to that in Fig. 4 above. Membranous vesicles are often seen opposite the tips of microvillar processes. These interdigitations may be a means of intercellular communication (Arnold and Williams-Arnold, 1976). 28,000 X.


1
2
3
4
5
1 µm

reticulum present in cleavage stages is of the smooth variety and ribosomes are distributed evenly throughout the cytoplasm individually rather than as polysomes. The Golgi complex is quite extensive. Cross sections of blastomeres reveal an average of six to ten dictyosomes which appear to be distributed in no particular pattern. Multivesiculate bodies are rarely seen during early cleavage, but may be seen in areas of furrow vesiculation, for example, in late phases of cytokinesis.

The blastomeres of early through late cleavage develop no organized intercellular junctions, but commonly form 20 nm to 25 nm non-junctional appositions (PLATE XVIII, Fig. 1). At the apex of most of these appositions filopodia extend from one cell to another. These filopodia contain bundles of oriented microfilaments (PLATE XVIII, Figs. 1 and 2). Filopodia also form non-junctional appositions with adjacent cells. Various interdigitations and projections of one cell into another, similar to those reported in later stages (Arnold and Williams-Arnold, 1976), are seen throughout cleavage (PLATE XVIII). Arnold and Williams-Arnold (1976) have suggested that these processes may play a role in inter-cellular communication.

3.5.2. Fine Structure of the Late Cleavage Blastoderm

A number of changes occur in the blastoderm after eighth cleavage. One of the first, which is evident even in thick epon sections, is a marked increase in the RNA content of the presumptive middle-layer cells. The difference in RNA content between blastoderm and presumptive middle-layer cells is seen by electron microscopy as a difference in numbers of ribosomes per cell (PLATE XIX, Fig. 1).

Subsequent to ninth cleavage the blastomeres in the outer layer of the blastoderm begin to develop more structured intercellular junctions. The initial junction observed is the belt desmosome (Zonula Adherens). These junctions appear between cells at their apices and form a stable bond surrounding the apex of each cell (see Staehelin, 1974). The continuity of these junctions was confirmed by observation of serial sections of blastoderms at this stage. At first the membranes in the area of junction formation display an apparent thickening as an electron dense material is deposited on their cytoplasmic faces (PLATE XVIII, Fig. 1). At the same time there is an accumulation of a finely granular material between the membranes. At this time the intermembrane distance ranges from 20 nm to 25 nm. In some cases microfilamentous material is seen associated with the membranes in the area of junction formation.

PLATE XIX

Germ Layer Segregation

Fig. 1. Micrograph of the margin of the blastoderm during germ layer segregation. This illustrates the leading lamellipodium of an overlapping cell. The overlapping cell (OLC) is less electron opaque due to a lower ribosome and membrane content. The overlapping lamellipodium extends over the presumptive middle-layer (ML) making non-junctional contact with those underlying cells. 3,300 X.

Fig. 2. Tip of lamellipodium (boxed area of Fig. 1). The lamellipodium makes a 20 nm non-junctional contact with middle-layer cells. Lamellipodia typically contain a fine fibrous cytoplasmic network and arrays of 70 nm to 100 nm vesicles (V). The cortex of the middle layer displays a network of microfilaments in the area of the leading lamellar contact (arrows).

Fig. 3. High magnification micrograph of an adjacent section illustrating the leading lamella of the overlap cell and the cortical microfilaments in the middle-layer cell. Note the array of vesicles in the lamellipodium and the former area of contact (arrow).


10 µm
1
1 µm
2
3

Below the area of desmosome formation the intermembrane distance ranges from 10 nm to 20 nm. This region develops into a fairly extensive septate junction. The sequence of events with regard to septate junction formation in Loligo is the same as that described for sea urchins (Gilula, 1973). At first the membranes are separated by a variable intermembrane space, are not necessarily parallel, and are not bridged by septa (PLATE XVIII, Fig. 1). The intermembrane distance is progressively more constant at 15 nm as septa appear in increasing numbers. Septa are, in the electron microscope, seen as electron opaque bars which bridge the intermembrane space. They are about 5 nm thick, 15 nm in width, and appear between the membranes with a 15 nm center to center spacing (PLATE XVIII, Figs. 2 and 3). The maximum number of septa per junction was not determined definitely. However, in ninth cleavage embryos, as many as 30 bars were counted. This contrasts with the number which Gilula (1973) reports for sea urchins, but may reflect the condition early in junction development (Staehelin, 1974).

3.5.3. Fine Structure of the Overlapping Cells

Apart from their overall shape and possession of elongate cell processes, overlap cells appear similar to other blastomeres. Their centripetal (with regard to the geometry of the blastoderm) borders are rounded whereas the

opposite sides extend vegetally over the presumptive middle-layer cells as large tapered processes (PLATE XIII, Fig. 1; PLATE XIX). From the leading edges of these processes extend smaller lamellipodia which, in turn, possess fine filopodial processes. The filopodia make 20 nm non-junctional appositions with neighboring cells. The appositions of the overlap and middle-layer cells are in all aspects non-junctional. No tight or maculae adherens junctions were observed in cleavage or early gastrulation stage embryos.

Lamellipodia of overlap cells were found to range in length to about 15 μm and from about 0.25 μm to 0.50 μm in thickness. These lamellipodia contain few visible microfilaments and no microtubules were ever observed within such processes (PLATE XIX; PLATE XX, Fig. 1). Microfilaments exist as a fine network either dispersed within the microspikes or associated with the plasma membrane.

Overlapping cell lamellipodia form extensive non-junctional appositions with the underlying presumptive middle-layer cells as described above. Plate XX, Fig. 1 illustrates a lamellipodium with a terminal microspike. The leading edge of the lamellipodium is more hyaline than the rest of the cell. Multivesiculate bodies (MVB) are often found within the lamellipodia, but also frequently in the underlying cells in the area of overlapping cell contact. Multivesiculate bodies in cleavage stage embryos are

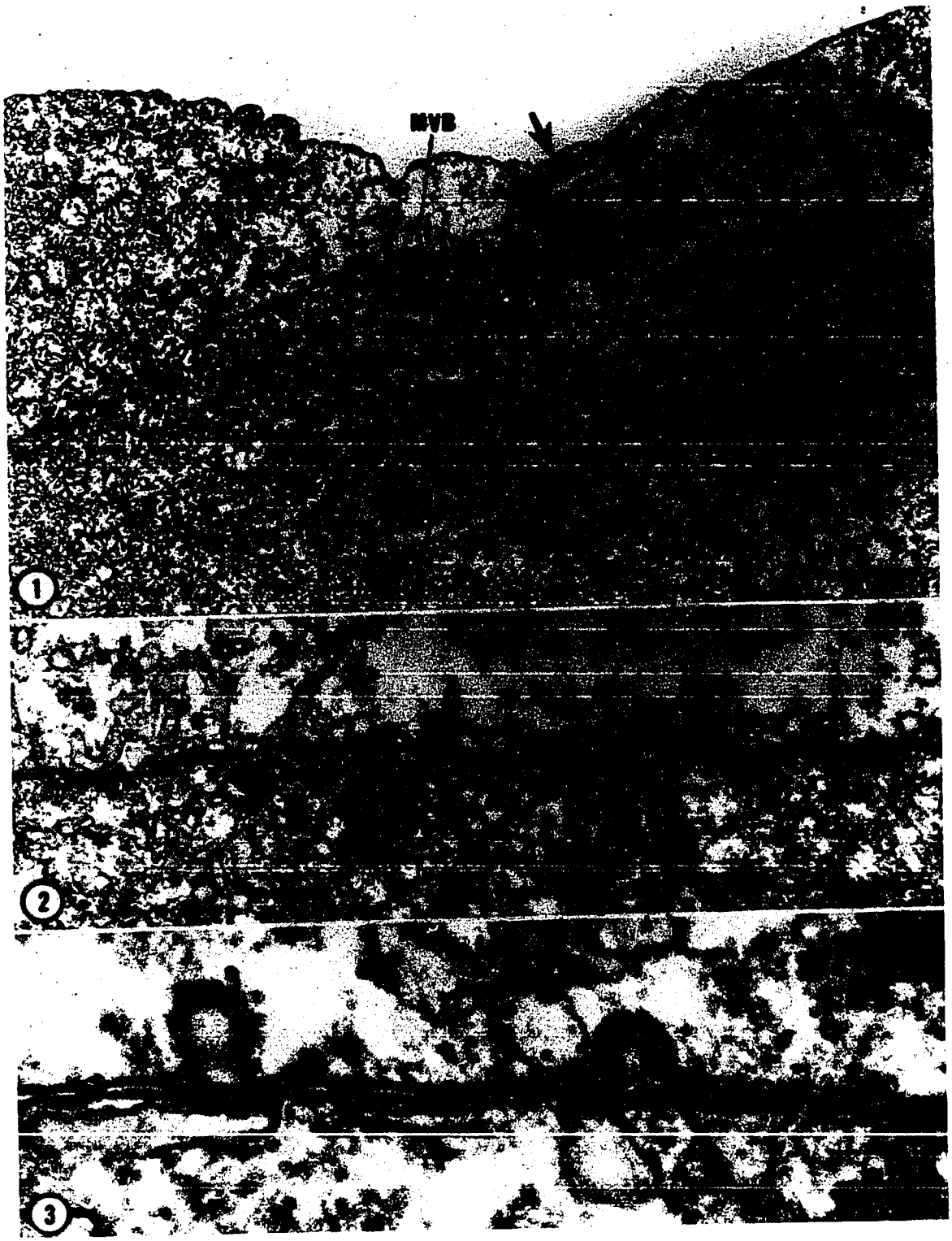
PLATE XX

Cell Overlapping

Fig. 1. Electron micrograph of an overlapping cell lamellipodium with terminal microspike. The lamellipodium is relatively hyaline whereas the microspike contains filaments and vesicles (arrow). The microspike extends from the margin of the lamellipodium and contacts the underlying presumptive middle-layer cell at several points along its length. A network of microfilaments is seen in the cortex of the middle-layer cell in areas of microspike contact (arrowheads). Multivesiculate bodies (MVB) are typical constituents of the lamellipodium cytoplasm. Membranous vesicles are typically seen along the non-junctional apposition of the overlap and presumptive middle-layer cells (double headed arrow). 8,700 X.

Fig. 2. Micrograph illustrating the association of membranous vesicles with the overlap cell membrane in the areas of apposition (Fig. 1 double headed arrow). Vesicles are 15 nm to 20 nm in diameter and usually closely applied to the cell membrane. The upper cell is the overlapping cell. Note the relative abundance of ribosomes in the middle-layer cell. 28,000 X.

Fig. 3. Micrograph illustrating the fusion of vesicles with the membrane in a section taken adjacent to that of Figs. 1 and 2 above. The vesicle at the left is tightly apposed to the cell membrane while the vesicle at the right has fused with the membrane. As with Fig. 2 above the overlapping cell is above in this figure. 127,000 X.


MVB
1
2
3

assemblages of 50 nm to 70 nm diameter membranous vesicles surrounded by another unit membrane. Although they are common features of late cleavage blastodermal cells, their function there is unknown at this time.

Filopodia typically contain numerous 70 nm to 100 nm membranous vesicles arrayed along their major axes (PLATE XIX, Fig. 3; PLATE XX, Fig. 1). The presence of these vesicles suggests a possible mechanism for cell membrane expansion in these actively extending processes. Bray (1973) has suggested from similar observations a mechanism for membrane assembly in neuronal growth cones. In growth cones vesicles are believed to be moved along the axon by means of a polarized actinomyosin system. The vesicles then fuse with the plasma membrane at the tip of the lengthening cell process. No evidence of vesicular fusion was observed in filopodia in Loligo blastomeres although arrays of vesicles were common.

Membranous vesicles are, however, often seen lying along and fusing with the overlap cell membrane in the area of contact with middle-layer cells somewhat centripetal from the leading edge (PLATE XX). These vesicles range in diameter from 150 nm to 200 nm and are lined along their inner surfaces by a slightly opaque material (PLATE XX, Fig. 2). This class of vesicles is not derived from multivesiculate bodies, but is more in the size range of Golgi produced vesicles. The 150 nm to 200 nm vesicles are

typically found tightly apposed to (PLATE XX, Fig. 2) or fusing with (PLATE XX, Fig. 3) the overlapping cell plasma membrane. The incorporation of these vesicles into the cell membrane in an area away from the expanding margin raises the question of the possible function of these vesicles. This question will be dealt with later in the discussion of overlapping cell movements

Microfilaments are seen only infrequently within the cytoplasm of overlapping cells. However, relatively dense arrays of microfilaments having a diameter of about 6 nm are found in the cortices of middle-layer cells in the area of their contact with overlapping cells (PLATE XXI). Plate XXI, Fig. 1 illustrates an overlap cell just prior to ninth cleavage. This cell is in mid-anaphase of mitosis, although the mitotic figure is out of the field in this micrograph. The leading edge of the overlap cell has broken its contact with the presumptive middle-layer cell and has retracted as the cell rounds up prior to cytokinesis. In the area of former contact (PLATE XXI, Fig. 1, bracket) the surface of the presumptive middle-layer cell is raised as several small papillae (PLATE XXI, Figs. 1 and 2, p). Within the cell cortex in this area is a network of 6 nm to 7 nm filaments (PLATE XXI, Fig. 2). These filaments appear to be arrayed in various directions although many are aligned along the axis of overlap (PLATE XXI, Fig. 3). This network of microfilaments varies in thickness, but

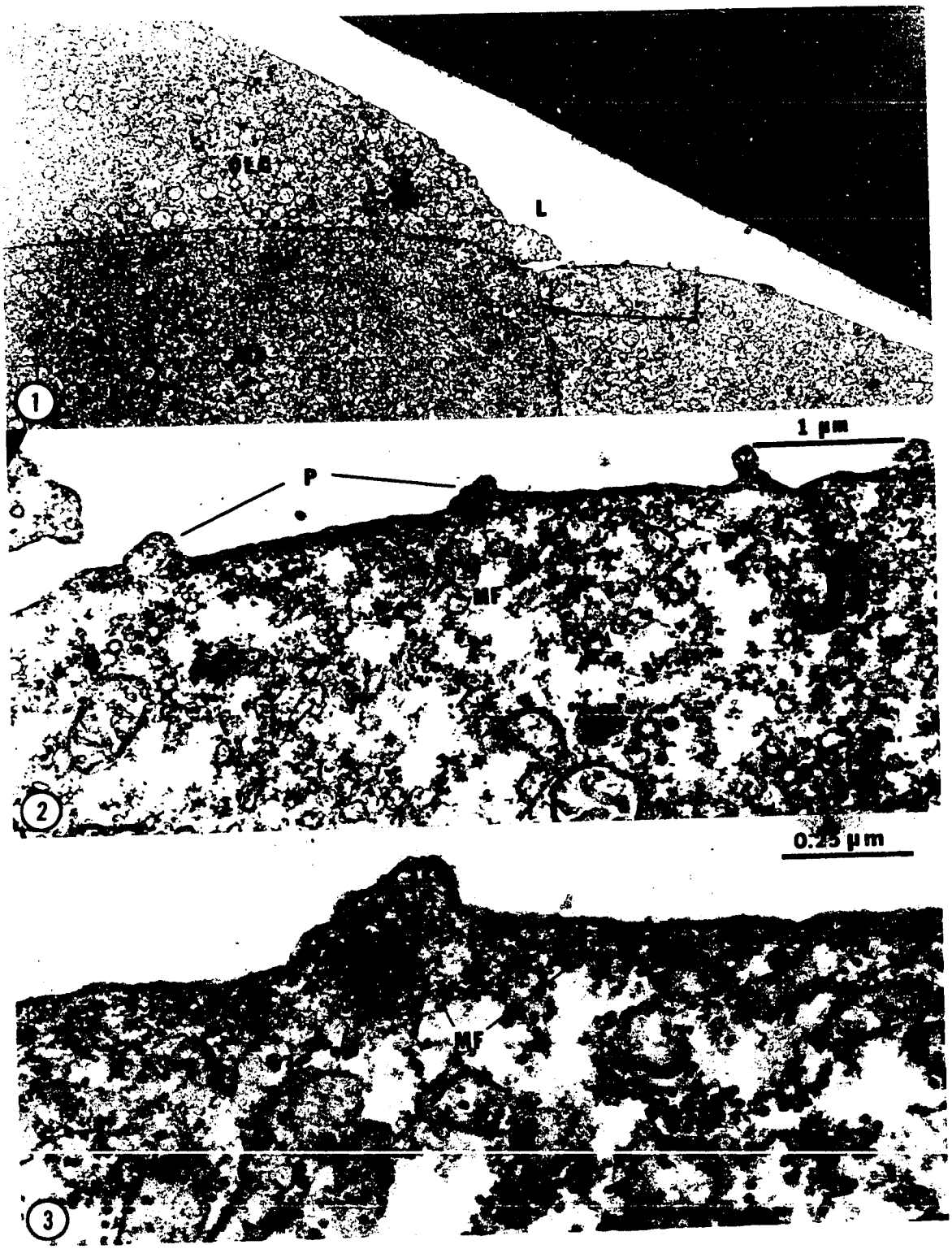
PLATE XXI

Germ Layer Segregation

Fig. 1. Overlapping cell. This micrograph illustrates an overlap cell (OLC) during mitosis. The mitotic figure is outside the frame to the left. The cell is in mid-anaphase. The marginal lamellipodium (L) of the overlapping cell (OLC) has retracted as the cell rounds up prior to cytokinesis. Note the area of the underlying middle-layer cell (ML) formerly in contact with the extended lamellipodium (bracket). Within this area are several short papillae or surface protrusions. The cortex in this area contains a network of microfilaments. 4,000 X.

Fig. 2. Higher magnification micrograph of the bracketed area of Fig. 1 showing in greater detail the surface papillae and the microfilamentous network (MF). The surface papillae are former points of contact with the overlap cell lamellipodium seen at the left. 24,600 X.

Fig. 3. High magnification micrograph illustrating the surface papillae and cortical microfilaments. This papilla contains several membranous vesicles and numerous filaments. The cortical microfilaments are 6 nm to 7 nm in diameter and appear to be oriented in all directions. However, numerous filaments can be seen running parallel to the axis of overlapping. 85,000 X.


L
1
1 µm
P
MF
2
0.25 µm
MF
3

averages about 140 nm thick. The microfilaments extend into the papillae which may also contain numbers of membranous vesicles (PLATE XXI, Fig. 3). Similar cortical arrays of microfilaments are seen in Plate XIX and Plate XX, Fig. 1.

3.5.4. The Secondary Epibolic Phase

Subsequent to segregation of the middle layer and establishment of the yolk syncytium, the blastoderm begins to spread over the yolk in a second phase of epiboly. The blastomeres produced by ninth cleavages of blastocones moved up onto the margin of the blastoderm during formation of the yolk syncytium. Shortly after eleventh nuclear division they begin epibolic spreading by extending broad, highly flattened lamellipodia (PLATE XXII, Figs. 1 and 2). These cells move across the underlying middle-layer cells and onto the yolk syncytium where they assume a position around the margin of the blastoderm (PLATE XXII, Fig. 3). There they continue to extend flattened lamellipodia and to spread vegetally. The lamellipodia produced by these cells are much broader and thinner than those produced by overlapping cells during middle-layer segregation (compare PLATE XIX and PLATE XXII). During somewhat later stages (Stages 11 to 12) these marginal cells spread out to become very flattened and polygonal in outline (PLATE XXII, Fig. 4). These cells continue to extend and retract their

PLATE XXII

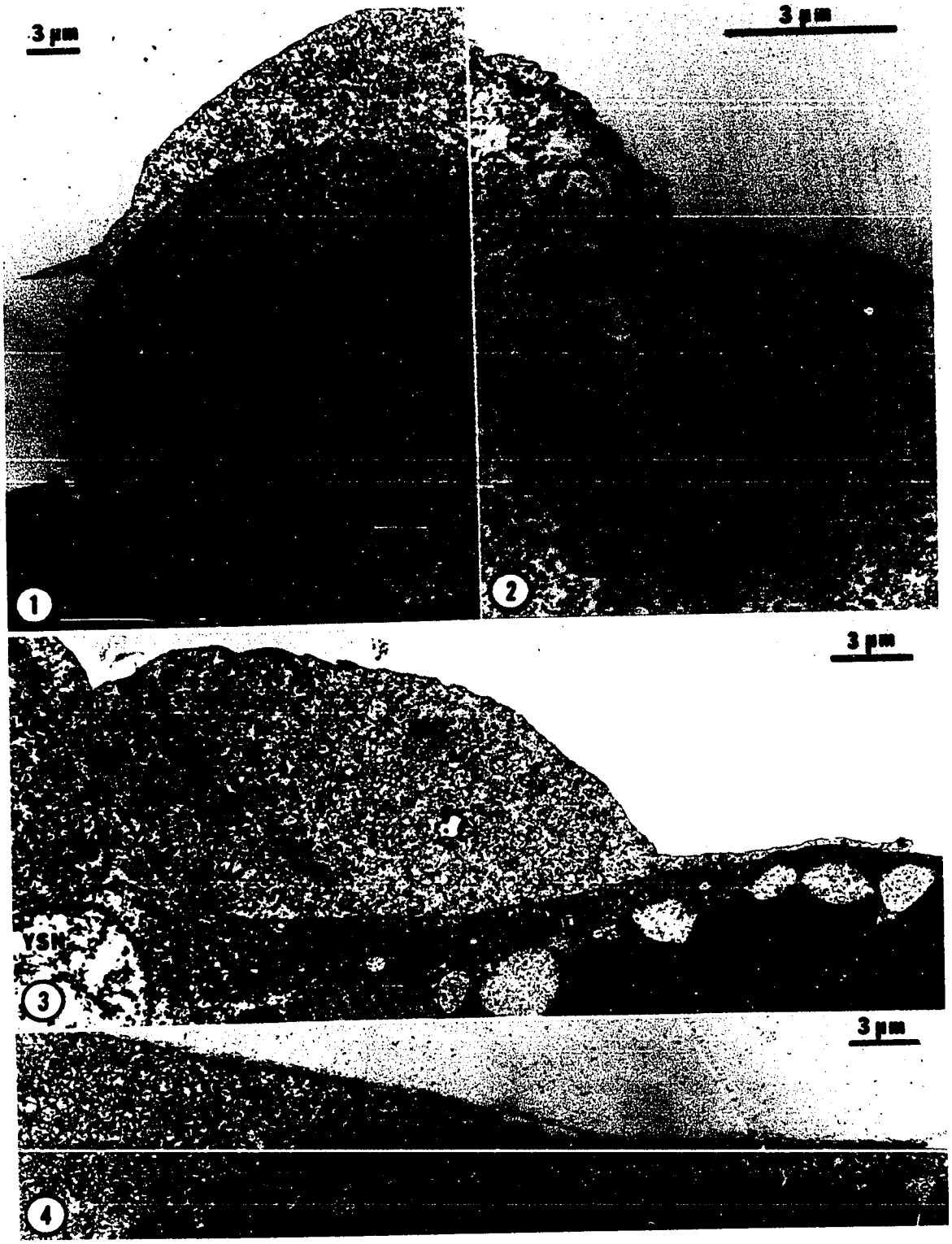
Stage 11 and 12 Epiboly

Fig. 1. Electron micrograph illustrating the first phase of epibolic spreading of the blastoderm (Stage 11). The marginal cells of the Stage 11 blastoderm extend lamellipodia that are usually thinner and broader than seen during overlapping. These cells move over the middle-layer cells and onto the yolk syncytium where they constitute the leading margin during epiboly. The lamellipodia often extend away from the middle-layer cells and into the intrachorionic space as illustrated here. Note the presence and location of microfilaments (MF) in the cortex of the middle-layer cell (ML). 2,700 X.

Fig. 2. Micrograph similar to Fig. 1 illustrating the initial phase of epiboly. Here the lamellipodium is much thinner than in the adjacent micrograph. Multivesiculate bodies (MVB) are typical components of the cytoplasm especially in the area of the lamellipodia. 9,200 X.

Fig. 3. Electron micrograph illustrating the margin of the blastoderm at Stage 12. The blastoderm is beginning its vegetal expansion. The marginal cells (MC) extend long lamellipodia which lie over the yolk syncytium. These lamellipodia establish no junctions with the yolk syncytium, but maintain a simple appositional contact. At this stage all yolk syncytium nuclei (YSN) lie beneath the blastoderm. 4,300 X.

Fig. 4. Micrograph illustrating another marginal cell lamellipodium at Stage 12 to 13. Here the lamellipodium is quite long (16.5 m) and thin (from 1.4 m at the base to 0.1 m near the tip). The epibolic spreading of the blastoderm constitutes the last phase of gastrulation. 3,700 X.


3 µm
3 µm
1
2
3 µm
YSN
3
3 µm
4

marginal lamellipodia throughout at least the initial phases of spreading of the blastoderm.

3.6. Experimental Inhibition of Cell Movement and Division

Embryos in various phases of Stages 9 and 10 were subjected to the inhibitors colchicine or cytochalasin B at several time/concentration combinations as outlined in Methods and Materials above. These inhibitors were used in an attempt to: 1) dissect the events of gastrulation as a means of determining the relative importance of the morphogenetic events of cell movement and cell division to the segregation of the middle layer; and 2) relate the basic mechanisms of cell movement in cephalopod gastrulation to theories of cell movement mechanisms in general.

3.6.1. Effects of Cytochalasin B

Time/concentration combinations greater than 0.2 μgm/ml for 30 minutes produced extensive short term and irreversible long term effects. High concentrations and/or extended treatment times completely inhibited normal development. Such treatments almost invariably yielded embryos consisting of a cap of cells situated at the apex of the yolk mass. The variation of long term effects in relation to the stage of application of short pulses of cytochalasin B has been analyzed by Arnold and Williams-Arnold (1974) and so will not be detailed here. Of greater concern are the effects of cytochalasin B on the early phases of gastrulation.

Tne period from sixth cleavage through germ layer segregation to the beginning of epiboly is encompassed by Arnold's (1965a) Stages 9 through 11. The major events of this period are: 1) the active overlapping of presumptive middle-layer cells; 2) ninth cleavage division, and tenth and eleventh nuclear divisions; 3) establishment of the yolk syncytium by means of coordinated nuclear migration and blastocone furrow retraction; and 4) epibolic cell movements. It therefore appeared necessary to determine more specifically the stage of development of embryos at the beginning of treatment in order to accurately assess the effects of the inhibitors. The effects of cytochalasin (as well as colchicine) differed markedly depending on the specific morphogenetic stages of embryos at the time of application. Data on cytochalasin effects presented here are therefore restricted to the effects of 0.2 μgm/ml cytochalasin B applied for ten to thirty minutes at very specific embryonic stages.

3.6.1.1. Middle-Layer Segregation

Embryos at mid- to late eighth cleavage were treated with cytochalasin B at 0.2 μgm/ml for 10 minutes to determine the effect of this inhibitor on cell movements of overlapping. Within the first 5 to 7 minutes of treatment the visible detail of the cleavage furrows decreased almost to invisibility (PLATE XXIII, Fig. 2). At the same time, overlapping

PLATE XXIII

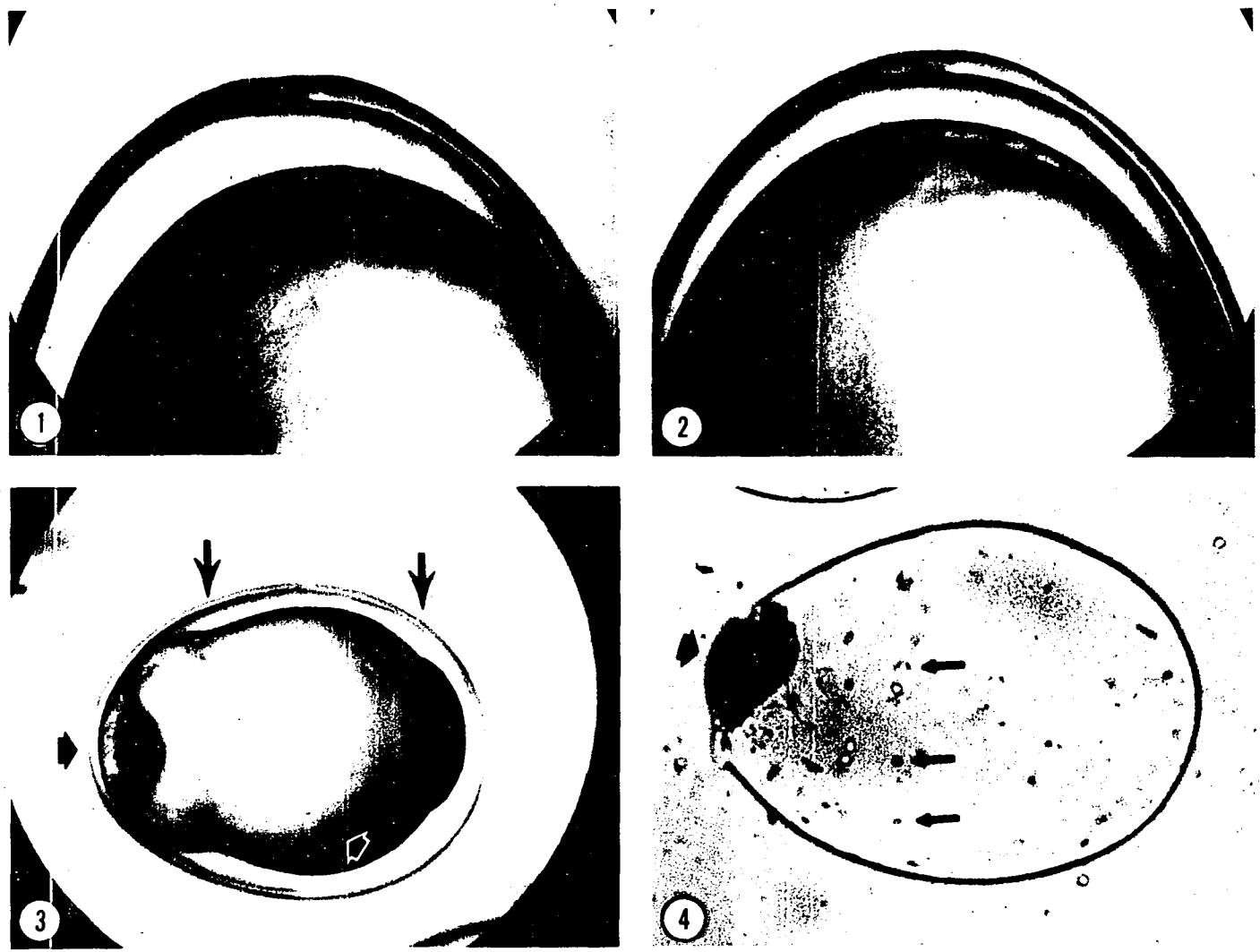
Effects of Cytochalasin B

Fig. 1. Normal mid-Stage 10 *Loligo pealei*. Blastomeres are distinct and the cleavage furrows are under tension. ca. 80 X.

Fig. 2. Immediate effects of cytochalasin B. This is a *Loligo pealei* embryo about 30 minutes after a 10 minute pulse with 0.2 μg/ml cytochalasin B at eighth cleavage. The blatomeres are indistinct and cleavage furrows are not easily seen. ca. 80 X.

Fig. 3. *Loligo pealei* embryo five days after treatment with 0.2 μg/ml cytochalasin B for 20 minutes at ninth cleavage. The blastomeres have collected as a clump of cells at the animal apex (broad arrow). Yolk syncytium nuclei can be seen as small depressions over the surface of the yolk (open arrow). Note the two rings of constriction (arrows). The vegetal ring is at the position of the blastoderm margin in the controls (Stage 17). ca. 37 X.

Fig. 4. FAA-Feulgen treated embryo from an experiment similar to that shown in Fig. 3. The clumped blastomere nuclei are visible at the animal apex (broad arrow). Yolk syncytium nuclei are seen arrayed in circumferential rings just above the equator of the egg and between these rings and the blastoderm (arrows). The specific staining of DNA confirms that the small depressions in the yolk in Fig. 3 are indeed yolk syncytium nuclei. Rings of constriction comparable to those seen in Fig. 3 were lost due to fixation procedure. ca. 45 X.


1
2
3
4

lamellipodia retracted. These effects were reversed upon removal of the cytochalasin. Furrows became more definite within 5 to 10 minutes and overlapping cell movements and cytokinesis were observed within 30 minutes.

Treatment of eighth cleavage embyros for 30 minutes with 0.2 μgm/ml cytochalasin B, in addition to the above effects, produced numerous multinucleate blastomeres. In some cases the multinucleate character of the blastomeres persisted and development was arrested. In most cases, however, the blastomeres did not remain multinucleate and the embryos developed normally to hatching. A few cases were also noted in which development appeared normal, but the organs were reduced in size (e.g. see Arnold and Williams-Arnold, 1974; Fig. 6, Class 2).

Examination of cytochalasin treated embryos by electron microscopy revealed that the microfilamentous bands at the bases of cleavage furrows became disassociated from the furrow membranes. The furrows retracted toward the surface of the blastomeres and the microfilamentous bands took the form of irregular masses of granular material (PLATE XXIV, Figs. 1 and 2). Furrow retraction occurred with newly formed furrows and in lateral and undercutting furrows of blastocones, but not with completed furrows.

The short term effects of cytochalasin treatment of embryos at ninth cleavage were similar to those seen at

PLATE XXIV

Effects of Cytochalasin B

Fig. 1. Electron micrograph of the blastoderm surface of an embryo fixed after a 20 minute pulse of cytochalasin B at 0.2μgm/ml. The cleavage furrow in the cell at the center has retracted toward the surface and the microfilaments of the furrow base are found as a mass of primarily granular material (MF). Some filaments are still visible, however (see Fig. 2 below). The cellular debris lying above this cell is probably the remains of the longitudinal folds of plasma membrane which are usually found within the furrow base (see Arnold, 1969). Other than the breakdown of the contractile band of microfilaments and the resultant retraction of the furrow, the cells have the appearance of untreated cells. The nucleus (N) and cytoplasmic constituents appear as in control embryos. 6,000 X.

Fig. 2. Higher magnification of a portion of the cell surface of Fig. 1 above. The microfilamentous material (MF) appears as fine granules. However, some filaments are still present (arrow). Numerous multivesiculate bodies (MVB) are seen associated with this area. Note also the appearance of the remnants of the longitudinal folds. 24,300 X.

Fig. 3. Light micrograph showing the effect of localized treatment with cytochalasin B at Stage 12. This is an embryo about 20 hours after a localized 10 minute pulse of 2.0μgm/ml cytochalasin B. The embryo is now at about Stage 13, but development appears to be delayed generally. The middle layer is not yet complete such that the central yolk papilla (YP) is still present. In the treated area the blastoderm has not spread in concert with the nuclear migration of the yolk syncytium. In this area the marginal cells are not even with the edge of the remainder of the blastoderm (B). The yolk syncytium nuclei (YSN) are thus not covered by the blastoderm in this area. The line of constriction is weakly evident. ca. 17 X.

1
2
3

eighth cleavage; that is, furrows and overlapping lamellipodia retracted. Long term effects were somewhat varied. In most cases 10 minute treatment with 0.2 μgm/ml cytochalasin produced minimal long term effects. Approximately 12% of the embryos treated at this stage developed organs of reduced size (see Arnold and Williams-Arnold, 1974; Fig. 6, Class 2.)

Treatment of ninth cleavage embryos for 30 minute at 0.2 μgm/ml had more severe long term effects. In this case the lateral and undercutting furrows of blastocones retracted to a greater degree than with a ten minute treatment. These furrow retractions released the blastocone nuclei into the yolk cytoplasmic layer, prematurely establishing the yolk syncytium. These effects were not reversed upon removal of the cytochalasin. The yolk syncytium nuclei eventually moved vegetally around the yolk mass while the blastomeres formed a clump of cells at the animal apex of the egg. (PLATE XXIII, Figs, 3 and 4). The time course of vegetal movement of the yolk syncytium nuclei correlated well with the time course of spreading of the blastoderm over the yolk.

3.6.1.2. Yolk Syncytium Formation

Treatment of embryos with cytochalasin B at 0.2 μgm/ml during tenth and eleventh nuclear division had variable effects depending upon the specific stage of the embryo at

the time of treatment and upon the length of treatment. The immediate effects of treatment at tenth division were similar to those at other stages. Furrow definition was reduced and newly formed furrows relaxed (PLATE XXIII, Fig. 2; PLATE XXIV, Figs. 1 and 2). In addition, post mitotic migration of the yolk syncytium nuclei was inhibited and the lamellipodial cell processes of marginal cells were retracted. Subsequent to the removal of the cytochalasin after ten minutes treatment the yolk syncytium nuclei moved up under the margin of the blastoderm, as well as vegetally, and the blastoderm renewed epibolic spreading. In most cases these embryos developed normally. After thirty minutes treatment, however, the yolk syncytium nuclei moved in a vegetal direction only and the blastoderm did not renew its epibolic spread. The blastomeres of these embryos eventually formed a mass of cells at the animal apex as in the case of extended treatment of ninth cleavage embryos (see PLATE XXIII, Fig. 3). The yolk syncytium nuclei continued to move vegetally preceded by a line of constriction that moved vegetally on a time course identical to that of the margin of the blastoderm in control embryos. Plate XXIII, Fig. 3 illustrates such an embryo when the controls were in Stage 17 (Arnold, 1965a). The yolk syncytium nuclei have a distribution similar to that of a normal embryo at Stage 17. The line of constriction at the vegetal pole corresponds to the position of the blastoderm margin at Stage 17.

Attempts were made to discover the cellular basis of the line of constriction using transmission electron microscopy. The results obtained were inconclusive. However, spontaneously occurring abnormalities morphologically similar to the above result of cytochalasin B treatment occur infrequently in spawns of "end of the year" females. Observation of these abnormal embryos with the transmission electron microscope revealed bands of microfilaments in the cortex of the yolk cytoplasmic layer in regions of circumferential constriction similar to those of cytochalasin treated embryos (not illustrated).

In an initial attempt to identify the relationship between the ring of constriction and the spreading of the blastoderm after middle-layer segregation, an experiment was performed in which four Stage 12 embryos were regionally treated with cytochalasin B by the method of Arnold and Williams-Arnold (1976). In all embryos treated by this method a "cleft" formed in the margin at the point of application (PLATE XXIV, Fig. 3). In the treated region, spreading of the blastoderm was inhibited while the remaining portion continued to spread. In contrast to the inhibition of blastomere movement of yolk syncytium nuclei was not inhibited and the nuclei appeared to migrate normally. The constriction did not appear as prominent in the treated area. Embryos treated in the above manner developed to what approximated Stage 15 then became very abnormal and died.

In summary, the effect of cytochalasin B during middle-layer segregation is that of inhibiting cell movements of overlapping. During the formation of the yolk syncytium, cytochalasin B inhibits nuclear movements and the cell movements of epibolic spreading.

3.6.2. Effects of Colchicine

Embryos of Loligo pealei were treated with colchicine at a concentration of 10^{-4}M during various phases of Stages 9 through 10. Colchicine treatment totally inhibited mitotic division, an effect which was not reversible. Colchicine does not inhibit cell motility so was used here to differentiate, by contrast with cytochalasin effects, the relative functions of cell division and cell movement in middle-layer segregation.

Embryos treated with colchicine at seventh to ninth cleavage developed two-layered blastoderms, but did not develop further and eventually died (PLATE XXV). Colchicine treatment inhibited all divisions so that the blastomeres remained large in comparison with control embryos. Active cell movements were not inhibited and overlapping of certain marginal cells eventually placed these cells between the overlapping cells and yolk cytoplasmic layer and resulted in a two-layered blastoderm (PLATE XXV, Figs. 1 to 5). Embryos treated with colchicine up to sixth cleavage did not develop two-layered blastoderms. In embryos treated at tenth or eleventh division, a two-

PLATE XXV

Effects of Colchicine

Fig. 1. This is a late cleavage (ca. eighth) L. pealei embryo prior to treatment with 10^{-4}M colchicine. The blastoderm is a single layer of cells. ca. 120 X.

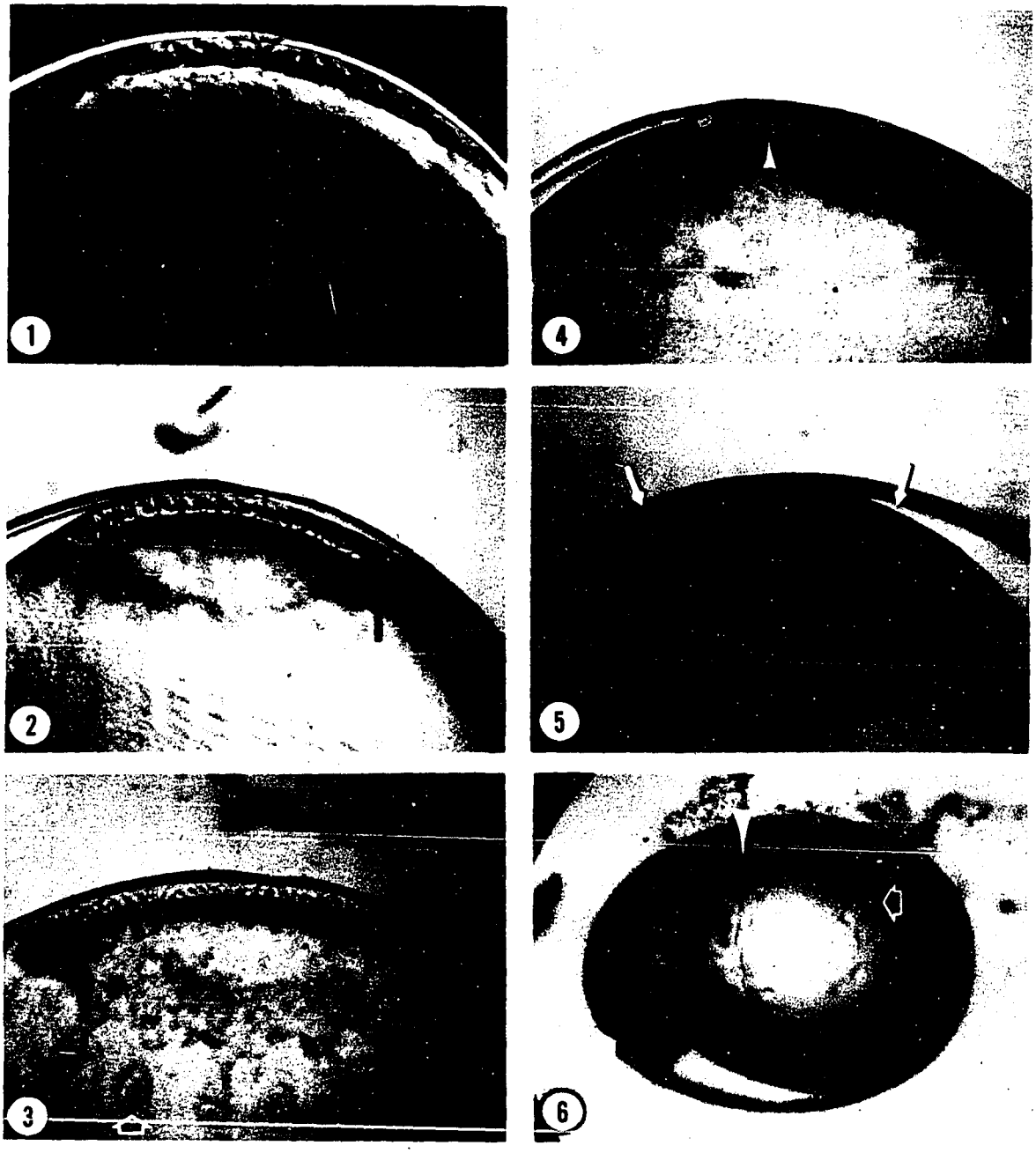
Fig. 2. Loligo pealei embryo seven hours and fifteen minutes after a twenty minute pulse with 10^{-4}M colchicine. Note the large size of the blastomeres and that the blastoderm is bilayered at its margin (arrow). The ring of cells in the middle area has created a depression in the yolk. ca. 120 X.

Fig. 3. Control for Fig. 2. Normal embryo seven hours and fifteen minutes after the time of treatment of the embryo in Fig. 2. Note that the blastomeres are much smaller than those in Fig. 2 and that a ring of cells has been produced in the middle area at the edge of the blastoderm. Note the yolk syncytium nuclei forming (open arrow). ca. 120 X.

Fig. 4. Control embryo after nineteen hours. This embryo is now in late Stage 11. The middle layer is well formed and has produced a large yolk papilla (arrowhead). ca. 120 X.

Fig. 5. Colchicine treated embryo after nineteen hours. Note that the middle-layer cells have become flattened (arrows). The embryo has undergone little morphological change since T=7:15 (Fig. 2). ca. 120 X.

Fig. 6. Loligo pealei embryo about seven days after treatment with 10^{-4}M colchicine at eleventh nuclear division. Most of the blastomeres have piled up at the animal apex (solid arrow). The yolk syncytium nuclei can be seen as small depressions scattered over the yolk surface (open arrow). Note the ring of contraction seen in the yolk surface (arrowhead). ca. 37 X.


1
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layered blastoderm developed and the yolk syncytium nuclei formed prior to treatment moved vegetally around the yolk mass (PLATE XXV, Fig. 6). The blastoderms of these embryos eventually formed a clump of cells at the animal apex and the yolk syncytium nuclei became evenly distributed throughout the syncytial cytoplasm.

4. DISCUSSION AND CONCLUSIONS

4.1. Cleavage: Establishment of the Blastoderm

The description of cleavage in Loligo pealei presented here confirms and expands upon the previous descriptions of Watase (1891) and Arnold (1965, 1971). Previous descriptions of development of this species have followed cleavage only as far as seventh cleavage. I have expanded upon the earlier descriptions to include eighth and ninth cleavages as well as the two divisions subsequent to the onset of gastrulation. The principal purpose for extending the description of cleavage is to detail the precise timing and events of the first phase of gastrulation.

The first through the eighth cleavages divide the original blastodisc into progressively smaller cells producing what is termed the blastoderm. This term is somewhat misleading because no true blastula is formed in cephalopods (Arnold, 1971). Some writers have attempted, however, to find structures in cephalopod embryos that are homologous to structures in embryos of other organisms.

Fioroni (1974), for example, insists that a space (Subgerminalhole) exists between the blastoderm and the yolk cytoplasmic layer in cephalopods which corresponds to the blastocoel of other molluscs. I feel, however, that the space seen by these writers is an artifact of fixation. The marginal blastomeres are more tightly adherent to the underlying layers than those of the central part of the blastoderm as is seen by dissecting away the outer layer of the blastoderm. Only the marginal cells adhere so tightly that attempts to remove them result in the rupture of their membranes. This marginal adhesion probably allows the blastoderm to act as a small osmometer. In a hypo-osmotic fixative the small intercellular space between the blastoderm and yolk syncytium would be inflated. On the other hand, the term blastoderm is useful as well as descriptive and is retained.

In addition to being the end of cleavage in the strictest sense of the word, eighth cleavage produces those cells which will give rise to the middle layer (presumptive middle-layer cells). The group of presumptive middle-layer cells is comprised of a population of 48 to 52 blastomeres arrayed in a ring near the perimeter of the blastoderm. The onset of gastrulation in Loligo is signaled by the beginning of an overlapping of these presumptive middle-layer cells by the population of cells in the centripetally adjacent ring. In addition, the cytoplasm of the presumptive middle-layer cells

shows signs of differentiation.

The cytoplasmic differentiation of the presumptive middle-layer cells in Loligo appears by transmission electron microscopy as a progressive increase in the numbers of ribosomes, mostly as polysomes. In addition, the presumptive middle-layer cells exhibit a differential uptake of basic dyes beginning at eighth cleavage. While the stains used are non-specific and the increased staining cannot be attributed conclusively to an increase in RNA, the affinity of RNA for basic dyes is documented (see Pearse, 1968). The association of an increased rate of ribosomal RNA synthesis with the onset of gastrulation has been confirmed in a number of embryos (for review see Gurdon, 1974). For example, an increase in RNA content has been found coincident with gastrulation in the gastropod Ilyanassa by Collier (1968, 1975). Collier's data concern the total RNA content of whole embryos and thus does not represent the differentiation of a specific population of cells as implied by the increased staining and ribosome content seen here. While the observations presented here do not prove that a specific differentiation of these cells has occurred, they point out a potentially interesting avenue of inquiry.

4.2. Cell Division and Movement in Middle-Layer Segregation

Ninth cleavage divides the presumptive middle-layer cells in such a manner as to produce a ring of cells two cells wide between the blastoderm and the yolk cytoplasmic layer. This cleavage, along with the cell movements of overlapping described above, is one of the two major morphogenetic processes giving rise to the middle layer of cells. In addition, ninth cleavage plays an essential role in gastrulation in this species because it produces the nuclei which give rise to the yolk syncytium as well as the blastomeres which will become the leading edge of the blastoderm during the second phase of epiboly.

Ninth cleavage and the overlapping of presumptive middle-layer cells are both dynamic phenomena which have historically been observed only in static fixed and sectioned embryos. It is no doubt for this reason that the theories of delamination (Ussow, 1880; Sacarrao, 1953; Arnold, 1971; Fuchs, 1973) and immigration (Korschelt, 1892; Fioroni, 1974) have been upheld until now. Certainly the orientation and cleavage plane of the presumptive middle-layer cells at ninth cleavage, if seen in histological section, would prompt either of the above theories.

The pattern of cleavage in Loligo pealei is relatively consistent through ninth cleavage. Because of this, attempts were made to construct a cell lineage for this species. Two

lineages were constructed; one used the numbering system commonly used for spiralians (Conklin, 1907), and used by De Leo (1972) for the squid Sepiola rondeletii; the second lineage attempted to record the location and divisional relationships of blastomeres within each quadrant of the blastoderm. Both lineages were abandoned, however, because the number of cell divisions subsequent to cleavage and the presence of the middle layer prevent the clear determination of the fates of individual or even groups of cells. In addition, the intent of lineage construction has usually been to provide a comparative map of the "prelocation" (Lillie, 1895) of adult structures within the egg and early embryo. Cephalopods, however, have been shown to be regulative (Ranzi, 1932; Arnold, 1965b; Arnold and Williams-Arnold, 1974, 1976). Arnold and Williams-Arnold (1974, 1976) have shown that the organogenetic pattern exists as an inductive pattern in the egg cortex thus precluding mosaicism. For these reasons a detailed lineage is not presented here.

The time-lapse cinemicrographic observations of middle-layer segregation presented above show that the presumptive middle-layer cells are almost completely overlapped by the active extension of flattened processes from the adjacent ring of cells prior to ninth cleavage. Active overlapping of the presumptive middle-layer cells effectively places them between the yolk cytoplasmic layer and the remaining blastomeres. I have, therefore, shown that the segregation

of the middle layer is a result of active cell movements as Sacarrao (1953) suggested.

The appearance of active cell movements in Loligo with time-lapse is similar to the ruffling seen with time-lapse in cultured fibroblasts (Abercrombie, et al., 1970) and motile activities of epithelial cells in culture (DiPasquale, 1975a). The marginal cells of epithelial sheets in culture exhibit a variety of cell surface activities. These are (1) extension and retraction of filopodia; (2) extension and withdrawal of flattened lamellipodia; (3) extension of lamellipodia accompanied by filopodial activity; (4) ruffling of the lamellipodia; (5) blebbing; and (6) particle movement away from the leading edge of the lamellipodium (DiPasquale, 1975a).

The overlapping cells during active overlapping are in contact laterally, form junctions with centripetally adjacent blastomeres and spread as a coherent sheet of cells. This is a case of epiboly, the spreading of a sheet of prospective ectodermal cells over the middle-layer cells of the early embryo (see Trinkaus, 1969, 1976). Marthy (1976) has recently reported that, in Alloteuthis media, middle-layer segregation conforms to my earlier description (Singley, 1974) of epibolic overlapping during germ layer segregation in L. pealei. Preliminary studies of Euprymna scolopes indicate that middle-layer segregation occurs by a similar process (Arnold, et al., 1972 and unpublished observations).

In summary, time-lapse cinemicrography has provided the proper perspective for the study of gastrulation in cephalopods. The image of germ layer segregation in Loligo pealei as a dynamic process of epiboly involving active, oriented cell movements contrasts sharply with the historical concepts of delamination (Ussow, 1881; Sacarrao, 1953; Arnold, 1971) and immigration (Korschelt, 1892; Fioroni, 1974). In light of the evidence from time-lapse studies presented here, the delamination and immigration theories are no longer tenable.

4.3. Yolk Syncytium Formation

The results presented above provide a more thorough description of the formation of the yolk syncytium and some insight into the mechanisms involved. The data from time-lapse cinemicrography in particular confirm that the yolk syncytium is established as a result of the incorporation of blastocone nuclei into the yolk cytoplasmic layer as originally described by Vialleton (1888) and recently confirmed for several octopods (Fuchs, 1973; Fioroni, 1974). My studies have shown more specifically that in Loligo pealei these nuclei are derived by the mitotic division of blastocone nuclei preceding ninth cleavage.

Time-lapse cinemicrography further reveals that nuclei actively migrate out of the blastocones as meridional and undercutting furrows of the blastocones retract. The mechanism of this nuclear movement is unknown. Similar

forms of nuclear migration have been seen, for example, prior to pronuclear fusion in a sea urchin (e.g. Longo and Anderson, 1968), during early embryogenesis in insects (e.g. Zalokar and Erk, 1976), prior to mitosis of the secondary nuclei in Acetabularia (Boloukhère, 1970), during chick lens development (Zwaan, et al., 1969; Pearce and Zwaan, 1970), in virally induced syncytia from cultured kidney cells (Holmes and Choppin, 1968), and in periblast nuclei of Fundulus during epiboly (Trinkaus, 1951). Although microtubules have been observed associated with nuclei in some of these cases of nuclear migration (Longo and Anderson, 1968; Boloukhère, 1970; Holmes and Choppin, 1968), they do not appear to be required for nuclear movement in cases where this question has been tested experimentally (e.g. Kessel, 1960; Holmes and Choppin, 1968). Instead, nuclear migration in some of these systems appears to be microfilament (actin) mediated as judged by its sensitivity to cytochalasin B (e.g. Zalokar and Erk, 1976). I have shown here that the movement of blastocone nuclei after ninth cleavage is insensitive to 10^{-4}M colchicine, but is inhibited by 0.2µgm/ml cytochalasin B. This suggests that nuclear migration during yolk syncytium formation in Loligo pealei is mediated by an actin based contractile system.

It has been suggested that during the second phase of epiboly, yolk syncytium nuclei are continually produced by

division from the margin of the blastoderm (Korschelt, 1892; Arnold, 1971). I have found no evidence for such a derivation of yolk syncytium nuclei. In L. pealei there are 24 or 26 nuclei which migrate vegetally to form the yolk syncytium. Subsequent to this migration they undergo two mitotic divisions before coming to lie beneath the blastoderm. Thus the number of yolk syncytium nuclei at Stage 12 should be 96 or 104 as a result of these two mitotic divisions. The numbers of yolk syncytium nuclei counted in two FAA-Feulgen treated, Epon embedded, and serially sectioned embryos of Stages 11 to 12 were 93 and 98. A count of 89 yolk syncytium nuclei was found in a single Stage 16 embryo. Counts of numbers of yolk syncytium nuclei in whole-mounted FAA-Feulgen treated embryos of various stages all fall within the range of 99 to 103. My preliminary data suggest that the two mitotic divisions of the original 24 or 26 nuclei produce the entire complement of yolk syncytium nuclei and that no further mitotic divisions occur. The number of yolk syncytium nuclei present throughout development has not been documented for any cephalopod species. The figures presented here are preliminary and are used only as an illustration of the probable manner of yolk syncytium nucleus replication in L. pealei. In other species the number of nuclei produced is not necessarily the same as that found in Loligo pealei and the final number would necessarily depend on the number of nuclei

making up the initial population.

Fuchs (1973) has suggested that the yolk syncytium nuclei in the octopod Eledone increase in number by means of amitotic divisions. He based this proposition on the facts that, 1) he has never seen a mitotic figure within the yolk syncytium, 2) the shape of some nuclei were suggestive of an amitotic division, and 3) the close proximity of certain nuclei suggested a recent division. Certainly the yolk syncytium nuclei of Loligo have a shape that is quite variable and they are generally highly flattened in comparison with blastodermal nuclei (Arnold, 1971). In addition, some yolk syncytium nuclei seen in FAA-Feulgen treated embryos have an "amoeba-like" shape. This amoeboid form often gives the impression that amitosis is occurring and sections through individual yolk syncytium nuclei sometimes give the appearance of amitotic divisions. All of this, however, is only circumstantial evidence for amitosis. Amitosis to my knowledge is found normally only in the division of the macronucleus of some ciliate protozoans. The lone report I have found of amitosis occurring in a non-ciliate is that of Conklin (1903) which describes the amitotic division of ovarian follicle cells of a cricket.

During the initial two mitotic divisions of yolk syncytium nuclei a circumferential transitory furrow is formed as described above. The transitory nature of this furrow is essential to the maintenance of the syncytial

character of this cytoplasmic layer. The maintenance of the syncytial character of this layer is, in turn, essential to the subsequent movements of the nuclei within it. It is probably also essential to the layer's development as an embryonic digestive and circulatory organ (Boletzky, 1967; 1970; Arnold, 1971). The microfilamentous band formed in conjunction with transitory furrowing may also play a role in cell movement during the secondary epibolic phases as discussed below.

The stimulus for the formation of transitory furrows has not been investigated here. Formation of transitory furrows is, no doubt, influenced by asters during the mitotic divisions of the yolk syncytium nuclei. Rappaport (1974) has shown that the asters influence the localization and formation of cleavage furrows. Evidence of a similar astral influence during transitory furrow formation in Loligo is that the furrow deviates from horizontal in areas where mitosis is oblique to the dorsal-ventral axis of the embryo. Oblique divisions produce an undulation in the circumferential furrow.

4.4. Electron Microscopy: Questions of Fixation

In considering the results of observations of cell movement with electron microscopy it is necessary to recognize that some apparent cellular substructure may be the artifactual result of fixation. Differences in the degree of membrane vesiculation (Spooner, et al., 1974)

and the abundance of surface protrustions (Bard, et al., 1975) in motile cells have been attributed to differences in fixation. Fixations using osmium tetroxide, with or without prior fixation with glutaraldehyde, for long periods at room temperature and near neutral pH have recently been shown to cause the destructions of actin filaments *in vitro* (Maupin-Szamier and Pollard, 1977). It is thought that osmium cleaves the actin polymer into small peptides.

I have found some marked differences in the amount of cytoplasmic vesiculation present in *Loligo* and *Octopus cyanea* embryos depending on the type of fixation procedure used. While the use of glutaraldehyde as a fixative has gained wide acceptance in recent years and appears to produce the "best" fixation in numerous tissues, all glutaraldehyde fixation schedules I have used on embryos of *Loligo pealei*, *Euprymna scolopes*, and *Octopus cyanea* produced large numbers of apparently abnormal vesicles and some membrane distortion. In several cases the membranes appeared to have delaminated. Cacodylate buffered glutaraldehyde fixation of *L. pealei* embryos produced large amounts of amorphous, electron dense material in the cytoplasm. Colidine buffered glutaraldehyde in sea water (Method of Arnold, in press) produced moderately good fixation of a few cells within the blastoderm, but produced massive vesiculation in the remaining cells. The well-

fixed cells were all entering prophase of mitosis. This disparity of fixation within a single blastoderm is possibly the result of differences in membrane permeability which appears to vary through the cell cycle.

Veronal acetate buffered osmium tetroxide fixation (Palade, 1952) gave the most consistent and best overall results. The principal variability in ultrastructure with this procedure was encountered when the pH of the fixative was allowed to increase to or above 7.0. At higher pH there was more vesiculation and microfilaments were absent. This lack of microfilaments is consistent with the results of Maupin-Szamier and Pollard (1977). Fixation with the veronal acetate buffer system at pH 6.8 for no more than 10 minutes was found to be adequate.

4.5. Scanning Electron Microscopy

Results of scanning electron microscopy confirm and extend time-lapse observations that overlapping cell margins actively extend broad, flattened and filiform processes during blastodermal spreading. The morphology of motile cell processes produced by blastodermal cells during germ layer segregation in Loligo pealei closely resemble the processes in numerous other cell types both in vivo and in vitro (e.g. Nelson and Revel, 1976, 1975; Bard, et al., 1975).

In general, the results of scanning electron microscopy show that the overlapping cells during germ layer

segregation in Loligo pealei are specifically differentiated from the other blastomeres by their possession of flattened elongate cell processes which extend vegetally over the adjacent ring of presumptive middle-layer cells.

4.6. Transmission Electron Microscopy

4.6.1. Cleavage Blastoderm

The general appearance of the cleavage blastomeres in L. pealei is much like that of other embryos at early stages (e.g. Trinkaus and Lentz, 1967). The cytoplasm is unspecialized and contains a limited variety of organelles; one sees no rough endoplasmic reticulum for example. That, however, is not unexpected considering that in the normal time course of development protein synthesis is not "turned on" to a large extent until gastrulation (see Davidson, 1968). In addition, the blastomeres of early cleavage stages show no junctional specializations. A lack of junction formation during cleavage stages seems to be common in a number of other embryos as well (e.g. Lentz and Trinkaus, 1971; Gilula, 1973; Trelstad, et al., 1967).

During late eighth and early ninth cleavages the blastomeres begin to develop adherens and septate type junctions at their apices (for definitions see Staehelin, 1974). In sea urchins septate junction formation begins during the early blastula stage (Gilula, 1973) and in Fundulus desmosomes begin to form at the onset of epiboly

(Trinkaus and Lentz, 1971). It has been suggested that junction formation may be induced by the tension placed on the blastoderm during epiboly in Fundulus (see Trinkaus, 1976). The occurrence of a depression in the yolk beneath the ring of middle-layer cells in Loligo while the surface of the blastoderm remains smooth suggests that some tension exists in the blastoderm of this embryo also. Little is known about the development of junctions in invertebrates but the studies of Gilula (1973) on the sea urchin indicate that the morphology of junction development seen there is similar to that seen in Loligo.

4.6.2. Overlapping Cells

The ultrastructural morphology of overlapping cells in Loligo pealei as revealed by transmission electron microscopy is, in most respects, similar to that of other blastomeres with the exception that overlapping cells possess cell processes typical of motile cells (e.g. Trelstad, et al., 1967; Abercrombie, et al., 1970; Bard, et al., 1975). The lamellipodial and filopodial processes seen in Loligo, unlike those seen for example in chick endothelial cells (Bard, et al., 1975) or epithelial cells in culture (DiPasquale, 1975a and b), lack large numbers of oriented microfilaments and these processes in Loligo make no specialized contacts at their leading edges with underlying cells. The absence of visible microfilaments within active cell processes in Loligo may be

due either to the fact that few filaments are normally present or that the filaments present were degraded during fixation (Maupin-Szamier, and Pollard, 1977). With regard to the former possibility, evidence from mammalian tissue cells and the slime mold Physarum, for example, indicates that a major proportion of the actin present in the endoplasm is in the unpolymerized (G) form (Bray and Thomas, 1976; Isenberg and Wohlfarth-Botterman, 1976). Thus only a few filaments may be necessary to support these cell processes in Loligo.

In well-fixed embryos microfilaments appeared to have a specific distribution within overlapping and presumptive middle-layer cells. The concentration of microfilaments observed in the cortex of the presumptive middle-layer cells immediately below the area of contact with overlapping cell processes is comparable to observations of intermediate contact formation between fibroblasts (Heaysman and Pagrum, 1973; Lloyd, et al., 1976) and gap junction formation between Novikoff hepatoma cells (Johnson, et al., 1974). This distribution of microfilaments in Loligo has even greater similarity to the distribution of filaments seen in areas of contact between the yolk cytoplasmic and enveloping layers of Fundulus during epiboly (Betchaku and Trinkaus, in prep.). The specific localization of microfilaments in the cortices of cells in areas of cell contact is consistent with evidence of microfilament-mediated cell surface modulation

(e.g. Edelman, 1976). According to models of cell surface modulation, surface adhesive sites could be moved over the surface of cells by means of their trans-membrane attachment and interactions with actin filaments in the cell cortex. Surface modulation is discussed in somewhat greater detail below.

In general the results of transmission electron microscopy provide some insight into the cellular basis of cell movement during the phase of overlapping.

4.7. Experiments with Inhibitors

4.7.1. Cytochalasin B

The most immediate effect of cytochalasin B treatment was seen as a "smoothing" of the surface of the blastoderm and a loss of visible definition of the blastomeres (PLATE XXIII, compare Figs. 1 and 2). This effect is probably due to the effects of cytochalasin B on the actin-based contractile system of the cells. The role of actin in cell motility and morphology is becoming increasingly apparent (e.g. see Allen and Taylor, 1975). Experiments with cell extracts (e.g. Pollard, 1976; Stossel and Hartwig, 1976) and purified actin (e.g. Kane, 1975, 1976) indicate that the cellular activity of actin as microfilaments is, in part, a result of actin's ability to form contractile gels. In addition, filamentous actin appears to be most concentrated in the cortices of cells (Pollard and Weihing, 1974). This information has provided a solid

physical basis for the construction of models of cell movement (Taylor, et al., 1973; Allen and Taylor, 1975).

Cytochalasin B apparently acts at or near the surface of cells to interfere with the microfilaments located in the cortex (Tannenbaum, et al., 1975). Cytochalasin B injected directly into cells causes the disruption, but not the depolymerization, of microfilaments (Luchtel, et al., 1976). Recent evidence from *in vitro* studies implies that the action of cytochalasin involves the inhibition and/or reversal of gelation of the actin-based contractile system (Hartwig and Stossel, 1976; Weihing, 1976a). This inhibition appears to be directed against the interactions of actin filaments with other proteins in the cytoplasm (Hartwig and Stossel, 1976). Thus the loss of blastodermal definition observed as the initial reaction to cytochalasin B treatment in *Loligo* may be the result of a gel-to-sol transformation reducing the surface tension of the blastomeres. A loss of surface tension would, in turn, reduce the small intercellular spaces and surface curvatures of the cells that normally interfere with the transmittance of light in such a way as to make the cell boundaries more distinct. Thus light passing through the blastoderm of a cytochalasin B treated embryo would be subject to less bending and retardation. Taking into consideration a number of other possible explanations, this seems the most plausible.

Recent evidence indicates that cytochalasin B binds at the cell surface and inhibits glucose transport (e.g. Lin and Spudich, 1975), nucleoside transport (Plagemann and Estensen, 1972; Plagemann, et al., 1975), and may have other non-actin related effects (Holtzer and Sanger, 1972). Cytochalasin B is used here to more or less specifically inhibit cell movement over short periods of time. Although other effects cannot be ruled out completely, I would argue that transport effects are of no consequence in most of the experiements performed here. First, there is little if any glucose, other form of sugar or nucleoside in the surrounding medium, sea water, and nutrition for the embryo is provided by its yolk. Second, the effects of cytochalasin on cell movement in other systems were found to be unrelated to the inhibition of monosaccharide and nucleoside transport. (Yamada and Wessells, 1973; Taylor and Wessels, 1973).

4.7.1.1. Effects on Overlapping Movements

The effect of cytochalasin B on cell movement in Loligo pealei occurs rapidly and reversibly. When cytochalasin is applied during the early epibolic phase (cell overlapping) of gastrulation, activity of the cells' margins is inhibited and overlapping is stopped. In embryos where application is continuous from late eighth into ninth cleavage the marginal cells fail to migrate far enough to make contact with the yolk syncytium and thus fail to form their normal association with the vegetal migration of the yolk syncytium

nuclei. In such cases the blastoderm remains a single layer. The effect on cell motility is reversible, however, and the blastodermal cells later migrate to form a clump at the animal apex (PLATE XXIII, Figs. 3 and 4). This result indicates that formation of the early physical relationship between vegetal migration of the yolk syncytium nuclei and epibolic spreading of the blastoderm is essential for epibolic spreading to occur normally.

In contrast with the results of cytochalasin treatment, application of colchicine at eighth, or even seventh, cleavage does not inhibit formation of a middle layer of cells even though blastomeres can no longer divide. The results of treatment with these two inhibitors at seventh to ninth cleavage indicate that cell movement is the major morphogenetic process accounting for segregation of the initial population of middle-layer cells. On the other hand, cell division is essential for the completion of the middle layer.

4.7.1.2. Effects on Yolk Syncytium Formation

The effects of cytochalasin B on yolk syncytium formation as outlined above indicate that the nuclear migration observed by time-lapse cinemicrography is mediated by an actin-based contractile system within the syncytial layer. That migration is mediated by actin rather than microtubules is consistent with the fact that colchicine does not inhibit these nuclear migrations. The cytoskeletal or contractile

system controlling these nuclear migrations appears to be similar to that which is suggested to control streaming of the egg cytoplasm after fertilization and nuclear orientation and distribution during cleavage (Arnold and Williams-Arnold, 1970, 1974, 1976). As yet, however, there is no morphological evidence for such a system.

Vegetal migration of the yolk syncytium nuclei appears to be independent of epibolic spreading of the blastoderm. Note, however, as stated above, that the inverse of this is not true. Disruption of the initial phases of movement disconnects these two processes in such a way that while the nuclei continue to move vegetally the blastodermal cells move to the animal apex. Yolk syncytial nuclei are also found to move independently during gastrulation in Fundulus. Trinkaus (1951) has shown by removing the blastoderm of Fundulus that epibolic spreading of the yolk syncytium layer is independent of the spreading of the blastoderm.

The results of cytochalasin application are consistent with the idea that spreading of the blastoderm in Loligo, as noted above, is, in contrast, dependent on the vegetal migration of the yolk syncytium nuclei. In fact, yolk syncytium nuclei are always found beneath the margin of the spreading blastoderm during later phases of epiboly (Arnold, 1971). Also consistent with the idea of dependent blastodermal spreading is the fact that when the blastoderm is manipulated with fine needles the upper layer of cells

adheres to the middle layer and later to the yolk syncytium only at its margin. Similar marginal adhesion is evident in sheets of epithelial cells (chick) spreading in culture (DiPasquale, 1975a and b) and between blastoderm cells and yolk syncytium layer during epibolic spreading in Fundulus (Trinkaus, 1976).

Another phenomenon of interest in regard to the processes of epiboly is the migration of the circumferential ring of constriction seen in embryos after treatment with cytochalasin B or colchicine during tenth nuclear division. The base of this depression was found to possess a band of microfilaments (not illustrated here) similar in appearance to the band found beneath overlapping cells and at furrow bases. Similar bands have been seen in naturally occurring abnormal embryos. This band moved vegetally on a time course similar to that of the blastodermal margin in controls. The band of constriction appears to correspond to the margin of yolk syncytium during vegetal movement. The independent movement of this band of microfilaments and marginal adhesion of the blastoderm suggest that blastodermal spreading may be dependent on and coordinated by movement of the yolk syncytium. That is, epiboly may be controlled within the yolk syncytium.

In the few cases where it was attempted, application of cytochalasin B to a localized area of the margin of the blastoderm in a Stage 12 embryo produced a small area in which the margin of the blastoderm was disassociated from

the margin of yolk syncytial nuclear migration (PLATE XXV, Fig. 6). Disruption of even a small region of the blastoderm margin, although the embryos continued to develop for two or three days, totally disrupted gastrulation and normal development. The results of localized application are also consistent with the idea that epiboly is controlled by the yolk syncytium.

The long-term effects of short pulses of cytochalasin B on organogenesis have been dealt with by Arnold and Williams-Arnold (1974, 1976) and so will not be discussed here. In addition, while cytochalasin B treatment during the various phases of gastrulation produces major long-term effects, these effects have little relation to questions concerning the mechanism of middle-layer formation and gastrulation movements.

4.7.2. Effects of Colchicine

Colchicine has been used here to differentiate the relative roles of cell movement and cell division during middle-layer segregation. Thus the effect of colchicine as an inhibitor of cell division has been discussed above in relation to the effects of cytochalasin B on cell movement.

It has been argued that colchicine cannot be claimed to be a specific inhibitor of microtubular function because colchicine also inhibits nucleoside transport (Wilson, 1975). These two forms of inhibition would appear, however, to be separable by experimental design. The inhibition of

nucleoside transport in HeLa cells was found to be competitive (Mizel and Wilson, 1972). Therefore it could be argued that removal of colchicine from the culture medium should eliminate the inhibition of nucleoside transport. In the experiments performed with Loligo, colchicine was applied for only a short time, and application was followed by several rinses in fresh sea water. Treated embryos were then maintained with daily changes of fresh sea water.

The apparent irreversibility of the inhibition of mitosis in Loligo, during short-term experiments, is consistent with data on the rate of reversible dissociation of colchicine-tubulin complex in vitro. The rate of reversible dissociation of the complex as determined for porcine brain tubulin and sea urchin tubulin in vitro corresponds to a half-life of approximately 36 hours (Garland and Teller, 1975). The reactions of colchicine with tubulin in Loligo are, no doubt, more complex. However, given that the rates of reaction are similar for Loligo tubulin in vivo, the observed irreversibility of the block of mitosis might be explained by the slow rate of dissociation of the colchicine-tubulin complex.

Regardless of the rates of association and dissociation of colchicine with tubulin, my observations show that colchicine blocked mitosis over the experimental period without inhibiting cell movement. I feel that my use of colchicine

is not unjustified and that its use has served the purpose of the experiments.

4.8. A Theory of Epiboly in Cephalopods

A number of observations made here, when considered together, suggest a possible mechanism for the organization and control of epiboly in Loligo. The following is provided as an outline of a testable model of that mechanism. The proposed model consists of the following four major components (see also Fig. 5): 1. A circumferential band of microfilaments is located within the cortex of the yolk cytoplasmic layer, and also within the cortices of the presumptive middle-layer cells during the early phase of epiboly. 2. A loose network of actin-like or possibly other filaments exists within the cortices of the presumptive middle-layer cells and the cortex of the yolk syncytium in addition to the concentrated band of microfilaments. It is not essential that this network be present continuously. Filaments could form precursor pools in response to the presence of the overlying cells. 3. Adhesion of the blastoderm to the underlying layer is mediated by specific molecules (possibly glycoproteins) which transit the membrane such that they are exposed at the outer surfaces of cells. On the inner surface of the plasma membrane these molecules are attached either directly or via a secondary protein(s) to the microfilamentous band. The adhesive molecules may be concentrated in specific areas via this attachment to cortical filaments.

4. The marginal cells of the blastoderm are motile and produce lamellipodia and filopodia which extend and make contacts (non-junctional) with the above adhesive areas. This behavior occurs in a cyclical fashion.

According to this model, as the microfilaments of the circumferential band are assembled, they move adhesive molecules such that when the band of microfilaments is completed a band of adhesive sites is organized on the surface of the plasma membrane of the yolk cytoplasmic layer (or presumptive middle-layer cell). Interaction between the band of microfilaments and the loose network of filaments within the yolk cytoplasmic layer causes the band to move vegetally, simultaneously moving attached trans-membrane adhesive molecules. This vegetal movement of the microfilamentous band is, according to the model, controlled at the level of microfilament-membrane interaction. Movement may be due to a sliding of the circumferential band of microfilaments in relation to other cortical filaments (see Durham, 1974), a streaming of the cytoplasm resulting from a sol-gel-contraction sequence as proposed in models of amoeboid movement (see Allen and Taylor, 1975), or to a cortical contraction parallel to the cell surface (Harris, 1976). Whatever the precise mechanism, the essential point is that the band of adhesive sites is moved as a unit.

During vegetal movement of the band of adhesive sites marginal cells of the blastoderm would extend filopodia and

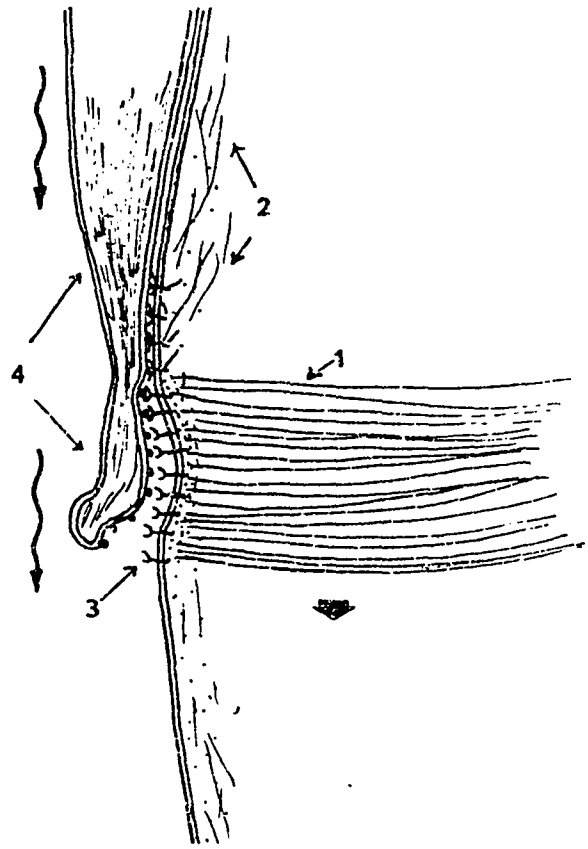

Fig. 5. A Model of Cephalopod Epiboly

The four basic elements of this model are: 1) a circumferential band of microfilaments (shown running perpendicular to the plane of the paper, but also shown turned into the plane of the paper); 2) a microfilamentous network located within the cell cortex; 3) adhesive molecules at the surface of the plasma membrane; 4) active cell extensions by the marginal cells of the blastoderm. As the filamentous band moves vegetally (broad arrow) the blastodermal margin is led in that direction as the cells extend, adhere and retract slender filopodia (wavy arrow). The surface adhesive sites transit the membrane and attach to the underlying filamentous band (3). These have corresponding reactive sites on the marginal cell surfaces. Interactions of the filamentous band with the cortical, filamentous network cause a vegetal movement of the band along with its attached adhesive molecules.

lamellipodia which would make discrete contacts with the adhesive sites, contract, and then disconnect. These events would occur in a cyclical manner across the membrane of the leading edge. During this activity the cytoplasm of the cells would flow into their motile processes yielding a net movement of the cells.

Thus as the band of adhesive sites moves vegetally, it would lead the blastoderm in the same direction. As the active marginal extensions retract locally the adhesive band would move forward in that same area. Re-extension of cell processes to the more vegetal location of adhesive sites in conjunction with cytoplasmic flow into the processes would move the cells forward to the same extent as the yolk syncytium.

The model of cephalopod epiboly which I have presented here is meant to suggest avenues of future research rather than as a representation of fact. Many aspects of this model are testable with currently available techniques. Others must await the development of new techniques. In general, however, this model attempts to portray epiboly in Loligo in terms of current knowledge cell movement.

4.8.1. Evidence for the Model; Literature on Biochemistry and Cell Movement

The model of cephalopod epiboly presented above is consistent with current information concerning functions of actin-like microfilaments in cell motility and cell surface

modulation. Actin in the form of microfilaments was first identified by Ishikawa, Bishoff and Holtzer (1969) using the heavy meromyosin binding technique. Actin filaments have now been identified in a vast array of animal and some plant species (see review of Pollard and Weihing, 1974). In many cell types, actin filaments are found to be especially prevalent in the cell cortex and in association with the plasma membrane (Pollard and Ito, 1970; Isenberg and Wohlfarth-Bottermann, 1976; Mooseker, 1976; Weihing, 1976b). Actin in its monomeric (G) form may also bind to the plasma membrane (Weihing, 1976b). Evidence further indicates that the association of actins with membranes is mediated by some secondary protein molecule or molecules (e.g. Mooseker, 1976; Tilney, 1976). Actin associations with certain heavy molecular weight proteins during the contraction of gelated cell extracts (e.g. Kane, 1975, 1976) and these associations are inhibited and reversed by cytochalasin B (Hartwig and Stossel, 1976).

Experiments with living cells, cell extracts and ectoplasmic ghosts of amoeboid cells (e.g. Taylor, et al., 1976; Taylor, 1976; Isenberg and Wohlfarth-Bottermann, 1976) indicate that the gelation and contraction of actin in the cortical cytoplasm may be the motive force of amoeboid movement (see Allen and Taylor, 1975). Such ideas are further reinforced by evidence indicating that pressure differences exist between discrete areas within the amoeboid

cell (see Allen, et al. 1971). The contraction of actin containing cytoplasm in the cell cortex is the basis for a number of models of amoeboid movement (see Allen and Taylor, 1975). Cell movement in developing systems must also, however, involve specific cell surface interactions (Trinkaus, 1976).

The model of cephalopod epiboly presented above relies on the fact that adhesive molecules (probably glycoproteins) transit the plasma membrane so that their amino-terminal, glycosylated sequences lie exterior to the membrane and the carboxy-terminal ends are internal and attach to a system of microfilaments. Evidence for a trans-membrane arrangement for glycoproteins is substantial. For example, the best established case is that for glycophorin. Glycophorin is the major glycoprotein of human erythrocytes and all evidence points to a trans-membrane arrangement (Segrest, et al., 1973). All of the carbohydrate containing amino-terminal sequences of glycophorin are found external to the cell and the carboxy-terminal end can be attacked enzymatically only after the inner surface of the membrane is exposed. Other glycoproteins also appear to have a trans-membrane arrangement. For example, certain HL-A antigens were found to consist of a hydrophilic region at the COOH-terminus and a penultimate hydrophobic region (Springer and Strominger, 1976). This molecular structure and the accessibility of the molecule to various reagents

strongly suggests a trans-membrane arrangement. Similarly, experiments on brush-border intestinal aminopeptidase suggest that this protein has a trans-membrane arrangement (Louvard, et al., 1976). During replication of Sindbis virus, nascent coat glycoproteins are sequestered by the lipid bilayer of host membranes (Wirth, et al., 1977). In the virus these glycoproteins are embedded in the virus' lipid bilayer envelope. Other evidence for the trans-membrane arrangement of glycoproteins is reviewed by Steck (1974) and Rothman and Lenard (1977).

Evidence of attachment of trans-membrane glycoproteins to cytoplasmic filaments has also been found in a number of cell types. For example, Ash and Singer (1976) have shown, with an immunofluorescent technique, that concanavalin A receptors in normal rat kidney cells form linear arrays 20 minutes after addition of the concanavalin A. These arrays of receptors were found superimposed on linear arrays of intracellular myosin-containing filaments. Other evidence for cytoplasmic-filament-membrane protein associations may be found in the reviews of Singer (1974), Nicolson (1976), and Edelman (1976).

Surface active sites such as antigens and concanavalin A binding sites are able to move laterally through the plasma membrane (e.g. Edidin and Weiss, 1972). The available evidence strongly indicates that the mobility of surface active sites is controlled via microfilaments and/or

microtubules linked to such sites at their cytoplasmic ends (for review see Nicolson, 1976; Edelman, 1976). Models attempting to explain surface site mobility propose a "surface modulating assembly" consisting of: 1) a set of glycoprotein receptors that transit the membrane and confer a specificity to the system; 2) various actin-like filaments and associated proteins are linked to the surface glycoprotein receptors at their inner faces and produce a coordinated movement of these surface active molecules; 3) microtubules provide anchorage of the receptors and allow the propagation of signals to and from the cell surface. The control of cell surface modulation may occur at the level of interaction between actin and other proteins (Edelman, 1976).

There is, in addition, circumstantial evidence from scanning electron microscope studies which suggests that cell contact may induce a form of cell surface modulation. For example, cell contact appears to induce the aggregation of intramembranous particles at sites of apposition (Scott, et al., 1973). This particle aggregation was found to be inhibited by very low concentrations of cytochalasin B. (Scott, et al., 1977).

Recent evidence suggests that cell motile behavior is controlled, in part, by the chemical behavior of specific cell surface glycoproteins and the chemical alterations which they undergo during cell-cell or cell-substrate

associations (e.g. Roth, 1973; Moscona, 1974; Burger, et al., 1975; Lloyd, 1975).

Trinkaus (1976) has recently formulated a theory of epiboly in Fundulus which is consistent with the evidence of the role of actin-like microfilaments in cell surface modulation and cell motility. Trinkaus and his coworkers have shown that epiboly in Fundulus occurs in two phases. During the first phase the margin of the enveloping layer is very active as revealed by time-lapse and the area of contact between this layer and the yolk syncytial layer is quite narrow. These observations are held to be consistent with the idea that the enveloping layer is actively spreading over the yolk syncytial layer during this phase. During the second phase on the other hand, the enveloping layer margin is quiescent and the contact zone becomes wider. Trinkaus (1976) suggests that the second phase does not involve active spreading of the cells, but that, on the contrary, the enveloping layer is being pulled passively over the autonomously spreading yolk syncytial layer. Other unpublished evidence is cited in support of this theory (Trinkaus, 1976). Trinkaus states, for example, that the leading edge of the enveloping layer shows surface activity during the first, but not the second phase. In addition, transmission electron microscopy reveals the presence of 5 nm to 7 nm filaments associated with the area of contact between the enveloping layer and the yolk syncytial layer. Trinkaus

(1976) discusses this model of Fundulus epiboly in relation to evidence for models of cell movement.

4.8.2. Evidence for the Model: A Study of Loligo

A band of microfilaments lying beneath the active edges of overlapping cells has been found with transmission electron microscopy. A similar band is also found in the yolk cytoplasmic layer immediately beneath the margin of the blastoderm during epibolic spreading. No other concentrations of microfilaments were observed.

Although no cortical meshwork of microfilaments was consistently observed, a lack of their presence is not inconsistent with the model presented here. This meshwork, according to the model, need consist of only a few filaments and would not necessarily be visible by electron microscopy.

No direct evidence for a band of adhesive sites has been presented here. Although surgical manipulation shows that marginal blastomeres adhere tightly to the yolk syncytium and presumptive middle-layer cells, adhesion cannot necessarily be attributed to a restricted band on the plasma membrane. In fact, the adhesiveness may reside instead solely in the surface of the marginal cell extensions.

The evidence presented above, from time-lapse cinemicrography and electron microscopy, shows that cell movement occurs during overlapping and in the marginal cells during spreading. This evidence is adequate proof of the

fourth component of the model presented here. Moving cells were observed to extend and retract filopodia and lamellipodia in a cyclical fashion and the degree of activity correlated with the rate of movement.

Evidence for the autonomous movement of the yolk syncytial layer in Loligo comes from studies with inhibitors presented above. That is, the migration of the yolk syncytial nuclei and the contractile band of microfilaments continues in the absence of its normal association with the blastoderm. This movement appears to be mediated by an actin-based system in that movement is inhibited in the presence of cytochalasin B.

In general the model presented here is consistent with the data on gastrulation in Loligo and with evidence for movement in other cells.

4.8.3. Suggested Further Investigation

The model of epiboly in cephalopods presented here suggests several areas for future investigation. Specifically, evidence is needed to show that the proposed adhesive sites exist and are different from other surface molecules. This might be revealed by the binding of specific ligands to the cells' surfaces. If the proposed sites are present, then it is of interest to reveal their means of attachment to the microfilamentous band if such exists. The morphology and manner of cell movement at the blastodermal margin need further elucidation. In

this regard a complete time-lapse cinemicrographic investigation of epiboly appears warranted. Finally, the mechanism of the control of vegetal movement needs clarification. The inhibitors colchicine and cytochalasin B could be used to answer some of the questions pertaining to this problem.

5. APPENDIX

5.1. A Reappraisal of Arnolds's (1965a) Staging Based on Observations from this Study

As a generalized description of gastrulation in cephalopods I propose the following description in terms of and as a revision of Arnold's (1965a) staging for Loligo pealei. Arnold's (1965a) stages are based on major morphological changes during embryogenesis and as such have long served as a guide in the study of cephalopod development. As information becomes available, however, it is clear that the original description is inadequate. Therefore, I have amended the descriptions of those stages to which the information contained in these researches pertains. The amended descriptions are as follows:

Stage 9. Sixth through eighth cleavages. Cleavage of the central blastomeres is largely asynchronous. Cleavage is the peripheral rings of blastomeres and in the blastocones is synchronous at sixth cleavage and develops as synchronous waves of division passing from anterior to posterior at eighth cleavage.

Stage 10. Ninth cleavage through eleventh nuclear division. After eighth cleavage the inception of middle layer segregation is signaled by the overlapping of a specific population of cells (the presumptive middle-layer cells). Ninth cleavage produces a ring of middle-layer cells by the division of these overlapped cells. After ninth cleavage the yolk syncytium (= yolk epithelium) is established by the immigration of the blastocone nuclei into the yolk cytoplasmic layer. The yolk syncytium nuclei undergo a mitotic division with the formation of a transitory furrow during tenth nuclear division. Eleventh nuclear division results in another doubling of the number of yolk syncytium nuclei and an increase in the number of middle-layer cells. The end of Stage 10 is marked by the complete superpositioning of the bilayered blastoderm over the population of yolk syncytium nuclei. The increase of middle-layer cells causes a depression in the yolk and produces a central yolk papilla above which the blastoderm consists of only the outer layer of cells.

Stage 11. The blastoderm commences the major epibolic phase coincidental with the completion of eleventh nuclear division. The middle-layer cells flatten and spread so as to complete the layer at the animal apex and thereby flatten the yolk papilla. The remaining blastocone daughter cells are incorporated into the blastoderm.

Stage 12. Completion of the middle layer of cells at the center of the blastoderm. The peripheral cells are greatly flattened, polygonal in outline, and have "ruffled membrane" extensions at their leading edges. These cells give rise to the external yolk sac as epiboly continues.

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