# MICROTUBULES IN THE FORMATION AND DEVELOPMENT OF THE PRIMARY MESENCHYME IN *ARBACIA PUNCTULATA*

## II. An Experimental Analysis of Their Role in Development and Maintenance of Cell Shape

LEWIS G. TILNEY and JOHN R. GIBBINS

From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138, and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543. Dr. Tilney's present address is Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania. Dr. Gibbins' present address is Department of Pathology, University of Sydney, Sydney, Australia.

ABSTRACT

To experimentally test the suggestion made in the preceding paper that the microtubules are involved in cell shape development during the formation and differentiation of the primary mesenchyme, we applied to the embryos two types of agents which affect cytoplasmic microtubules: (*a*) colchicine and hydrostatic pressure, which cause the microtubules to disassemble, and (*b*) $D_2O$, which tends to stabilize them. When the first type of agent is applied to sea urchin gastrulae, the development of the primary mesenchyme ceases, the microtubules disappear, and the cells tend to spherulate. With $D_2O$ development also ceases, but the tubules appear "frozen," and the cell asymmetries persist unaltered. These agents appear to block development by primarily interfering with the sequential disassembly and/or reassembly of microtubules into new patterns. The microtubules, therefore, appear to be influential in the *development* of cell form. On the other hand through a careful analysis of the action of these agents and others on both intra- and extracellular factors, we concluded that the microtubules do rather little for the *maintenance* of cell shape in differentiated tissues.

## INTRODUCTION

In the first paper in this series we demonstrated that the cells of the primary mesenchyme undergo a predetermined sequence of shape changes during their formation and subsequent differentiation. These form changes culminate in the production of a mesenchymal syncytium in which the $CaCO_3$ skeleton of the pluteus is deposited. At each stage in this sequence microtubules are distributed in the cytoplasm of the cells in such a way so that they could determine the shape of the cell and thus be responsible ultimately for the orientation and shape of the pluteus skeleton.

That microtubules are involved in the development of cell shape was suggested by Byers and Porter (1964) in the case of the elongation of the primordial lens cells of the chick. Subsequently, many investigators have speculated that these elements may act as cytoskeletal agents (Bikle et al., 1966; Fawcett and Witebsky, 1964; Porter et al., 1964; Silveira and Porter, 1964; de Thé,

1964; and many others), but little direct evidence of their function has been presented. It was not until recently that Tilney (1965) showed that the axopodia of *Actinosphaerium nucleofilum* were unable to reform if the microtubules were kept in the disassembled state with hydrostatic pressure (Tilney et al., 1966), low temperature (Tilney and Porter, 1967), or colchicine (Tilney, 1968). When these agents were removed, regrowth commenced, and microtubules were invariably present in reforming axopodia.

In order to demonstrate that in developing multicellular systems the microtubules are associated with the *development* of cell shape, we treated embryos of *Arbacia* at the appropriate stages of development with antagonists against microtubules, namely colchicine and hydrostatic pressure. If our contention is correct that the microtubules are involved in the determination of the shape of the cell at each developmental stage, then the development of the primary mesenchyme should cease at the stage at which these agents are applied to the embryos; by maintaining the microtubules in the disassembled state, the formation of the next cell shape in the predetermined sequence would be interrupted. If, on the other hand, a different type of experimental agent could be applied to the embryos, an agent which would stabilize the microtubules and prevent their disassembly and thus their reassembly into new patterns, arrest in development should occur as well. A substance fitting these requirements is $D_2O$ which has been demonstrated by Gross and Spindel (1960) to block mitosis in sea urchin and annelid eggs. They suggested that this agent "appears to act by 'freezing' or 'stabilizing' the spindles." More recently Marsland and Zimmerman (1965) have confirmed these observations on isolated mitotic spindles and have shown that $D_2O$ is antagonistic to the solating effects of hydrostatic pressure (Marsland and Zimmerman, 1963, 1965). Furthermore, Inoué et al. (1963) have demonstrated that the birefringence of the mitotic spindle increases in the presence of $D_2O$. They have suggested (Inoué et al., 1965) that this agent favors the polymerization of the microtubule monomers into microtubules. (It is now well known that the spindle fibers are largely composed of bundles of microtubules: see Roth and Daniels, 1962; Robbins and Gonatas, 1964; de Thé, 1964; Harris, 1962; Ledbetter and Porter, 1963).

Also included in this report is a discussion of the role of the microtubules in the *maintenance* of cell form. It appears that, unlike the situation in the protozoan *Actinosphaerium nucleofilum*, the microtubules in metazoan tissue and even in other protozoa may do rather little in the *maintenance* of cell form. Experiments were carried out in which the embryos were depleted of calcium which, in addition to causing the disassembly of most of the microtubules, breaks down other mechanisms involved in the maintenance of cell form.

## MATERIALS AND METHODS

### *The Organisms*

Eggs and sperm were obtained from *Arbacia punctulata* by the voltage method (Harvey, 1952). The eggs were fertilized and allowed to develop at 20°C to the appropriate stage, at which time they were collected and treated with one of the following agents. Some embryos from each batch were allowed to develop to the pluteus stage in order to be sure that the development of the batch was normal.

### *Colchicine*

Fresh solutions of colchicine were made up each morning before use. Blastulae and gastrulae were added to small Petri dishes which contained 10 ml of $10^{-2}$, $10^{-3}$, $10^{-4}$, or $10^{-5}$ M colchicine in seawater. They were studied with the dissecting microscope and compared to untreated specimens. When greater detail was desired, several organisms were placed on a glass slide, covered with a cover slip, and viewed with a compound microscope. Bright-field, phase-contrast, and polarization microscopy were used for observations on living material. Embryos were fixed 3 hr after the addition of colchicine.

### *$D_2O$*

Artificial seawater was made up using $D_2O$ rather than $H_2O$. 70% $D_2O$ was used for all experiments. As with the colchicine, organisms were added to small Petri dishes containing the $D_2O$ seawater.

### *Calcium-Free Seawater*

Calcium-free seawater was obtained from the Marine Biological Laboratory chemistry department at Woods Hole (Cavanaugh, 1956). The embryos at the desired stage of development were aspirated from the finger bowls in which they had been developing and were centrifuged gently to concentrate them. The natural seawater was drawn off, and the embryos were resuspended in calcium-free seawater. This procedure was repeated twice with the resuspension into calcium-free seawater

each time. Embryos were left in the calcium-free seawater the desired length of time, usually from 45 min to 1 hr. Longer periods than this resulted in complete breakdown of the embryo into free-swimming cells.

### *Hydrostatic Pressure (7000 psi)*

IN VIVO OBSERVATION OF THE PRESSURIZED CELLS: The pressure equipment employed in these experiments has been described by Marsland (1950). The observation chamber, which allowed the organisms to be viewed continuously, was modified slightly from the original design of Marsland. It consisted of two parts, each having a clear sapphire window $\frac{3}{16}$ in. thick and $\frac{5}{8}$ in. in diameter. When the two parts were connected together by bolts, the two windows were automatically aligned so that light could be transmitted from above, through the specimen which was placed in the space between the two windows, and on through the lower window. The embryos could then be viewed through the objective lens of an inverted microscope. The two parts were sealed by means of an O ring. The pressure to the chamber was supplied by an external hydraulic line attached to the assembled chamber.

FIXATION DURING COMPRESSION: The apparatus used for this procedure was attached in series to the observation chamber. It has been described by Landau and Thibodeau (1962). Fixation was carried out 45 min after the onset of compression.

### *Procedures for Electron Microscopy*

Embryos were fixed, in the case of the colchicine, $D_2O$, and calcium-free seawater experiments, by adding enough pretreated glutaraldehyde to the container of embryos and the experimental agent to make the final concentration 6%. The glutaraldehyde was first treated with $BaCO_3$, centrifuged to remove the excess $BaCO_3$, and then adjusted to pH 7.6 with 1 N NaOH. The glutaraldehyde was prepared as outlined just before fixation, because upon standing it becomes more acidic.

The embryos treated with hydrostatic pressure were fixed under pressure in 6% glutaraldehyde pretreated as outlined above.

In every experiment, organisms were fixed for 30 min, washed briefly in several changes of seawater, and postfixed for 30 min in 1% $OsO_4$ dissolved in 0.1 M phosphate buffer at pH 7.2. The specimens were embedded in Epon 812 (Luft, 1961) after rapid dehydration in ethanol.

Thin sections were cut with a diamond knife on a Servall MT2, Porter-Blum ultramicrotome, stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963), and examined with a Siemens Elmiskop I or a Philips 200 electron microscope.

## RESULTS

### *Colchicine*

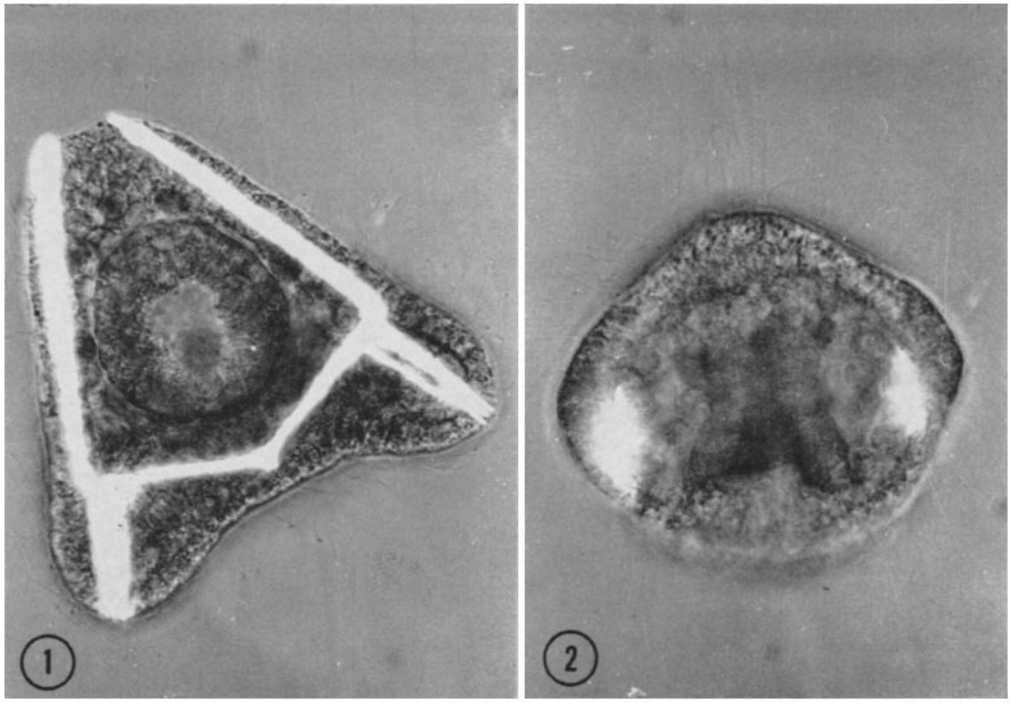
#### Effect upon Early Gastrulae

LIGHT MICROSCOPE OBSERVATIONS ON LIVING EMBRYOS: Alterations in the swimming behavior of the gastrulae are the first demonstrable effects of colchicine. Instead of swimming in a straight line as they rotate about their animal-vegetal axes, the embryos lose their capacity for directed movement but retain their rotary motion; in fact the velocity of the rotary movement increases. Coupled with the loss of directed movement is an apparent loss of orientation. No longer do the embryos lie parallel to the surface of the container; instead the animal pole makes contact with the substratum and spins around this point, simulating the behavior of a spinning top.

The immediacy of action of colchicine on motility is concentration-dependent. With the most concentrated solutions ($10^{-2}$ M) the effects upon motility are evident within 30 min, while at $10^{-5}$ M some embryos, even after 5–6 hr in this solution, still show some erratic linear movements. With longer periods in colchicine (up to 24 hr) the speed of the rotary movements gradually slows and finally ceases altogether.

When it is possible to compare the development of untreated with treated embryos, it is clear that colchicine causes a complete cessation of development. Not only does the archenteron fail to make oral contact, but also the inorganic skeleton discontinues its formation. The latter can be easily documented with the aid of a polarizing microscope (cf. Figs. 1 and 2). When the embryos are removed from the colchicine, the deposition of the skeleton recommences, but the skeletal pattern is abnormal and incomplete. The speed at which developmental arrest occurs is concentration-dependent. With concentrations as low as $10^{-5}$ M, normal development is inhibited, but it takes several hours to cease.

Unfortunately, in living *Arbacia* the mesenchymal cells are difficult to study due to the highly refractile yolk granules and echinochrome pigment granules in the ectodermal cells. In the transparent eggs of the sand dollar (*Echinarachnius parma*) where gastrulation is very similar to that of *Arbacia*, it is easy to observe on living embryos that colchicine causes a reduction in the number of mesenchymal processes and in archenteron




Figs. 1 and 2 are light micrographs taken with a Zeiss polarizing microscope. Both embryos were taken from the same batch of eggs which had been fertilized at the same time and were allowed to develop at the same temperature. They were both photographed 25 hr after fertilization. × 170.

FIGURE 1 Untreated embryo. The skeleton is well developed in this prism stage and is strongly birefringent. In the center of the embryo one can clearly make out the developing anal region.

FIGURE 2 This gastrula was treated for 3 hr with colchicine at a concentration of $10^{-3}$ M. At the time of application this embryo and the one depicted in Fig. 1 were at the same stage, midgastrula. As can clearly be seen, development appears completely arrested (cf. Figs. 1 and 2). Not only has oral contact not been established, but the birefringent calcium carbonate skeleton has not continued to be deposited.

height. The cells already associated into the cable when the embryos are placed in the colchicine do not separate, but those unconnected to each other at the time of application of the colchicine do not associate during treatment. Similar observations on *Arbacia* after colchicine treatment necessitate looking at stained sections from fixed and embedded embryos.

FINE STRUCTURE: In the first paper in this series the ectodermal cells of the blastulae and early gastrulae were described in some detail. It was found that in the apical end of the cell the microtubules converge towards the basal body of the cilium. The number and distribution of the microtubules in this region assured us that any section cut through the basal body would, by necessity, pass through microtubules (see Fig. 3 of preceding paper). Thus in this study the effect of colchicine on microtubules could be accurately determined by carefully examining this region. If portions of primary mesenchyme cells were examined, it would be more difficult to determine the action of colchicine on the microtubules, since in these cells the distribution of these elements is more complex. For reasons of convenience, therefore, the apical pole of the ectodermal cells was analyzed in some detail in order to determine the effects of this agent on the microtubules.

1. *The Ectoderm.* (a) *Cell shape:* The ectodermal cells assume a cuboidal form (Fig. 3). Unlike that of the untreated embryos, the center

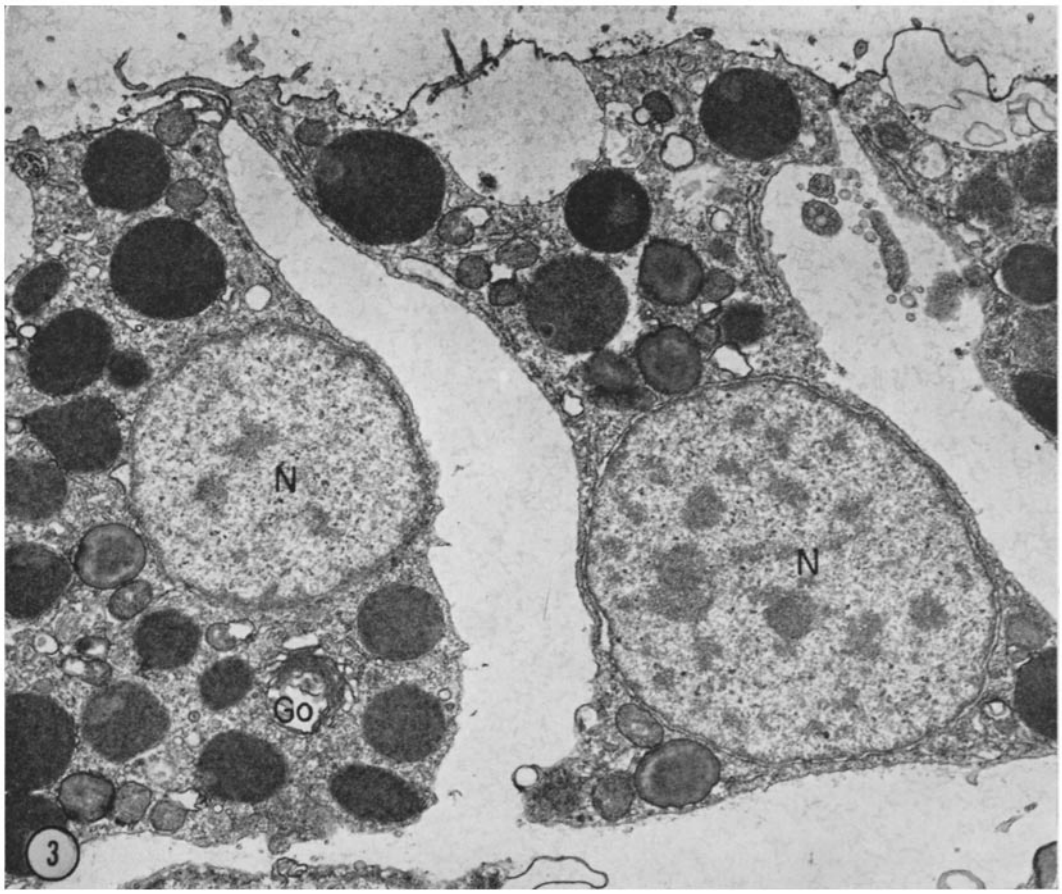



FIGURE 3 Section cut through several ectodermal cells of an early gastrula which had been treated with colchicine ($10^{-3}$ M) for 3 hr. Except for a slight reduction in cell height the over-all shape of these cells is not altered. Note the location of the nuclei (*N*) and the Golgi apparatus (*Go*) which in the cell on the left is basal to the nucleus. × 11,000.

of the apical surface of these cells is raised about a micron above the rest of the apical surface to make a "platform." In the center of this platform, which is circular and measures approximately 2 μ in diameter, is the basal body from which the cilium extends into the surrounding sea-water (Fig. 4). Short microvilli project from the lateral margins of the platform and from the apicolateral surfaces of the cell (Figs. 3 and 4).

(b) *Basement membrane, hyaline layer, desmosomes:* The basement membrane, the hyaline layer, and the septate desmosomes do not differ from those in untreated ectodermal cells.

(c) *Distribution of cytoplasmic constituents other than the microtubules:* The nuclei take on variable locations in the cell, sometimes basal other times apical (Fig. 3). The Golgi zone does not appear rigidly fixed in position either. Instead of consisting of a number of stacks of flattened cisternae arranged in the form of a cone whose apex points towards the base of the cilium, the Golgi complex now appears as a scattering of individual flattened cisternae; some are apical in distribution (Fig. 4), others are positioned laterally or even basally (Fig. 3). In the region normally occupied by the Golgi complex we frequently see yolk granules and mitochondria, constituents normally excluded from this region.

(d) *Microtubules, the basal body, and related structures:* The cilium, the basal body, its associated centriole, and rootlet system are not

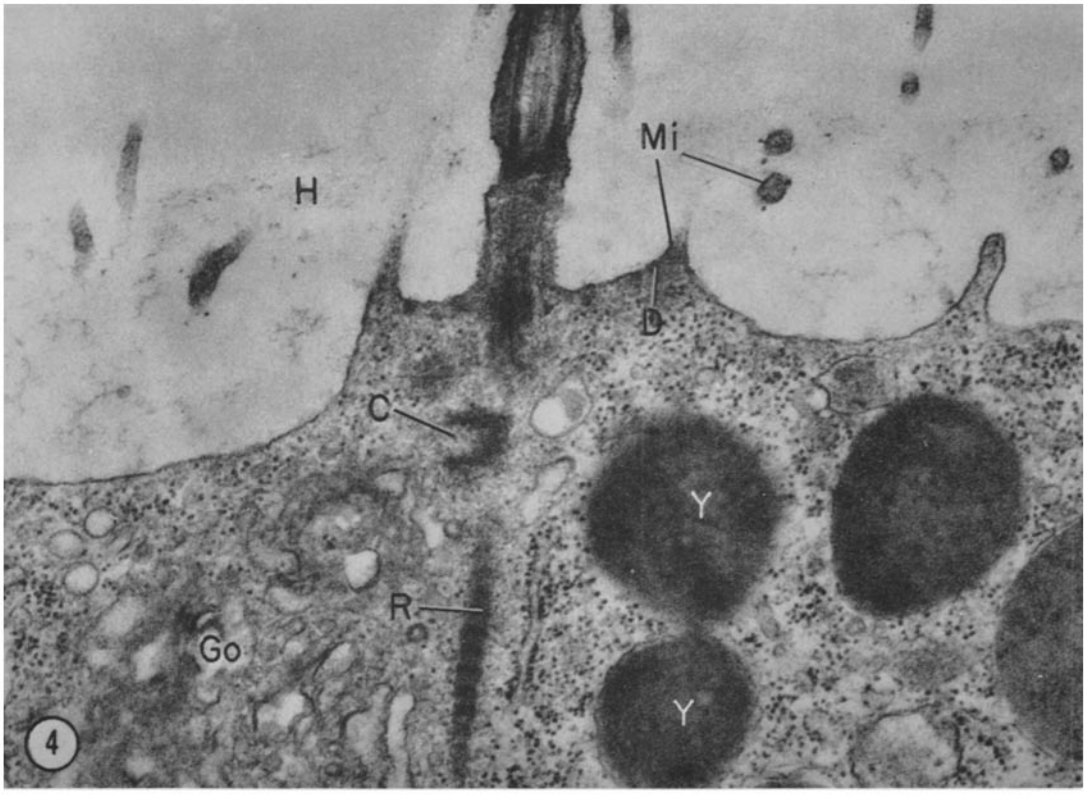
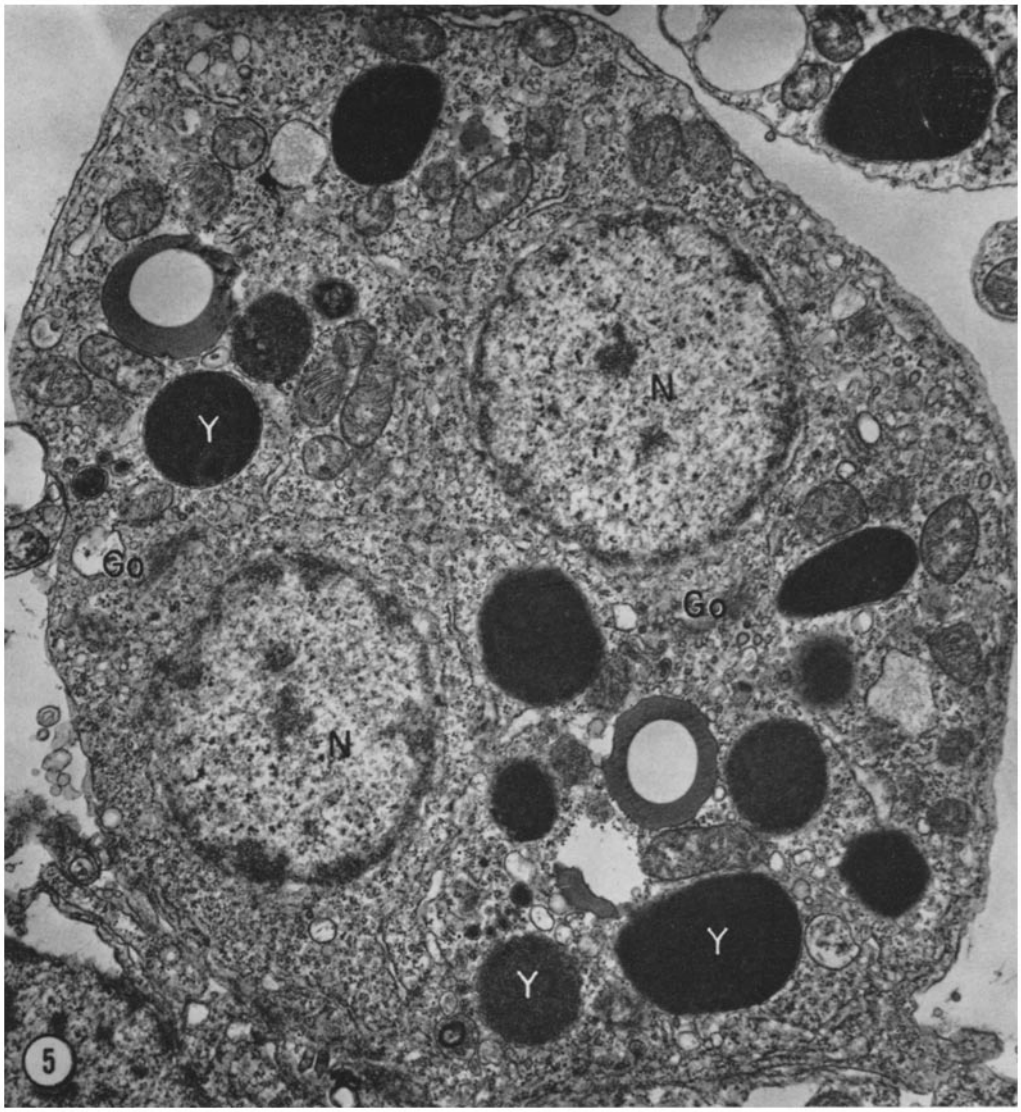



FIGURE 4 Higher magnification of the apical end of an ectodermal cell from an early gastrula which had been treated with colchicine for 3 hr. The basal body of the cilium is situated in the center of a segment of cytoplasm which is raised above the rest of the apical cell surface. This raised region has been referred to in the text as the platform. Extending from the lateral margins of the platform are portions of microvilli (*Mi*). These extend into the hyaline layer (*H*). Surrounding the basal body and actually occupying much of the platform is an amorphous material which appears to be confined to this region. Embedded in it are a number of small densities (*D*). Beneath the basal body is an oblique section through a centriole (*C*). A portion of the Golgi apparatus (*Go*) is present to one side of the centriole, with yolk granules (*Y*) on the other side. A portion of the ciliary rootlet (*R*) can be seen beneath the centriole. Perhaps most significant is the conspicuous absence of microtubules. × 25,000.

altered by colchicine. However, the cytoplasmic microtubles which, in untreated embryos, converge on satellites associated with the basal body are completely missing in embryos treated with $10^{-4}$, $10^{-3}$, and $10^{-2}$ M colchicine. With $10^{-5}$ M colchicine their number is reduced, but they do not disappear entirely.

On the inner surface of the plasma membrane in the region of the platform there are numerous small densities (Fig. 4). These structures, some of which are connected to the basal body by short rootlet-like connections, appear to be arranged in two concentric rings. Those on the outermost ring are situated near the microvilli. The distribution of these cortical densities does not differ from that of the untreated cells. Also, limited to the region of the platform and enmeshing the small densities, is an amorphous material (Fig. 4). The distribution of this material is identical with that seen around the basal bodies of untreated embryos.

2. *The Primary Mesenchyme.* (a) *Cell shape:* These cells appear nearly spherical, and the number of cell extensions is reduced. If the mesenchymal syncytium has already formed at the time of application of the colchicine, it remains. Several



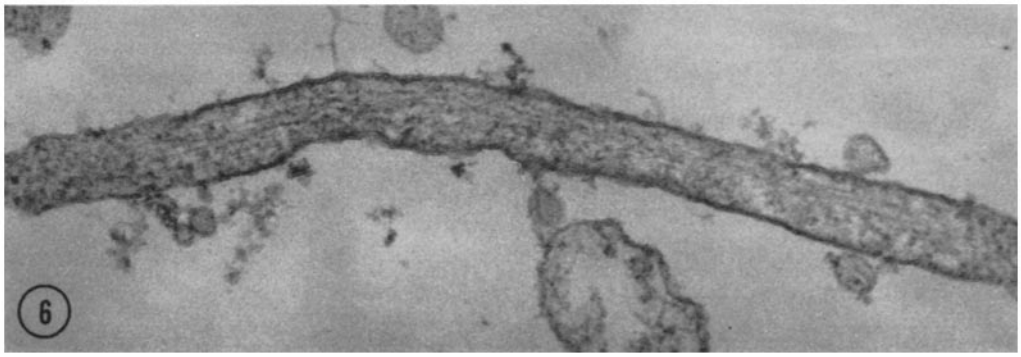




FIGURE 5 Primary mesenchymal cell from an early gastrula treated with colchicine for 3 hr. Two cell bodies have apparently fused, for two nuclei (*N*) are present each of which has an associated Golgi element (*Go*). Many of the pseudopodia have been withdrawn. Yolk granules (*Y*), mitochondria, and elements of the rough-surfaced endoplasmic reticulum are common. Microtubules are not found. × 25,000.

FIGURE 6 One of the few primary mesenchyme processes which remain after the embryos have been treated for 3 hr with colchicine. Within these remaining processes one generally finds 50-A filaments and small numbers of ribosomes. Inconspicuous extracellular fibrils are frequently found connected to these processes. × 72,000.

instances were found in which the cell bodies of adjacent primary mesenchyme cells in the syncytium had fused (Fig. 5). The extracellular fibrils remain throughout the blastocoel.

(b) *Organelle distribution exclusive of the microtubules:* No change was found in the distribution of organelles in the cytoplasm of the cell bodies or in the cytoplasm of the cable. Within the remaining filopodia, bundles of 50-A filaments were found (Fig. 6).

(c) *Microtubules:* Microtubules were not found.

#### Effect upon the Blastula (Hatching Stage)

The first effect of the colchicine upon the embryo is on its swimming behavior. In concentrations of $10^{-4}$ M or greater, development ceases; in $10^{-5}$ M, development continues until early gastrulation, and primary mesenchyme cells enter the blastocoel and begin to aggregate into the cable syncytium. Beginnings of a $CaCO_3$ skeleton are deposited, but this does not continue. This concentration of colchicine is not sufficient to block mitosis.

### *Hydrostatic Pressure (7000 psi)*

#### Effect upon Early Gastrulae

LIGHT MICROSCOPY: Shortly after pressure is applied, movement slows, and many organisms begin to rotate in small circles, apparently incapable of linear movement. Within 1 hr about 50% of the embryos have stopped completely, and most of the remainder are rotating slowly in tight circles.

Changes other than those which affect the motility begin 10–15 min after the application of hydrostatic pressure. One of these changes is the appearance of small herniations on the surface of many of the embryos, apparently caused by the extrusion of individual cells from the surface. With increased time under pressure, more and more cells are liberated.

But what is of even greater significance to this report is that development of intact embryos is completely arrested. Not only does the skeleton cease to form, but the archenteron fails to move across the blastocoel.

After 2–3 hr, the pressure was released, and the embryos were allowed to recover. In most cases, following a treatment of this duration the animals did not recover. In a relatively small percentage, motility was regained and development proceeded normally. These recovering embryos were followed to the pluteus stage. At least part of the death in the pressure "bomb" after these long periods should be attributed to anoxia.

FINE STRUCTURE: 1. *The Ectoderm.* (a) *Cell shape:* There are variations in the shape of the ectoderm from embryo to embryo. In all cases, however, the ectodermal cells undergo a degree of spherulation (Figs. 7 and 8) such that there is an over-all shortening of the cell and a rounding of both the apical and basal surfaces. The apical surface platform which appeared during the colchicine treatment is not formed in these cells. The basal body with its attached cilium on its distal end and ciliary rootlet on its proximal surface remains intact. The microvilli in most embryos appear to fragment into a series of beads which remain enmeshed in the hyaline layer. The basal surface remains in contact with the basement membrane although frequently the area of contact is reduced.

(b) *Basement membrane, hyaline layer, and desmosomes:* Hydrostatic pressures of the order employed in this study in general do not affect the morphology of these structures. However, the hyaline layer and the outer surfaces of the apical and lateral plasma membranes show marked affinities for heavy metal stains (Figs. 7 and 8). Prior to the application of pressure, these regions do not stain strongly, but in cells fixed during compression these regions take up the stain very strongly, so strongly in fact that at very low magnifications the lateral margins of the individual cells are clearly demonstrated. Interestingly enough, the greater affinity for heavy metals is not shown by the intracellular membrane systems such as the Golgi apparatus or ER (endoplasmic reticulum). The staining of the hyaline layer is confined largely to its outer surface and is particulate, as though individual spots in this layer possess great affinity for the heavy metals.

(c) *Organelle distribution:* The nucleus is generally located near the center of the cell, but in some cases it is displaced towards the apical cell surface. When the latter is the case, the Golgi apparatus is also displaced so that it lies parallel to the apical cell surface; it is always found apical to the nucleus. The echinochrome pigment granules, identifiable in fixed preparations as membrane-limited vacuoles of no appreciable density, have frequently disappeared.

(d) *Microtubules:* With 6000 psi or higher

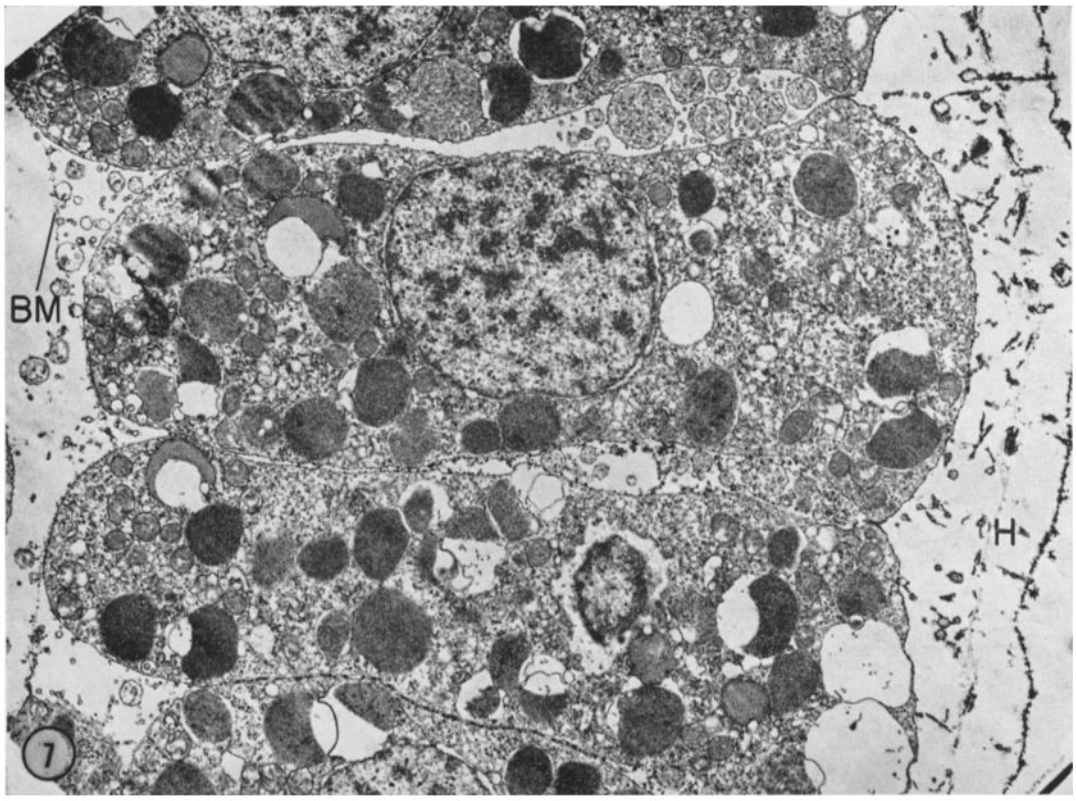



FIGURE 7 Portion of the ectoderm of a gastrula treated with 7000 psi for 45 min. Both the apical and basal surfaces have undergone marked rounding, becoming almost hemispherical. Most of the microvilli have disappeared. The desmosomal connections remain. The hyaline layer (*H*) and the basement membrane (*BM*), although rather insubstantial, are, nevertheless, present. × 9,000.

the cytoplasmic microtubules completely disappear. The basal body, centriole, small densities, ciliary rootlet complex, and the 9 + 2 configuration of the cilium are unaffected (Fig. 8). The terminal web which surrounds the basal body can still be identified.

2. *Primary Mesenchyme.* The pseudopodia are reduced in number, and the cells tend to spherulate. The number of cells in the blastocoel is very large in some embryos as if some of the ectodermal cells had entered this cavity. Cable syncytia were not encountered, although several cases of apparent fusion of individual primary mesenchyme cells were seen. Microtubules and filaments were not found.

#### CELLS FIXED 10 MIN AFTER THE RELEASE OF PRESSURE

Microtubules were present in normal orientations in the primary mesenchyme and ectodermal cells. The cells are still rounded although pseudopodial processes, within which are microtubules and filaments, have begun to reform from some of the mesenchymal cells (Figs. 9 *a* and *b*).

In time normal cell shape returns, and development, at least in some of the embryos, proceeds normally.

### *Calcium-Free Seawater*

#### GASTRULAE

Early gastrulae, when left in calcium-free seawater, soon break down into their component cells. The cilium of each of these cells continues to beat for a short time so that all that remains of a once fully organized embryo is a number of ciliated cells. Within a single batch of eggs treated for a given length of time there is considerable variation in the degree of cell separation as well as in many of the characteristics which will be

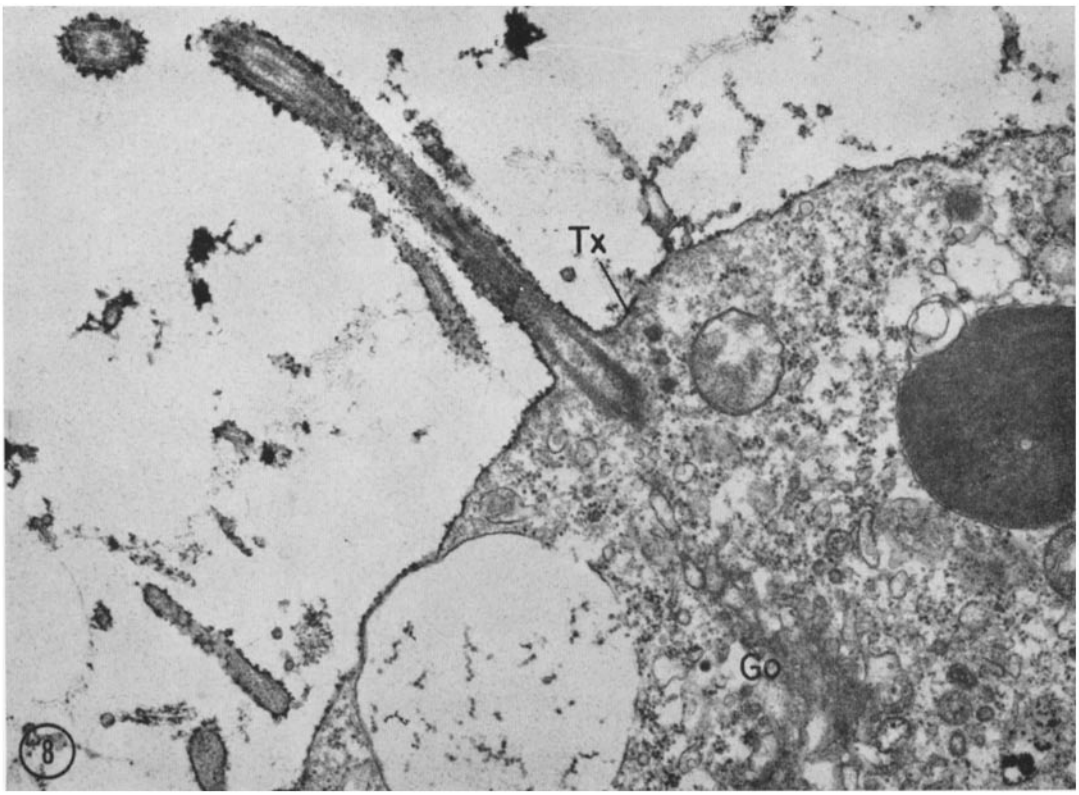



FIGURE 8 Higher magnification of the apical portion of an ectodermal cell from a gastrula treated with 7000 psi. The cilium extends from the center of the apical cell surface. An amorphous material (*Tx*) which appears to correspond to the terminal web in intestinal cells found on either side of the basal body of this cilium can still be recognized. Beneath it is a portion of the Golgi apparatus (*Go*). The echinochrome pigment granules remain in the cortex of the cell. × 25,000.

discussed below. The embryos were fixed just prior to, or in an early stage of, cell separation.

FINE STRUCTURE: 1. *Cell Shape.* In Fig. 10, which is a low-magnification electron micrograph through an entire gastrula, one can easily recognize that the cells are considerably shorter and more rounded than normal. A number of very irregular bulbous pseudopodia appear to engulf the basal end of the cilium, almost as if climbing up it. Thus we encountered many instances, such as illustrated in Fig. 11, where a naked 9 + 2 configuration was found in the apical cell cytoplasm. Elements which we normally associate with the cell cortex, namely the basal body and its associated small densities, are now located deeper in the cytoplasm. The microvilli disappear as well.

The mesenchymal cable also loses its shape, apparently collapsing over what skeleton has formed (Fig. 12). Its borders are very irregular. Some of the cell bodies of adjacent mesenchymal cells have fused (Fig. 10).

2. *Basement Membrane, Hyaline Layer, and Septate Desmosomes.* The hyaline layer and the basement membrane completely disappear in most of the cells after 45 min, while only some of the desmosomal connections vanish in the same period. The degree of desmosomal disappearance varies from cell to cell, but with time it is complete.

3. *Organelle Distribution.* Of greatest interest is the contents of the pseudopodia which project from both the apical and basal surfaces of the ectodermal cells (Figs. 10 and 11). These bulbous processes are filled with ribosomes and an occasional small vesicle, but they are almost free

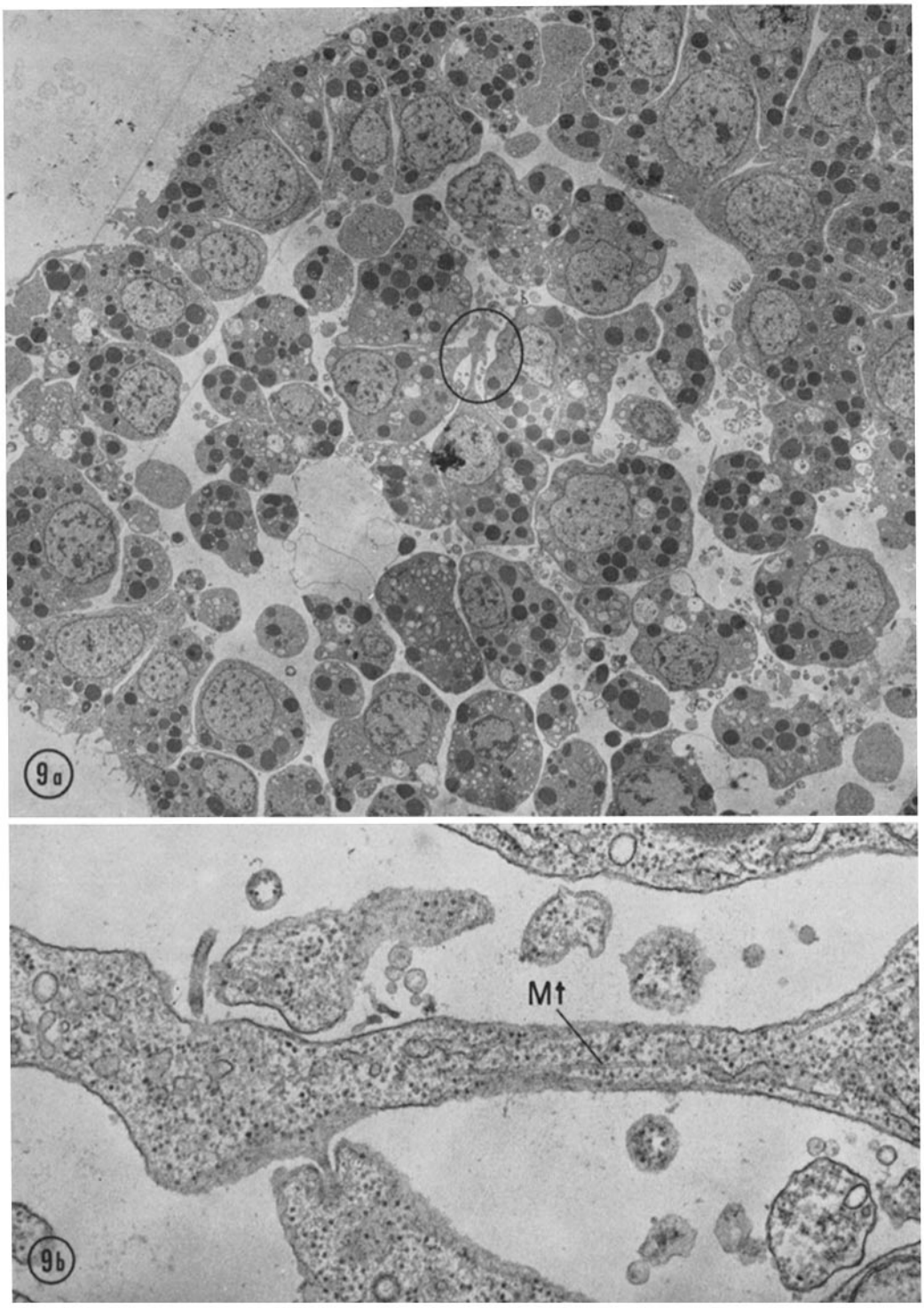



FIGURE 9 *a* Micrograph taken of a section cut through an early gastrula which had been treated with 6500 psi for 45 min; the embryo was fixed 15 min following the release of pressure. Some of the mesenchymal cells have reformed their pseudopodia. The circle indicates a pseudopodium which is shown at higher magnification in Fig. 9 *b*. × 2,000.

FIGURE 9 *b* Microtubules (*Mt*) are present in this pseudopodium. × 40,000.

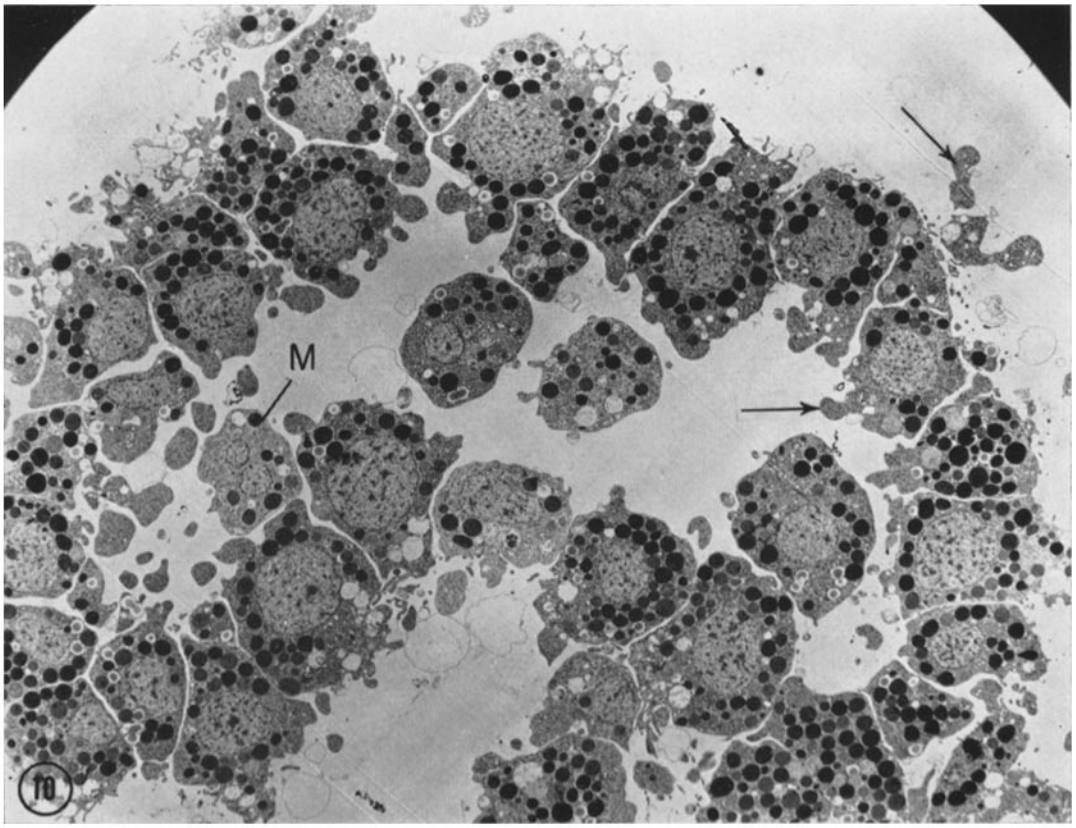



FIGURE 10 Section through an entire gastrula which had been treated for 45 min with calcium-free seawater. Most prominent are changes in the shape of the cells from columnar to nearly spherical. Often small protuberances (see arrows) extend from their free surfaces, apical or basal, which contain ribosomes, but seldom larger formed elements such as mitochondria and yolk granules. Desmosomal connections, formerly encountered on the apicolateral margins of the ectodermal cells, have largely disappeared, as have the basement membrane and the lateral infoldings between adjacent cells. The mesenchymal cells have rounded up as well, and we frequently find cells containing two nuclei (see the cell marked by the letter *M*). × 2,000.

of the larger formed elements of the cytoplasm, namely the yolk granules and the mitochondria.

4. *Microtubules and Cilium.* There is considerable variation from embryo to embryo in the number of microtubles present. In most instances, however, these tubular elements, normally present in the cytoplasm, are decreased in number. As already pointed out, the apical cell cytoplasm is frequently seen to flow over part of the cilium so that portions of the 9 + 2 complex of microtubules which makes up the ciliary axoneme are frequently present within the cytoplasm of the cell (Fig. 11). Few, if any, microtubules are present in the apical or basal pseudopodial processes.

## *$D_2O$*

### The Effect on Early Gastrula

Almost immediately after the embryos are placed in $D_2O$ the ciliary beat ceases, and most of the organisms settle to the bottom of the dish. A few embryos continue to move, although slowly, for 10–15 min, but after that they completely stop. If these organisms are viewed at higher magnifications, an individual cilium, here and there, undergoes sporadic twitches.

Development ceases immediately after the application of $D_2O$. What is remarkable, however, is that upon replacing the $D_2O$-seawater with

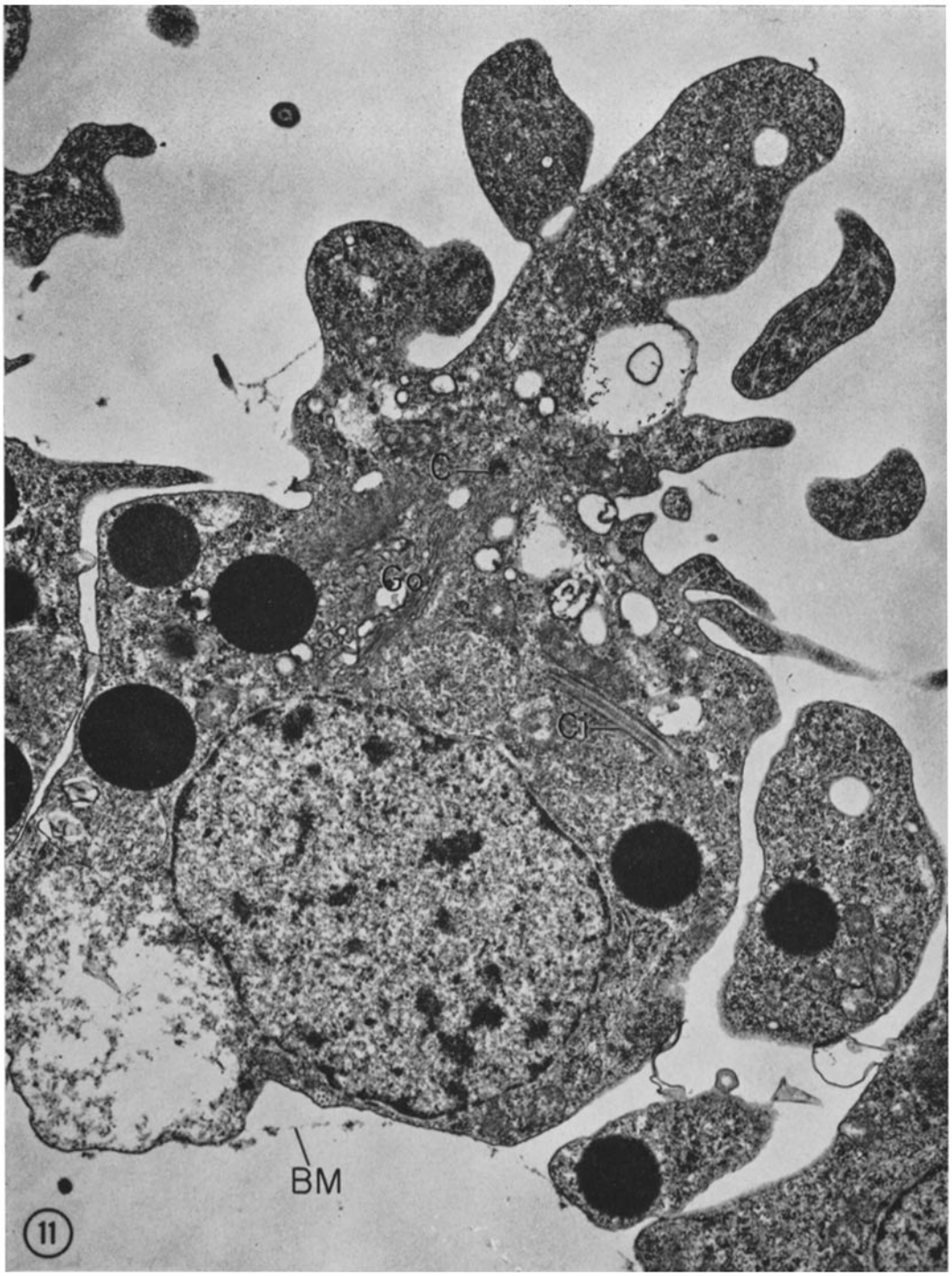



FIGURE 11 Higher magnification of an ectodermal cell from a gastrula which has been treated with calcium-free seawater for 45 min. Extending out from the free surface is a ribosome-rich protrusion. Near the base of this protrusion is a centriole (*C*). Directly beneath this structure is a portion of the Golgi apparatus (*Go*). To one side of the Golgi apparatus one can distinguish a portion of the ciliary axoneme (*Ci*). It is not membrane-bounded and so cannot represent an invagination of the cell surface. Microtubules are present in this cell, particularly around the centriole, but in most cells of similarly treated embryos they are reduced in number. The desmosomes, the hyaline layer, and the lateral infoldings are no longer present; the basement membrane (*BM*) is less apparent. × 15,500.

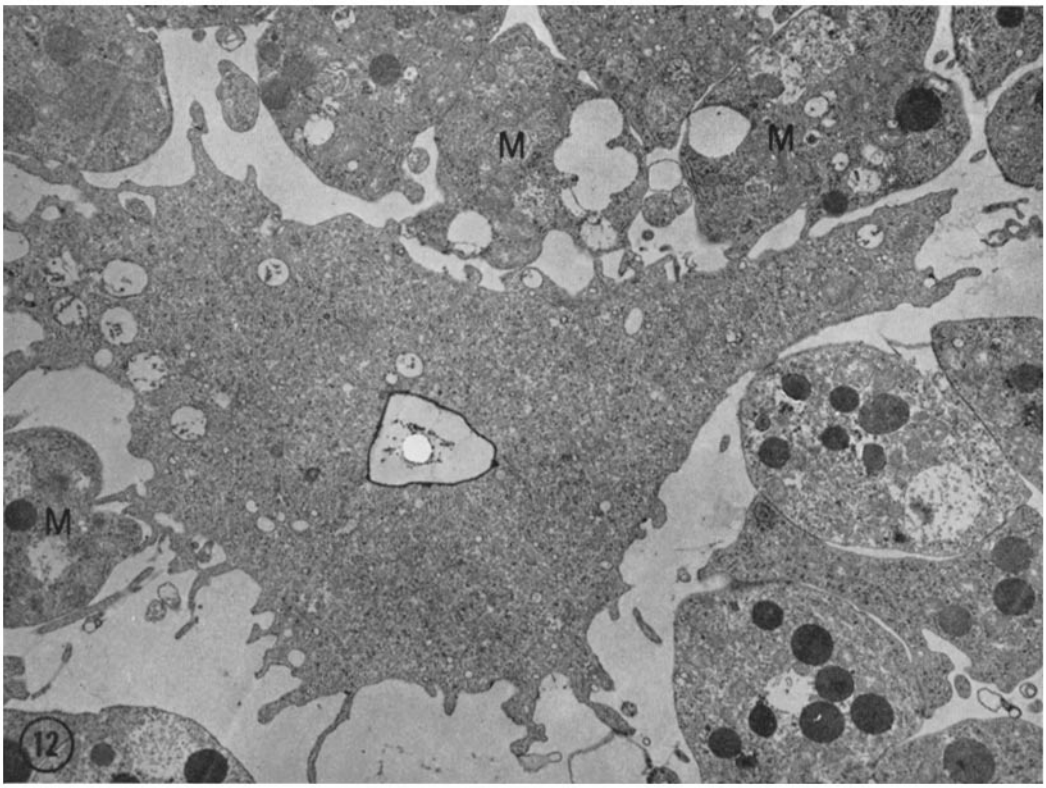



FIGURE 12 Transverse section through the mesenchymal cable from a gastrula treated for 45 min with calcium-free seawater. In the center of the cable is a vacuole which contains the developing skeleton. The cable itself seems to have lost most of its former shape, now appearing as a mass of cytoplasm which is rich in ribosomes but is poor in many other organelles and inclusions. This cable is attached to the cell bodies of adjacent mesenchymal cells by slender stalks. These cells are identified by the letter *M*. × 6,000.

normal seawater, even after periods as long as 12 hr, normal coordinated ciliary beat reappears, and development continues from the stage at which it was interrupted.

FINE STRUCTURE: Except for slight increases in the length of the ectodermal cells and an occasional fusion of adjacent cells (mesenchymal), specimens fixed during the administration of $D_2O$ show a morphology essentially unchanged from that of the untreated controls fixed at the time of application of the $D_2O$ (Fig. 14). More specifically, cell shape (Fig. 13), the distribution of organelles (Figs. 13–15), the presence of mesenchymal processes (Figs. 15 *a* and *b*), and the number and organization of microtubules (Figs. 14, 15 *a*) are not appreciably changed. The only demonstrable change is one that is extremely difficult to quantitate. This involves the disappearance in these cells of much of the density of the ground substance. It appears as if the embryos were badly fixed, but experimentally treated embryos and controls fixed in the same solutions at the same time and processed identically show that this could not be the case. What happens in the $D_2O$ to give this effect is not clear.

## EFFECT ON BLASTULA

When free-swimming blastulae are immersed in $D_2O$-seawater, the swimming movements cease abruptly, and the organisms sink to the bottom of the dish. Development fails to proceed, and primary mesenchyme cells do not form.

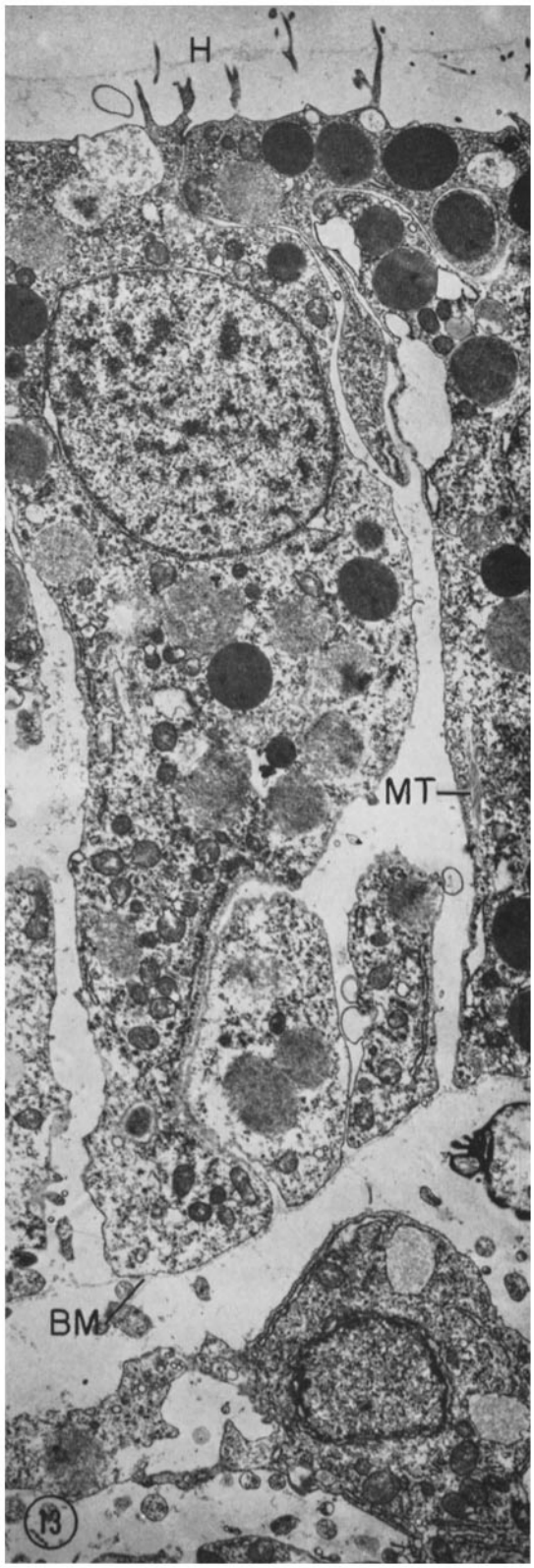



FIGURE 13 Low magnification through a portion of the surface of a gastrula treated for 2 hr with $D_2O$. The ectodermal cells appear identical with those present in untreated embryos, except that they appear more elongated. Neither the organelle distribution nor the extracellular coats, such as the hyaline layer (*H*) or the basement membrane (*BM*), appear altered. Bundles of microtubules (*MT*) run parallel to the long axis of these cells. × 11,000.

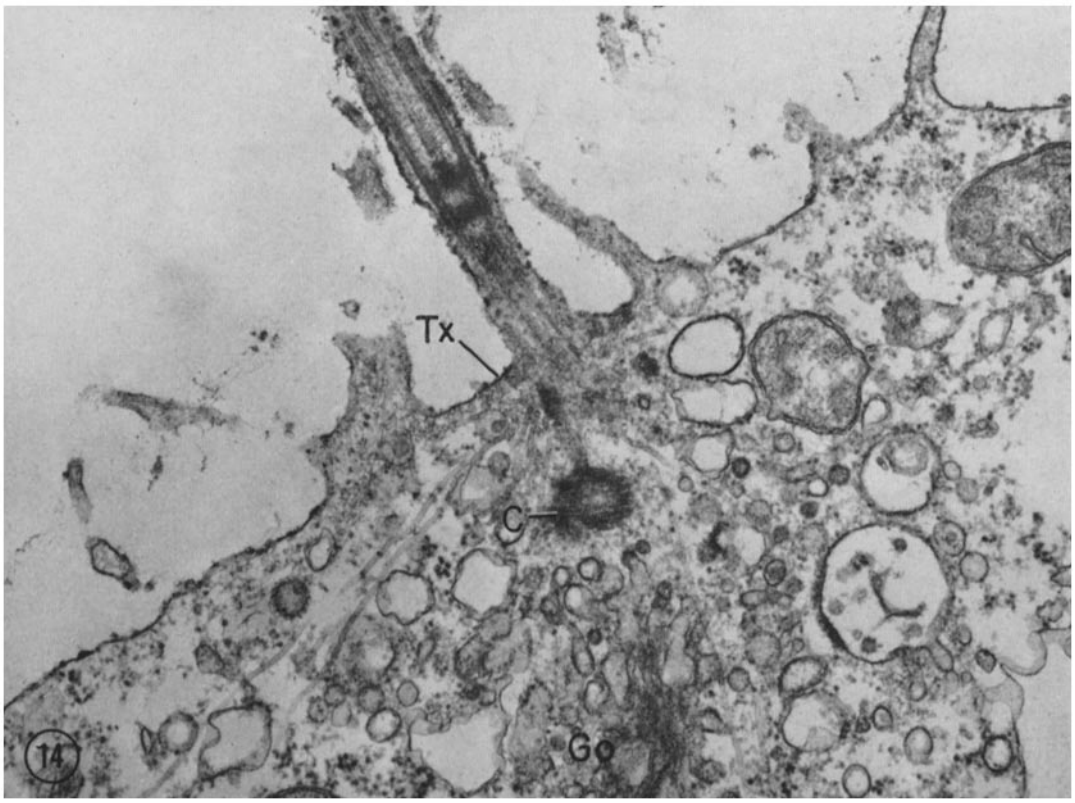
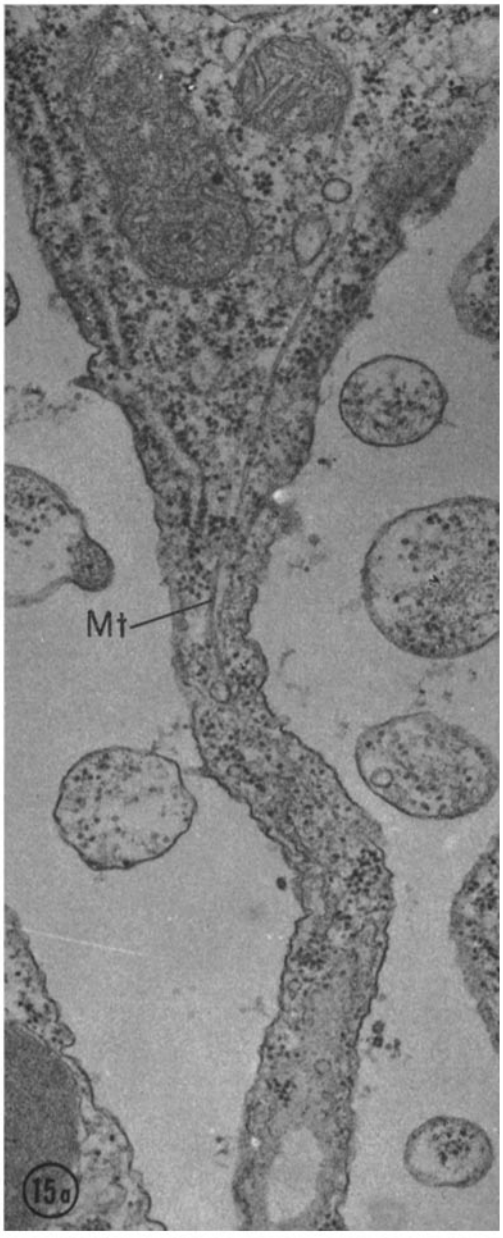


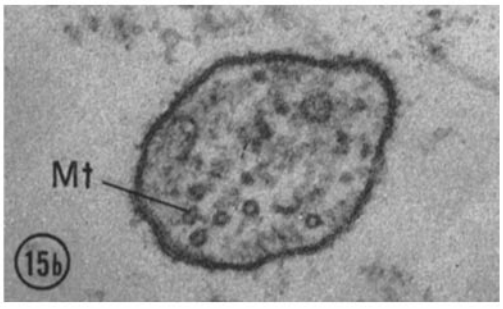

FIGURE 14 Higher magnification of the apical surface of an ectodermal cell treated for 2 hr with $D_2O$. Organelles and inclusions appear in normal configurations and in normal numbers. On either side of the basal body is the terminal web (*Tx*). Directly beneath it are large numbers of microtubules appearing to focus on regions adjacent to the basal body. Beneath the basal body is the centriole (*C*) and a portion of the Golgi apparatus (*Go*). The microtubules are found in numbers and distributions similar to that in untreated cells. × 37,000.

## DISCUSSION

### *The Role of Microtubules in the Development of Cell Shape*

The interpretation which was presented in the first paper in this series was that the microtubules are involved in the *determination* of the shape of the cell at each stage in the sequence of shape changes which individual primary mesenchyme cells undergo during their formation and differentiation. This interpretation is fully supported by the experiments reported in this paper. When agents which cause the tubules to disassemble and remain disassembled, namely colchicine and hydrostatic pressure, are applied to the embryos, development of the primary mesenchyme is completely inhibited. Alternatively, when deuterated seawater, an agent which stabilizes or freezes the microtubules (Gross and Spindel, 1960; Inoué et al., 1963, 1965), is applied to the embryos, development is also arrested. These agents could, therefore, block development by primarily interrupting the sequential disassembly and reassembly of the microtubules. We would suggest that in the experiments with deuterated seawater the cell shape cannot change because the microtubules are prevented from breaking down in order to reassemble into new patterns; in the case of the experiments with colchicine and hydrostatic pressure, the microtubules cannot reform so that cells, dependent on microtubles for shape expression, cannot progress to the next stage in the developmental




sequence. If these agents are removed, development continues, as one might expect, from the stage in the sequence at which the agent was applied. Thus if the sequence of the shape changes is interrupted, development can only continue from that stage at which it was interrupted; it cannot jump a stage in this sequence.

Since these agents affect the microtubules of the mitotic spindle, one might argue that blockage of development is due to an interruption of mitosis. This is certainly not the case, for in the stages examined the primary mesenchyme cells do not undergo mitosis. Rather they are confined to exploratory movements around the blastocoel, aggregation to form the cable syncytia, and skeleton formation (see the previous paper for more detailed coverage). Since spindle microtubules cannot be involved, the effect of these agents on the development of the primary mesenchyme must be the result of their action on the cytoplasmic microtubules.

This conclusion, that the development of cell shape is related to the assembly and distribution of the microtubules, is strengthened by the studies on the protozoan *Actinosphaerium nucleofilum*, in which the microtubules making up the axoneme appear to be intimately involved in the production of cell shape (Tilney, 1965, 1968; Tilney et al., 1966; Tilney and Porter, 1967). Thus in a metazoan system, as well as in a protozoan system, the microtubules appear to play an important role in the development of cell asymmetry.

If this is the correct interpretation, then the *control* of the distribution of microtubules in cells becomes of great interest. A discussion of the control of microtubule distribution is presented in the first paper in this series (Gibbins et al., 1969).

### *Microtubules, Gel Strength, and the Plasmagel*

The extensive studies of Marsland and his students over the last 30 yr have defined protoplasmic gels (Marsland, 1956). These gels are formed by an endothermic reaction characterized by an increase in volume (Marsland, 1956). Thus low temperature and hydrostatic pressure

FIGURES 15 *a* and 15 *b* Mesenchymal pseudopodia of embryos fixed after a 2 hr treatment with $D_2O$. Microtubules (*MT*) appear normal in distribution and numbers. 15 *a*, × 43,000; 15 *b*, × 110,000.

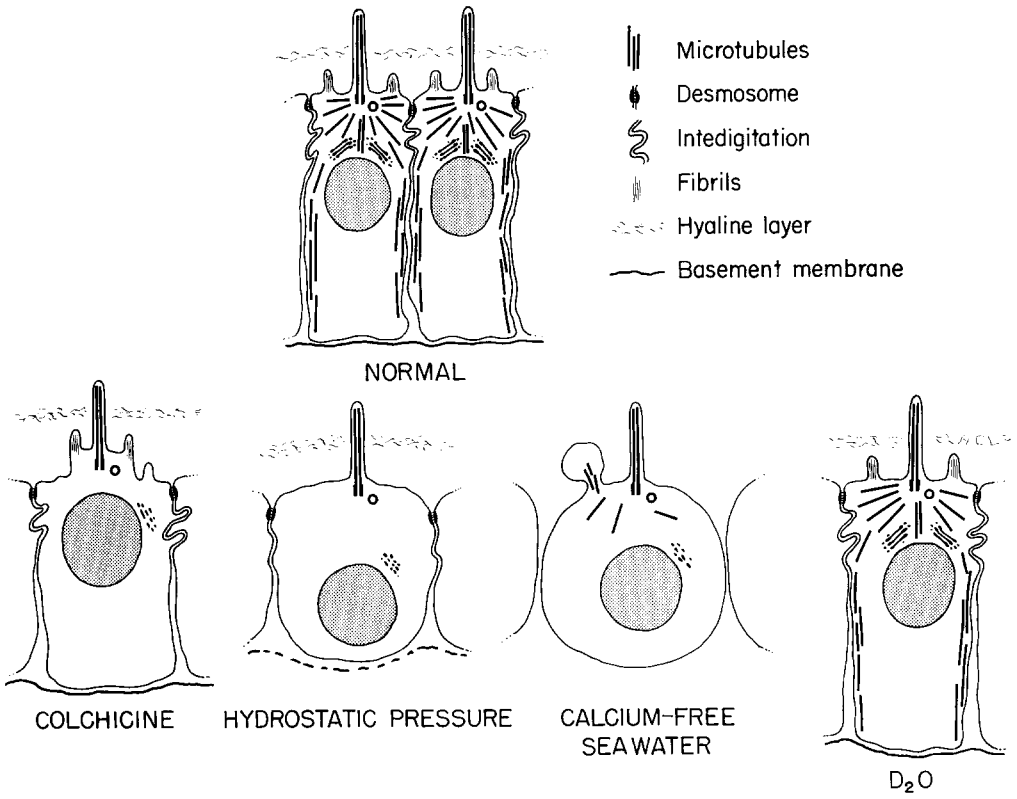



FIGURE 16 This figure depicts diagramatically the shape of the ectodermal cells when they are subjected to colchicine, hydrostatic pressure, calcium-free seawater, and $D_2O$. Also included in this figure is information on the effect of each experimental agent on the presence of microtubules, desmosomes, interdigitations between cells, the hyaline layer, and the basement membrane. Even though the microtubules have disappeared during colchicine and under hydrostatic pressure and have been reduced in number during treatment with calcium-free seawater, the shape of the cell in the presence of each agent appears to be more dependent on the other factors just mentioned than on the microtubules. In the case of colchicine treatment, which does not appear to effect the desmosomes, the cell interdigitations, the hyaline layer, and the basement membrane, cell shape is most nearly like that of untreated cells. When most of these factors are broken down, the cells assume a nearly spherical outline.

act to dissociate the gels. More recently it has been shown that microtubules tend to depolymerize at low temperatures (Tilney and Porter, 1967) or under hydrostatic pressure (Tilney et al., 1966), and thus it has been suggested that the microtubules are an integral part of the plasmagel.

If one compares the literature on the distribution of microtubules with the literature on the distribution and characteristics of the plasmagel as defined by the behavior of the cell or cell organelle under hydrostatic pressure, one finds that there are many cases, besides the above-mentioned protozoan, where gel distribution and dimensions can be correlated with the presence of microtubules. The mitotic spindle in intact dividing cells (Robbins and Gonatas, 1964; de Thé, 1964; Roth, 1964; Pease, 1946), the isolated mitotic apparatus (Kane and Forer, 1965; Zimmerman and Marsland, 1964), the melanophores of *Fundulus* (Bikle et al., 1966; Marsland, 1944), and the tentacles of *Tokophrya* (Kitching and Pease, 1939; Rudzinska, 1965) are some examples.

Colchicine, as well as hydrostatic pressure, causes a breakdown of the microtubules and has been shown to shift the equilibrium from the gelated to the solated state (Malawista, 1965;

TABLE I

*Table Summarizing Changes in Extracellular Structures during Experimental Conditions*

| | Colchine | Pressure | Ca-free | $D_2O$ |
|---|---|---|---|---|
| Extracellular factors: | | | | |
| Desmosomes | Present | Present | Absent | Present |
| Hyaline layer | Present | Present, although altered | Absent | Present |
| Basement membrane | Present | Present | Reduced | Present |
| Lateral infoldings | Reduced | Reduced | Absent | Present |
| Extracellular fibrils | Present | Reduced | Absent | Present |
| Skeleton | Present | | Present | Present |
| Intracellular factors: | | | | |
| Microtubules | Absent | Absent | Reduced | Present |
| Filaments | Present | Absent | Reduced | Present |
| Terminal web | Present | Present, yet less dense | Absent | Present |

Tilney, 1968). When colchicine, known to break down many of the spindle microtubules in dividing cells and the microtubules that make up the axonemes of *Actinosphaerium* (Tilney, 1968), is applied to dividing cells at the same time as hydrostatic pressure, the two agents act synergistically. Thus an amount of pressure or a concentration of colchicine, inadequate to cause mitotic arrest, when applied with the other agent completely inhibits mitosis (Marsland, 1966).

The work presented in this report is consistent with the interpretation that the microtubules represent an important factor in the development of oriented asymmetric gels as was suggested by Tilney et al. (1966) and Porter (1966). Hydrostatic pressure and colchicine would cause the cells to undergo a gel-to-sol transformation, and one manifestation of this transformation would be the alteration in the intracellular location of organelles and inclusions in at least some of the cells. For example, the Golgi apparatus is displaced, sometimes even being found in the basal end of the cell. At the same time yolk granules and mitochondria, constituents normally excluded from the cytoplasm immediately adjacent to the basal body, can frequently be found in this region during treatment with colchicine or hydrostatic pressure. $D_2O$, on the other hand, acts antagonistically to colchicine pressure (Marsland and Zimmerman, 1965), and low temperature (Marsland and Asterita, 1966); and it leads to an increase in the gel strength of the cytoplasm, reversing the gel-to-sol transformation. In fixed preparations of cells treated with $D_2O$ we have demonstrated that the microtubules appear normal in numbers and in distribution. Furthermore, no alterations in either cell shape or organelle distribution could be found.

### *The Role of Microtubules in the Development of Cell Polarity*

Wilson (1925), in his classical book, The Cell in Development and Heredity, states (p. 106), "Fundamentally both the nature and the origin of polarity are unknown. We know only its visible expression, which in most cases is both structural and functional, appearing on the one hand in a polarized grouping of the cell components, on the other in differences of functional or metabolic activity with respect to the axis marked off." It is interesting that our concept has not changed in the years that have elapsed since 1925; in fact, little has been added which could amplify this and other statements on cell polarity. In defining polarity much has been made of the position of the Golgi apparatus, at least in secretory cells, and of the centrosphere in many nonsecretory cells.

It seems worthwhile to report, therefore, that colchicine and hydrostatic pressure tend to randomize the position of the Golgi apparatus but do not affect the position of the basal body or the centriole (centrosphere). Since the microtubules appear to terminate or originate, in many

cases, on the centriole or satellites associated with the centriole (see Gibbins et al., 1969; de Thé, 1964; Robbins and Gonatas, 1964), it seems possible that these structures might be important in patterning the development of cell polarity as defined by Wilson. The microtubules, by shaping the plasmagel, might be one of the cytoplasmic agents active in expressing polarity. The microtubule is apparently not the only structure involved in this function, for in some differentiated cells one finds few microtubules yet the cells are polarized.

### *The Role of Microtubules in the Maintenance of Cell Shape*

The functions of microtubules in the separate roles of development and maintenance of cell form have not been clearly differentiated in the literature; in most instances the microtubules have been referred to as cytoskeletal elements (Bikle et al., 1966; Fawcett and Witebsky, 1964; Porter et al., 1964; Silveira and Porter, 1964; de Thé, 1964). Unfortunately, the word "cytoskeletal" tends to imply only the second of these two possibilities, that is relating the microtubules to the maintenance of cell form. As we shall see, in a metazoan tissue and in some single-celled organisms these tubular elements are not the only structures involved in maintaining a particular cell shape. In fact, they probably play a rather insignificant role in the maintenance of cell form, compared to their involvement in this role in the heliozoan, *Actinosphaerium nucleofilum* (Tilney, 1965).

What other components could act to maintain cell shape in metazoan tissues, and how do we know that microtubules play only a minor role? One feature that metazoan tissues have in common is the association of individual cells to form tissues. These cell-to-cell associations are generally maintained for relatively long periods and are achieved by the following two general mechanisms: (*a*) direct cell-to-cell adhesive devices, and (*b*) indirect cell connections via extracellular connective tissues. For example, in an epithelial sheet such as the ectoderm of *Arbacia*, there are at least four such devices by which these cells are connected to each other: the hyaline layer, the septate desmosomes, the lateral interdigitations of adjacent cells, and the basement membrane. These connective devices could act to give form to the epithelial sheet so that if the ectodermal cells of a blastula undergo a gel-to-sol transformation without affecting the four extracellular devices just mentioned, one might expect a rather limited degree of cell spherulation. It is not surprising to discover, therefore, that colchicine, which does not appear to affect any of these extracellular factors (at least they remain morphologically unchanged), has little effect on cell shape. If, on the other hand, during gel-to-sol transformation, one or more of these intercellular factors are broken down, more marked changes in cell shape would be expected, and this, in fact, is just what was observed. As summarized in Table I, hydrostatic pressure reduces the lateral infoldings and solates the hyaline layer (Asterita and Marsland, 1964); cells tend to spherulate. Prolonged treatment with calcium-free seawater in which all the extracellular devices disappear causes almost complete spherulation.

There are, of course, intracellular components as well as the extracellular ones just mentioned which can be related to the maintenance of cell shape. Of these intracellular components, those we can recognize are the microtubules, the 50-A filaments, and an amorphous material that surrounds the basal body. The latter appears to correspond to the terminal web in other cell types. As shown diagrammatically in the drawing (Fig. 16), colchicine causes a slight reduction in cell length and a platform to appear on the apical cell surface, from which the cilium and some of the microvilli project. Hydrostatic pressure, on the other hand, causes a rounding of the apical surface and a marked reduction in the number of microvilli. Finally, calcium-free seawater causes a marked cell spherulation and a disappearance of the microvilli, yet it induces the appearance of rather irregular processes extending from both the apical and basal surfaces. Although all three agents break down microtubules, the 50-A filaments in microvilli are unaffected by colchicine but disappear with hydrostatic pressure and calcium-free seawater. Thus it appears that the 50-A filaments are influential in the maintenance of the microvilli.

From recent experiments on intestinal epithelial cells where the terminal web is more

developed than in *Arbacia* embryos, we now know that hydrostatic pressure breaks down this material and that concomitantly there is a distinct rounding of the cell surface (unpublished observations) similar to that observed in *Arbacia*. Thus it appears that the terminal web is influential in the maintenance of the shape of the apical cell surface.

In *Arbacia* the terminal web is present only in a small region around the basal body, not over the whole apical cell surface as is true for the intestinal epithelial cell. This region, which measures about 2 $\mu$ in diameter, corresponds exactly to the platform which appears during colchicine treatment. Since colchicine does not appear to affect the integrity of the terminal web, we interpret these colchicine-derived platforms as having resulted from the collapse of the apical cell cytoplasm peripheral to the terminal web, thus emphasizing the terminal web-stabilized portion which takes the form of the platform. We assume that the collapse of the rest of the apical cell cytoplasm is related to the disappearance of the microtubules, either directly or indirectly.

In contrast to the above-mentioned agents, deuterated seawater does not affect any of these factors (see the accompanying table), and likewise this agent has no effect on cell shape.

To summarize then, it appears that at least in terms of the ectoderm, the microtubules may play only a minor role in the *maintenance* of cell shape, this function being largely taken over by extracellular factors operative in tissue maintenance. Intracellular factors, namely the the 50-A filaments which account for the maintenance of the microvilli, and the terminal web which accounts for the maintenance of a portion of the apical cell surface, are also operative.

The degree of spherulation of the primary mesenchyme cells, on the other hand, depends not only on the agent applied but also on whether the cells have joined into the syncytium or are still unattached. The greatest degree of cell rounding occurs in cells treated with calcium-free seawater; the least occurs with colchicine. As seen in the table, the former results in the breakdown of the microtubules, the filaments, the skeleton, and the basement membrane; thus it is not surprising that even the cells in the syncytium are rounded. Colchicine, on the other hand, appears to affect only the microtubules. Thus during colchicine treatment unattached cells tend to spherulate, with the exception of those cells which have processes containing 50-A filaments. These extensions remain. Hydrostatic pressure causes a more marked spherulation of the unattached cells and in some instances a pinching off of bits of cytoplasm which remain in the blastocoel as round globules. A similar phenomenon was seen in the spaces surrounding the ectodermal cells.

The shape of the mesenchyme, therefore, appears dependent upon the filaments, microtubules, extracellular fibrils, the basement membrane, and the skeleton. Since colchicine, which appears to affect only the microtubules, has little effect on cell shape, with the exception of the retraction of some of the pseudopodia of the unattached mesenchymal cells, we must assume that the microtubules, as in the ectoderm, play a relatively minor role in the *maintenance* of cell shape.

It is worthwhile at this point to try to draw some tentative over-all conclusions as to the role of the microtubules in the maintenance of cell shape in other biological systems, since there exist good, fine-structural studies on the distribution of these elements and the other intra- and extracellular devices, as well as a growing literature on the effect of colchicine and hydrostatic pressure on cell shape. Clearly, in the heliozoan *Actinosphaerium nucleofilum*, the microtubules are intimately associated with the maintenance of the axopodia, since if colchicine or hydrostatic pressure is applied to these cells, the microtubules disassemble and the axopodia retract (Tilney et al., 1966; Tilney, 1968). In other protozoa the situation is not so clear. In ameba, for example, colchicine has no effect on cell shape (unpublished observations), but hydrostatic pressure causes complete spherulation of the cell. Furthermore, the presence of microtubules in the pseudopodia of these cells has not been demonstrated, whereas filaments varying from 50–150 A have been shown (Bowers, B. 1966. Personal communication; Wohlfarth-Botterman, 1964; Marshall and Nachmias, 1965; Griffin, 1965). Microtubule protein appears to be present in these cells because microtubules have been found in the mitotic spindle (Roth and Daniels, 1962) and in the cell body (Griffin, 1965). From this evidence tubules do not appear to be involved

in cell shape in ameba. In other protozoa such as, for example, the ciliates, *Blepharisma* and *Paramecium*, treatment of the cell with enzymes which affect the pellicle results in cell spherulation or, if doses of enzymes are small enough not to affect cell shape, there is a greater susceptibility of these enzyme-treated cells to hydrostatic pressure (Asterita and Marsland, 1961). Furthermore, colchicine does not appear to affect the shape of a related ciliate, *Tetrahymena* (Pitelka, 1963; Allen, R. 1965. Personal communication); this evidence suggests that the pellicle plays a greater role in the maintenance of cell shape than do the microtubules, assuming of course that the colchicine breaks down the tubules in these protozoa. Plant cells, of course, have cell walls which are most likely active in shape maintenance. If colchicine or hydrostatic pressure is applied to plant cells, the shape the cell had achieved at the time of application of these agents is not altered (Green, 1962, on *Nitella*; Marsland, 1938, on *Elodea*), although subsequent growth is abnormal. Although no fine structural studies with glutaraldehyde fixation have been carried out on pressure-treated chromatophores, it seems reasonable to expect that pressure would cause the disassembly of the microtubules. It is undoubtedly significant that pigment granule movement is interrupted (Sammon, J. 1966, Personal communication; Marsland, 1944). If, indeed, the microtubules have been broken down by the pressure, then we would suggest that the collagen which surrounds the chromatophores might be influential in the retention of the stellate form of this cell.

From the examples listed above (with the exception of *Actinosphaerium*) and from the results reported in this paper, it appears that the microtubules do rather little to *maintain* cell shape, although they are undoubtedly very important in the *development* of cell form. One wonders, therefore, what the microtubules do once the cell shape has been achieved. Are they merely kept in the cell as interesting relics, the intracellular version of a wisdom tooth, or do they take over other functions? Do they, for example, as has been suggested earlier in the discussion, serve to define regions of plasmasol and thus regulate streaming?

## *Ribosome-Rich Processes Induced by Calcium-Free Seawater and Their Possible Relationship to the Basal Lobes of the Presumptive Mesenchymal Cells*

In the first paper in this series we demonstrated that the presumptive primary mesenchyme cells produced from their basal surfaces a lobe which contained numerous ribosomes and short segments of rough-surfaced ER, but from which other formed elements of the cytoplasm appeared to be completely excluded. We postulated that the ribosomes in the cap on the surface of the newly formed mesenchyme cells and ultimately the ribosomes in the cable cytoplasm were derived from the ribosomes segregated in the basal lobe. Regardless of the validity of this postulate, it is a fact that at three stages in the development of the primary mesenchyme, ribosomes appear to be segregated. The mechanism for this segregation is not clear.

Calcium-free seawater appears to induce the formation of lobes similar to those already described. These experimentally induced lobes, however, are found not only on the basal surfaces of the ectodermal cells but on their apical surfaces. Since calcium-free seawater causes a reduction in microtubule number and since in the basal lobes of untreated presumptive mesenchyme cells there are relatively few tubules, we are forced to conclude that these elements play no role in segregating the ribosomes.

It is a pleasure to thank Mrs. Helen Lyman for her drawing.

Mr. Robert Chapman designed and built the observation chamber for the hydrostatic pressure experiments and assembled the apparatus.

This work was supported by grant No. 5T1-GM-707 from the National Institutes of Health, awarded to Professor Keith R. Porter.

*Received for publication 2 August 1967, and in revised form 28 August 1968.*

## BIBLIOGRAPHY

ASTERITA, H., and D. MARSLAND, 1961. The pellicle as a factor in the stabilization of cellular form and integrity: Effects of externally applied enzymes on the resistance of *Blepharisma* and *Parmecium* to pressure-induced cytolysis. *J. Cell. Comp. Physiol.* **58**:49.

ASTERITA, H., and D. MARSLAND. 1964. Pressure-temperature studies on the hyaline membrane of sea urchin eggs. *J. Cell. Comp. Physiol.* **64**:221.

BIKLE, D., L. G. TILNEY, and K. R. PORTER. 1966. Microtubules and pigment migration in the melanophores of *Fundulus heteroclitus* L. *Protoplasma.* **61**:322.

BYERS, B., and K. R. PORTER. 1964. Oriented microtubules in elongating cells of the developing lens rudiment after induction. *Proc. Nat. Acad. Sci. U.S.A.* **52**:1091.

CAVANAUGH, G. M. 1956. Formulae and Methods IV. of the Marine Biological Laboratory, Chemical Room. Woods Hole, Massachusetts. 4th edition.

DE THÉ, G. 1964. Cytoplasmic microtubules in different animal cells. *J. Cell Biol.* **23**:265.

FAWCETT, D. W., and F. WITEBSKY. 1964. Observations on the ultrastructure of nucleated erythrocytes and thrombocytes with particular reference to the structural basis of their discoidal shape. *Z. Zellforsch. Mikroskop. Anat.* **62**:785.

GIBBINS, J. R., L. G. TILNEY, and K. R. PORTER. 1969. Microtubules in the formation and development of the primary mesenchyme in *Arbacia punctulata.* I. The distribution of microtubules. *J. Cell Biol.* **41**:201.

GREEN, P. B. 1962. Mechanism for plant cellular morphogenesis. *Science.* **138**:1404.

GRIFFIN, J. L. 1965. Fixation and visualization of microfilaments and microtubules and their significance in the movement of four types of ameboid cells. *J. Cell Biol.* **27**:39A.

GROSS, P., and W. SPINDEL. 1960. Heavy water inhibition of cell division: An approach to mechanism. *Ann. N. Y. Acad. Sci.* **90**:500.

HARRIS, P. 1962. Some structural and functional aspects of the mitotic apparatus in sea urchin embryos. *J. Cell Biol.* **14**:475.

HARVEY, E. B. 1952. Electrical method of "sexing" *Arbacia* and obtaining small quantities of eggs. *Biol. Bull.* **103**:284.

INOUÉ, S., H. SATO, and R. W. TUCKER. 1963. Heavy water enhancement of mitotic spindle birefringence. *Biol. Bull.* **125**:380.

INOUÉ, S., H. SATO, R. E. KANE, and R. E. STEPHENS. 1965. Dynamic structure of the living spindle. *J. Cell Biol.* **27**:115. (Abstr.)

KANE, R. E., and A. FORER. 1965. The mitotic apparatus. Structural changes after isolation. *J. Cell Biol.* **25**(3):31.

KITCHING, J. A., and D. C. PEASE. 1939. The liquefaction of the tentacles of suctorian protozoa at high hydrostatic pressures. *J. Cell. Comp. Physiol.* **14**:1.

LANDAU, J. V., and L. THIBODEAU. 1962. The micromorphology of *Amoeba proteus* during pressure-induced changes in the sol-gel cycle. *Exp. Cell Res.* **27**:591.

LEDBETTER, M. C., and K. R. PORTER. 1963. A "microtubule" in plant cell fine structure. *J. Cell Biol.* **19**:239.

LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.

MALAWISTA, S. E. 1965. On the action of colchicine. The melanocyte model. *J. Exp. Med.* **122**:361.

MARSHALL, J. M., and V. T. NACHMIAS. 1965. Cell surface and pinocytosis. *J. Histochem. Cytochem.* **13**:92.

MARSLAND, D. A. 1938. The mechanism of protoplasmic streaming. The effects of high hydrostatic pressure upon cyclosis in *Elodea canadensis. J. Cell. Comp. Physiol.* **13**:23.

MARSLAND, D. A. 1944. Mechanism of pigment displacement in unicellular chromatophores. *Biol. Bull.* **87**:252.

MARSLAND, D. A. 1950. The mechanisms of cell division; temperature-pressure experiments on the cleaving eggs of *Arbacia punctulata. J. Cell. Comp. Physiol.* **36**:205.

MARSLAND, D. A. 1956. Protoplasmic contractility in relation to gel structures. Temperature-pressure experiments on cytokinesis and amoeboid movement. *Int. Rev. Cytol.* **5**:199.

MARSLAND, D. A. 1966. Anti-mitotic effects of colchicine and hydrostatic pressure; synergistic action on the cleaving eggs of *Lytechinus variegatus. J. Cell. Physiol.* **76**:333.

MARSLAND, D. A., and H. ASTERITA. 1966. Counteraction of the anti-mitotic effects of $D_2O$ in the dividing eggs of *Arbacia punctulata*: A temperature-pressure analysis. *Exp. Cell Res.* **42**:316.

MARSLAND, D. A., and A. M. ZIMMERMAN. 1963. Cell division: differential effects of heavy water upon the mechanisms of cytokinesis and karyokinesis on the eggs of *Arbacia punctulata. Exp. Cell Res.* **30**:23.

MARSLAND, D. A., and A. M. ZIMMERMAN. 1965. Structural stabilization of the mitotic apparatus by heavy water in the cleaving eggs of *Arbacia punctulata. Exp. Cell Res.* **38**:306.

PEASE, D. C. 1946. Hydrostatic pressure effects upon spindle figure and chromosome movement. II. Experiments on the mitotic divisions of *Tradescantia* pollen mother cells. *Biol. Bull.* **91**:145.

PITELKA, D. R. 1963. Electron Microscopic Structure of Protozoa. The Macmillan Company, New York.

PORTER, K. R. 1966. Cytoplasmic microtubules and their function. *In* Principles of Biomolecular Organization. G. E. W. Wolstenholme and M. O'Conner, editors. J. and A. Churchill Ltd., London. 308.

PORTER, K. R., M. C. LEDBETTER, and S. BADENHAUSEN. 1964. The microtubule in cell fine structure as a constant accompaniment of cytoplasmic movement. Proceedings of the 3rd European Regional Conference on Electron Microscopy. M. Titlbaum, editor. Academia (Publishing House

of the Czechoslovak Academy of Science), Prague. **B**:119.

REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.

ROBBINS, E., and N. K. GONATAS. 1964. The ultrastructure of mammalian cells during the mitotic cycle. *J. Cell Biol.* **21**:429.

ROTH, L. E. 1964. Motile systems with continuous filaments. *In* Primitive Motile Systems. R. D. Allen and N. Kamiya, editors. Academic Press Inc., New York, 527.

ROTH, L. E., and E. W. DANIELS. 1962. Electron microscopic studies of mitosis in Amoeba. II. The giant amoeba, *Pelomyxa carolinensis*. *J. Cell Biol.* **12**:57.

RUDZINSKA, M. A. 1965. The fine structure and function of the tentacle in *Tokophrya infusionum*. *J. Cell Biol.* **25**:459.

SILVEIRA, M., and K. R. PORTER. 1964. The spermatozoids of flatworms and their microtubular systems. *Protoplasma.* **59**:240.

TILNEY, L. G. 1965. Microtubules in the Heliozoan, *Actinosphaerium nucleofilum* and their relation to axopod formation and motion. *J. Cell Biol.* **27**: 107A.

TILNEY, L. G. 1968. Studies on the microtubules in Heliozoa. IV. The effect of colchicine on the formation and maintenance of the axopodia of *Actinosphaerium nucleofilum*. *J. Cell Sci.* **3**:549.

TILNEY, L. G., Y. HIRAMOTO, and D. A. MARSLAND. 1966. Studies on the microtubules in Heliozoa. III. A pressure analysis of the role of these structures in the formation and maintenance of the axopodia of *Actinosphaerium nucleofilum* (Barrett). *J. Cell Biol.* **29**:77.

TILNEY, L. G., and K. R. PORTER. 1967. Studies on the microtubules in Heliozoa. II. The effect of low temperature on the formation and maintenance of the axopodia. *J. Cell Biol.* **34**:327.

WATSON, M. L. 1958. Staining of tissue sections for electron microscopy with heavy metals. *J. Biophys. Biochem. Cytol.* **4**:475.

WILSON, E. B. 1925. The Cell in Development and Heredity. The Macmillan Company, New York.

WOHLFARTH-BOTTERMANN, K. E. 1964. Differentiation of the ground cytoplasm and their significance for the generation of the motive force of amoeboid movement. *In* Primitive Motile Systems. R. D. Allen and N. Kamiya, editors. Academic Press Inc., New York. 79.

ZIMMERMAN, A. M., and D. A. MARSLAND. 1964. Cell Division: Effects of pressure on the mitotic mechanisms of marine eggs (*Arbacia punctulata*). *Exp. Cell Res* **35**:293.