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

I. The Distribution of Microtubules

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ABSTRACT

Prior to gastrulation, the microtubules in the presumptive primary mesenchyme cells appear to diverge from points (satellites) in close association with the basal body of the cilium; from here most of the microtubules extend basally down the lateral margins of the cell. As these cells begin their migration into the blastocoel, they lose their cilia and adopt a spherical form. At the center of these newly formed mesenchyme cells is a centriole on which the microtubules directly converge and from which they radiate in all directions. Later these same cells develop slender pseudopodia containing large numbers of microtubules; the pseudopodia come into contact and fuse to form a "cable" of cytoplasm. Microtubules are now distributed parallel to the long axis of the cable and parallel to the stalks which connect the cell bodies of the mesenchyme cells to the cable. Microtubules are no longer connected to the centrioles in the cell bodies. On the basis of these observations we suggest that microtubules are a morphological expression of a framework which opeartes to shape cells. Since at each stage in the developmental sequence microtubules appear to originate (or insert) on different sites in the cytoplasm, the possibility is discussed that these sites may ultimately control the distribution of the microtubules and thus the developmental sequence of form changes.

INTRODUCTION

The importance of individual cell shape in determining the form of the sea urchin embryo and the importance of an orderly progression of changes in cell shape for normal morphogenesis are well established (see discussion by Gustafson and Wolpert, 1963). Although most investigators have postulated that these shape changes are controlled by changes in patterns of intercellular adhesion, one cannot rule out that intracellular mechanisms may operate as well. Recently developed methods for preserving cell fine structure (Sabatini et al., 1963), which are especially valuable for embryonic tissues, have made the investigator of developmental sequences especially aware of cytoplasmic microtubules.

One of the most dramatic of these sequences occurs during the formation and differentiation of the cells of the primary mesenchyme of echinoderm

embryos (Gustafson and Wolpert, 1963, 1967). As is well known, these cells arise by migration of certain vegetal pole ectodermal cells into the blastocoel. In the period immediately following this migration these cells develop pseudopodia and become motile, explore the wall of the blastocoel, and finally aggregate in a ring around the base of the developing archenteron. The pseudopodia of adjacent cells in a ring align and finally fuse to form a process common to the group. These common processes are referred to as "cables," and it is in these cables that the larval skeleton, which will reach its full development in the pluteus, is deposited.

Associated with these events there are some prominent changes in cell form. To learn more about these, we have investigated the fine structure of the primary mesenchyme cells during early embryogenesis to observe whether there is, in fact, a cytoplasmic component that could be held accountable for the determination of the changes in cell shape as well as for the ultimate form of the cable cytoplasm.

The earlier observations of Byers and Porter (1964) on cells in the posterior epithelium of the chick lens directed attention for the first time to the possible involvement of microtubules in the development of cell forms. These ideas have gained support in numerous subsequent studies (Porter, 1966; Tilney et al., 1966; Tilney, 1968; Tilney and Porter, 1967; and many others). It has been our aim, in turning to an examination of primary mesenchyme cells, to see (*a*) if here also microtubules were present and arranged in such a manner as logically to affect cell form and (*b*) to follow the changes in microtubule distribution and orientation in cells which, without apparent division, go through a sequence of changes while they are relatively free-living within the blastocoel of the embryo.

This is the first in a series of papers on the development of the primary mesenchyme. Other papers in this series will be concerned with experimental studies on these cells in an attempt to pinpoint still further the role of the microtubules in development.

MATERIALS AND METHODS

Eggs and sperm were obtained from *Arbacia punctulata* by the voltage method of Harvey (1952). The eggs were fertilized and allowed to develop to the appropriate stages when aliquots were fixed in glutaraldehyde. The glutaraldehyde used was treated with $BaCO_3$ and filtered, and the pH was adjusted to 7.6 with 1 N NaOH. This pretreated glutaraldehyde and seawater containing the embryos were mixed in proportions to give a final concentration of 6% glutaraldehyde. In this the embryos were fixed for 30–45 min, washed in several changes of seawater, and finally postfixed in 1% OsO_4 dissolved in 0.1 M phosphate buffer at pH 7.2. The specimens were embedded in Epon 812 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.

Sections 1 μ thick were cut and stained with 1% toluidine blue and azure II after the method of Richardson et al. (1960).

For analysis of skeletal deposition, whole embryos were studied with a Zeiss polarizing microscope.

RESULTS

Formation of the Primary Mesenchyme

There is extensive literature reporting the events of primary mesenchyme formation, aggregation, and skeleton deposition (for a detailed description reference may be made to the following reviews: Hörstadius, 1939; Dan, 1960; Okazaki, 1960; Gustafson and Wolpert, 1963; and Gustafson, 1964). Some disagreement exists as to whether all the primary mesenchyme cells form through direct transformation of ectodermal cells or if limited cell proliferation occurs in the population of primary mesenchyme cells once they have migrated into the blastocoel. Gustafson and Wolpert (1961) state, "During observation of many hundreds of cells in normal larvae, we have never seen division of primary mesenchyme cells in the blastocoel." This statement conflicts, however, with the observations of Agrell (1954) and of Okazaki (1960). It is possible, of course, that these contradictions are due to species differences. Certainly in *Arbacia punctulata*, the species reported here, we have not seen mitoses in the cells of the primary mesenchyme.

If mitosis does not occur in these cells following their release into the blastocoel, then each individual primary mesenchyme cell must independently complete a complex developmental program. As each cell passes through this program it will undergo an ordered sequence of shape changes. We have chosen to divide the sequence into five arbitrary stages, during each of which the cells of

the primary mesenchyme, or their immediate precursors, exhibit a particular cell shape.

The Blastula—Early

CELL SHAPE: During early embryogenesis mitosis has partitioned the contents of the spherical egg in such a way that each blastomere extends from the free surface of the blastula to its center (Fig. 1). Individual blastomeres are, therefore, thin conical cells, each possessing a portion of the original cortex of the egg. This is accomplished, as was pointed out by Wolpert and Gustafson (1961 *b*) and confirmed by us, through radial cleavages in which the long axis of the mitotic spindle is oriented tangentially to the egg surface. When these conical cells divide, they shorten, round up, and adopt a position near the surface of the embryo. After division the cells reextend to their conical form.

Phase-contrast microscopy of living blastulae shows clear separations along the lateral borders of individual cells below the terminal bar, indicating that in the living organisms the cells of the blastula are not closely adherent but rather are separated by a considerable extracellular space. This separation, while undoubtedly exaggerated by the shrinkage which results from fixation, dehydration, or embedding (Fig. 1), is at least representative of the native state. Only at their apicolateral margins, where septate desmosomes are found, are the blastomeres closely adherent (for micrographs of these desmosomal connections see Balinsky, 1959; Wolpert and Mercer, 1963). The apical region of each cell is further specialized in possessing a single cilium (Fig. 2).

As the blastula develops, the individual blastomeres shorten, and the blastocoel appears (cf. Figs. 1 and 6). At the same time a very thin basement membrane (similar to that illustrated in Fig. 4) is deposited beneath the basal surface of the cells. It delimits the developing blastocoel.

DISTRIBUTION OF CELL CONTENTS: The Golgi apparatus is located between the nucleus, which is situated near the apical end of the cell, and the free surface of the cell (Fig. 2). In thin sections this organelle appears as stacks of parallel, flattened cisternae. In a three-dimensional reconstruction it has the form of a funnel whose wide end sits on the nucleus and whose narrow end lies just beneath the basal body of the cilium. The apical region of this cell also contains a centriole which is oriented normal to the basal body yet at a distance of 0.2 μ from it. In some cells, which we believe to be primary mesenchyme precursors, there are at least two centrioles in addition to the basal body. Such centrioles are characteristically perpendicular to each other in one plane and perpendicular to the basal body in another.

DISTRIBUTION OF MICROTUBULES: In the apical end of the cell, most of the microtubules converge towards two or three points closely associated with the basal body (Fig. 2). Some of the

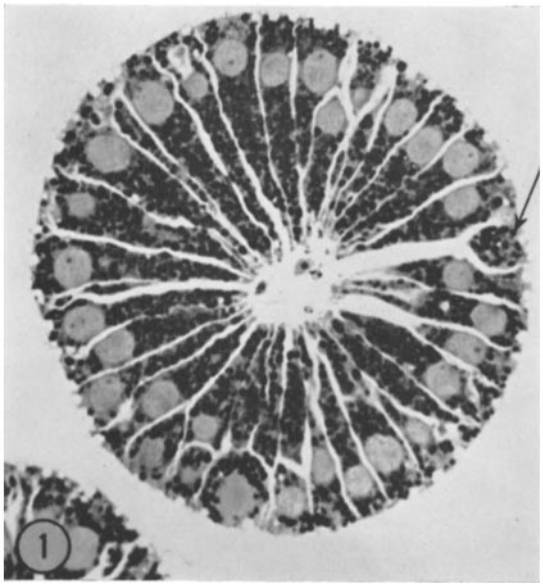


FIGURE 1 Light micrograph taken of a section cut through an early blastula. Each long conical-shaped blastomere has its nucleus located near its broad or apical end. Within each nucleus there are one or two nucleoli. Yolk granules are scattered throughout the cytoplasm. The basal surfaces of these cells make contact with the expanding blastocoel. The cell indicated by the arrow is in the process of, or has just completed division. Characteristically when mitosis occurs, the cell in question rounds up and adopts a position near the free surface of the blastula; its only attachment to its neighbors is at this surface. After division the daughter cells elongate and transform into the conical shape of the parent cell. × 150.

convergent tubules actually become confluent with these points which are perhaps better referred to as satellites (Figs. 3 *a* and *b*).

We do not know the exact number of satellites involved. The tubules diverge from this region; some lie parallel or nearly parallel to the apical cell surface; the majority, however, are oriented parallel to the long axis of the cell (Figs. 2, 5 *a* and *b*). In that part of the cell basal to the nucleus, the microtubules generally appear in groups of 2–10, often in arrays close to the cell surface.

The Midblastula and Events Culminating in the Formation of Primary Mesenchyme

By the midblastula stage, the fertilization membrane has broken down, and the embryo has become free-swimming. The vegetal pole cells destined to become primary mesenchyme undergo pulsatory movements just prior to their migration into the blastocoel. The newly formed primary mesenchyme cells seldom, if ever, enter the animal half of the blastocoel (Gustafson and Wolpert, 1961, 1963).

Although prior to the formation of the archenteron it is difficult to distinguish in thin sections the vegetal half of the blastula from the animal half, we have identified Figs. 6–9 as having been cut through the vegetal pole of an embryo fixed during the release of the primary mesenchyme into the blastocoel.

CELL SHAPE: During the course of blastulation the large, conical cells of the early blastula shorten by about 50% and assume a more columnar form (Fig. 6). Concurrently short evaginations ("basal lobes") from the basal surfaces of certain cells (Figs. 6–8) penetrate the thin basement membrane and lie naked in the blastocoel, attached to the cell only by slender stalks. Besides developing basal lobes, these mesenchyme precursors lose their cilia and extend numerous irregular projections from their apical surfaces (Figs. 7 and 9). At their lateral margins the cells interdigitate to a greater extent, essentially clasping each other (Fig. 7). The septate desmosomes disappear from the apicolateral margins.

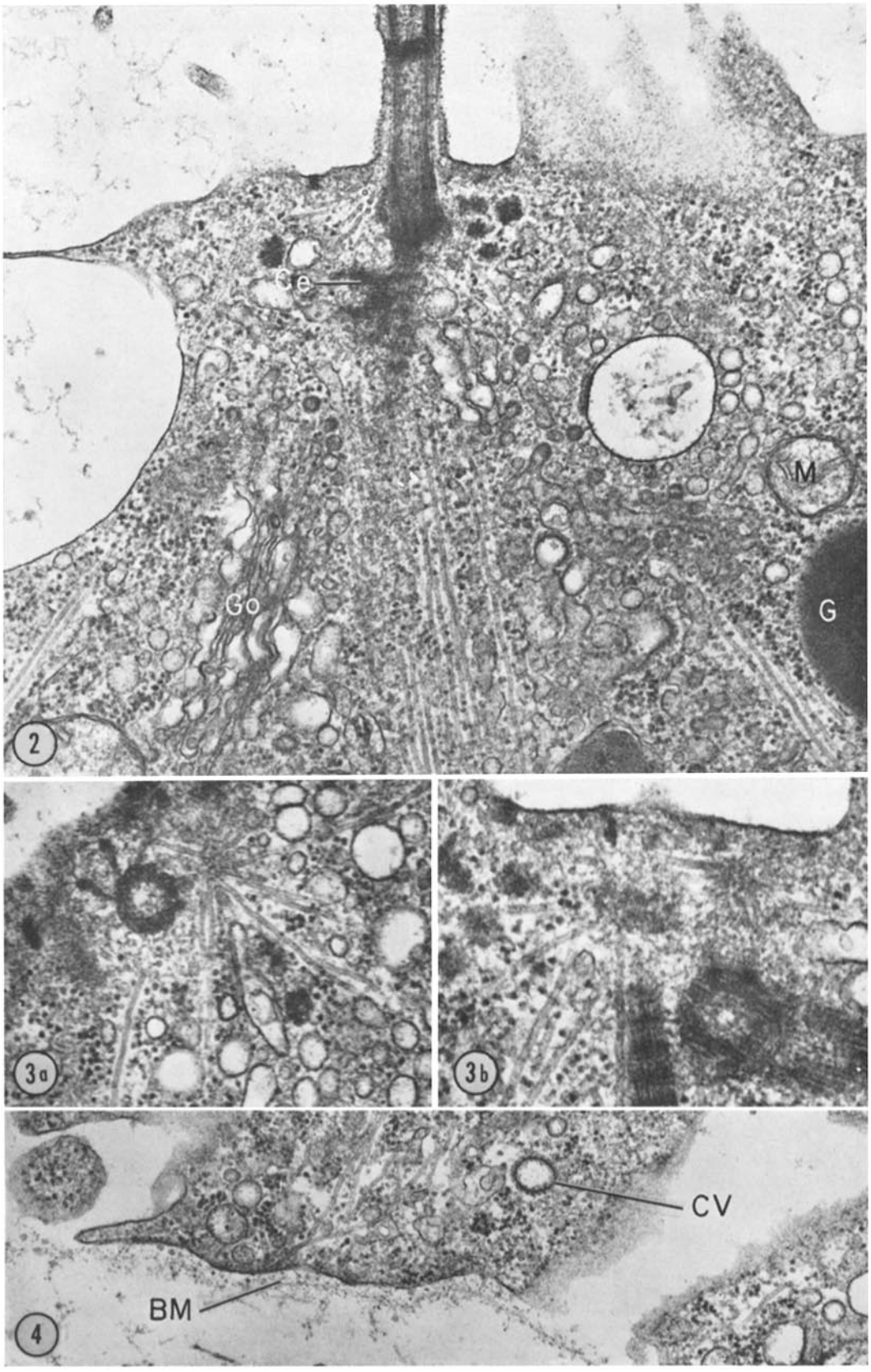
DISTRIBUTION OF CELL CONTENTS EXCLUSIVE OF MICROTUBULES: No important changes were observed in the ectodermal cells except for those at the vegetal pole. In the latter the Golgi apparatus, although still made up of

FIGURE 2 Section through the apical end of an ectodermal cell of an early blastula. A single cilium projects from its free surface. At the base of the cilium there is a basal body from which a portion of a rootlet can be seen extending basally. To one side of the rootlet a portion of a centriole (*Ce*) can be made out. The Golgi complex (*Go*), which is made up of a number of stacks of flattened cisternae and associated vesicles, takes the form of a cone whose narrow end points towards the basal body of the cilium and whose broad end opens on to the nucleus. Of greatest interest here are the microtubules which appear to converge around the basal body. These elements are very numerous and are frequently found in closely associated pairs. Sections of mitochondria (*M*) and yolk granules (*G*) are visible as well. × 39,000.

FIGURE 3 a Transverse section through the basal body of the cilium. Mircotubules appear to converge on a spot to the right of the basal body. We have termed this region a satellite. × 52,000.

FIGURE 3 b Longitudinal section through the apical end of an ectodermal cell in the region of the basal body. This section, although cut parallel to the basal body of the cilium, does not pass through it. Included in this section is a portion of the ciliary rootlet and two centrioles. Of greatest interest are two satellites on which the microtubules appear to converge. Note that they are not parallel to each other; one is situated nearer the free surface than the other. × 64,000.

FIGURE 4 Micrograph of the basal portion of a late blastula cell. A thin, finely fibrous layer, the basement membrane (*BM*), separates the ectoderm from the blastocoel. Microtubules are commonly encountered extending from these basal surfaces where they appear to originate (or terminate) on small dense zones closely associated with the limiting membrane. Coated vesicles (*CV*) are present in this region as well. × 36,000.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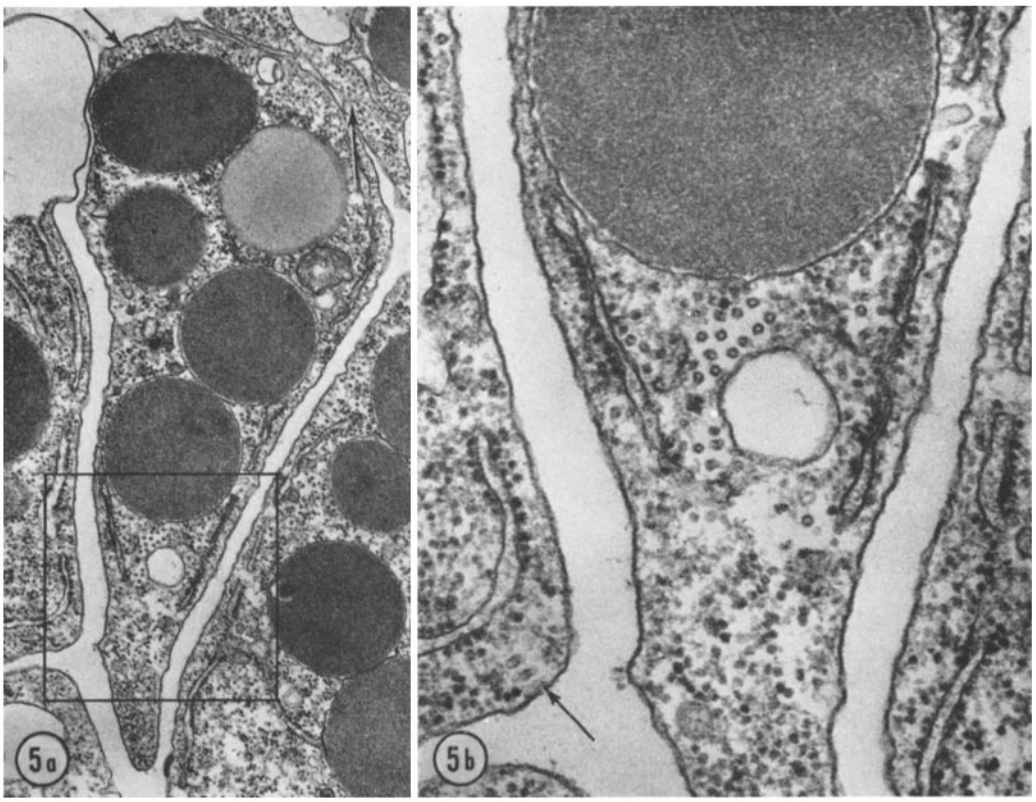


FIGURE 5 a Transverse section through the basal half of an early blastula cell. Within the cytoplasm are yolk granules, mitochondria, and rough-surfaced endoplasmic reticulum. The cisternae of the latter always lie parallel to the limiting membrane. The arrows indicate clusters of microtubules cut in transverse section. × 20,000.

FIGURE 5 *b* Higher magnification of a portion (within rectangle) of Fig. 5 *a*. Note the cluster of microtubules (arrow) × 60,000.

stacks of flattened cisternae, is no longer organized in the form of a cone. For the most part the cisternae lie parallel to the cell surface (Fig. 9), just above the nucleus. Of particular interest is the appearance of basal lobes illustrated in Figs. 6–8. Ribosomes and short segments of ER (endoplasmic reticulum) cisternae are prominent in these, while yolk granules and mitochondria are completely excluded (Figs. 7 and 8).

DISTRIBUTION OF MICROTUBULES: In vegetal pole ectodermal cells, the microtubules no longer focus on satellites around the basal body as they do in the other ectodermal cells, but instead they appear randomly distributed in the apical cytoplasm (Fig. 9). Some pass into the newly formed apical extensions, others pass into the basal lobes, and still others lie parallel to both the short and long axes of the cell.

Newly-Formed Primary Mesenchyme Cells

CELL SHAPE: Upon entry into the blastocoel the newly-formed primary mesenchyme cells assume a spherical outline (Figs. 6 and 10 *a*). Shortly thereafter pseudopodia grow out, and the cells begin to migrate actively around the blastocoel apparently by means of these pseudopodia (Gustafson and Wolpert, 1961).

DISTRIBUTION OF CELL CONTENTS: The cytoplasm of these newly-formed mesenchyme cells can be roughly divided into two discrete regions (Figs. 6 and 10 *a*). Of these, the larger contains the nucleus, the Golgi apparatus, all the yolk gran-

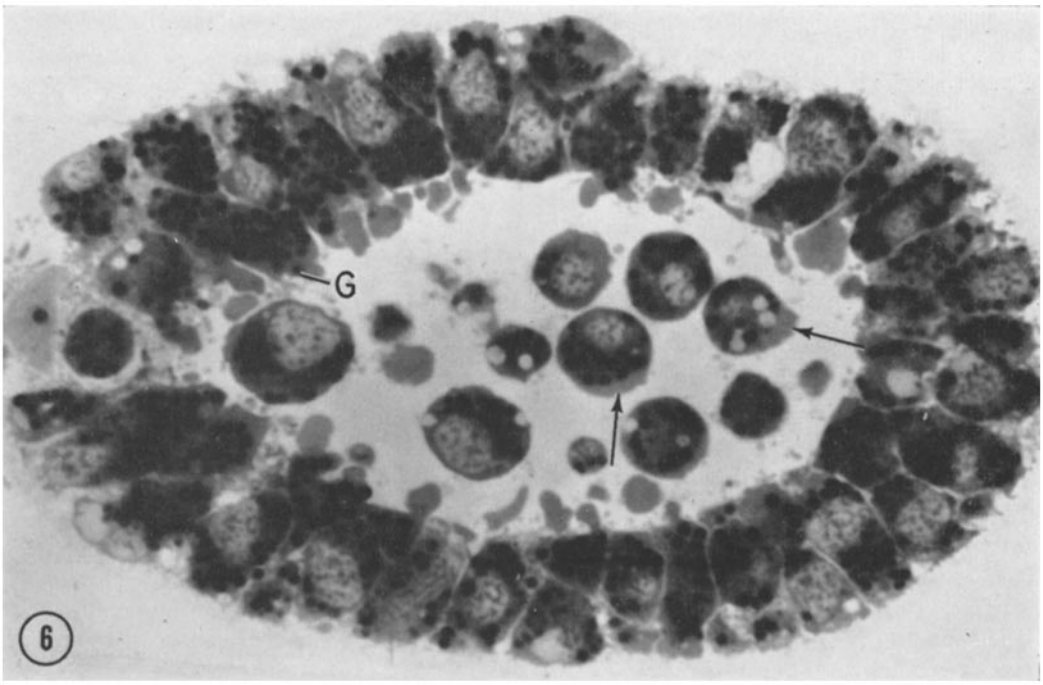


FIGURE 6 Section cut equatorially through the vegetal pole of a late blastula. Of particular interest are the bulbous pseudopodia or basal lobes extending from the blastocoel surface of the ectodermal cells. These lobes stain homogeneously and do not contain the large dense yolk granules (*G*) or other smaller, less heavily stained granules. Within the blastocoel proper are portions of at least nine newly-formed primary mesenchyme cells. These are nearly spherical in shape and show along one side or pole a homogeneous region (see arrows) similar in characteristics to the cytoplasm in the basal lobes × 300.

ules and mitochondria, and some of the ribosomes and rough-surfaced ER. The other region, which is smaller and yet easily separated from the former even with the light microscope (arrows in Fig. 6), is invariably located near one margin of the cell and thus appears as a cap relative to the rest of the cell. It characteristically contains mainly ribosomes and short segments of the rough-surfaced ER.

The cytocentrum occupies a position near the exact center of the larger mass of cytoplasm (Fig. 10 *a*). The Golgi complex consists of stacks of parallel and flattened cisternae which, in three dimensions, are arranged in the form of a funnel with its narrower end pointing towards the centriole and its wider end pointing towards the cap.

DISTRIBUTION OF MICROTUBULES: The microtubules are so distributed in the cell that they all appear to converge on the cytocentrum. In favorable sections through this region they appear to make direct contact with the centriole (Figs. 10 *b* and *c*) and to diverge in all directions as radi from this body, even extending into the ribosome-rich cap.

Differentiation of Primary Mesenchyme Cells into a Skeleton-Producing Structure

The nearly spherical cells of the newly-formed primary mesenchyme have arisen from the primitive blastomeres, therefore, by a definite sequence of changes in cell shape. From the slender conical form of the early blastula, they shorten to the cuboidal shape of the late blastula and then form distinctive apical and basal extensions just prior to their immigration into the blastocoel where they immediately become spherical. From this stage onwards the primary mesenchyme cells undergo an independent course of development, an essential part of which is the production of a variety of polymorphic forms resulting in the patterned skeleton of the pluteus.

As noted earlier, shortly after the newly-formed primary mesenchyme cells have entered the blastocoel, they form pseudopodia and begin to explore the surface of the blastocoel. Their pseudopodial activity has been analyzed by Gustafson and Wolpert (1961) and Okazaki (1960). According to these authors, the cells use their pseudopodia, which are up to 30 μ in length, in their exploratory migrations.

Primary Mesenchyme Cells During Their Migratory Phase

The morphology of the cell body, as distinct from the pseudopodia, is not greatly different from that already described in the newly-formed mesenchymal cells. The mitochondria frequently, although not always, cluster around the base of the pseudopodium and so are not as before randomly distributed in the cells. Of great interest, however, is the change from the previous stage in the distribution and orientation of microtubules. No longer do these elements converge on a *single point* in the cell as they did in the newly-formed mesenchymal cell. Instead they appear almost randomly oriented throughout the cytoplasm of the cell body, except in the pseudopodium where they always lie parallel to its long axis (Figs. 11 *b* and 12).

The pseudopodia possess besides microtubules a distinctive population of organelles. The most prominent are ribosomes, microtubules, short segments of ER, a few mitochondria, and special vesicles (Figs. 11 *a* and *b*, 12). Mitochondria, as mentioned above, are frequently clustered around the base of the pseudopodium.

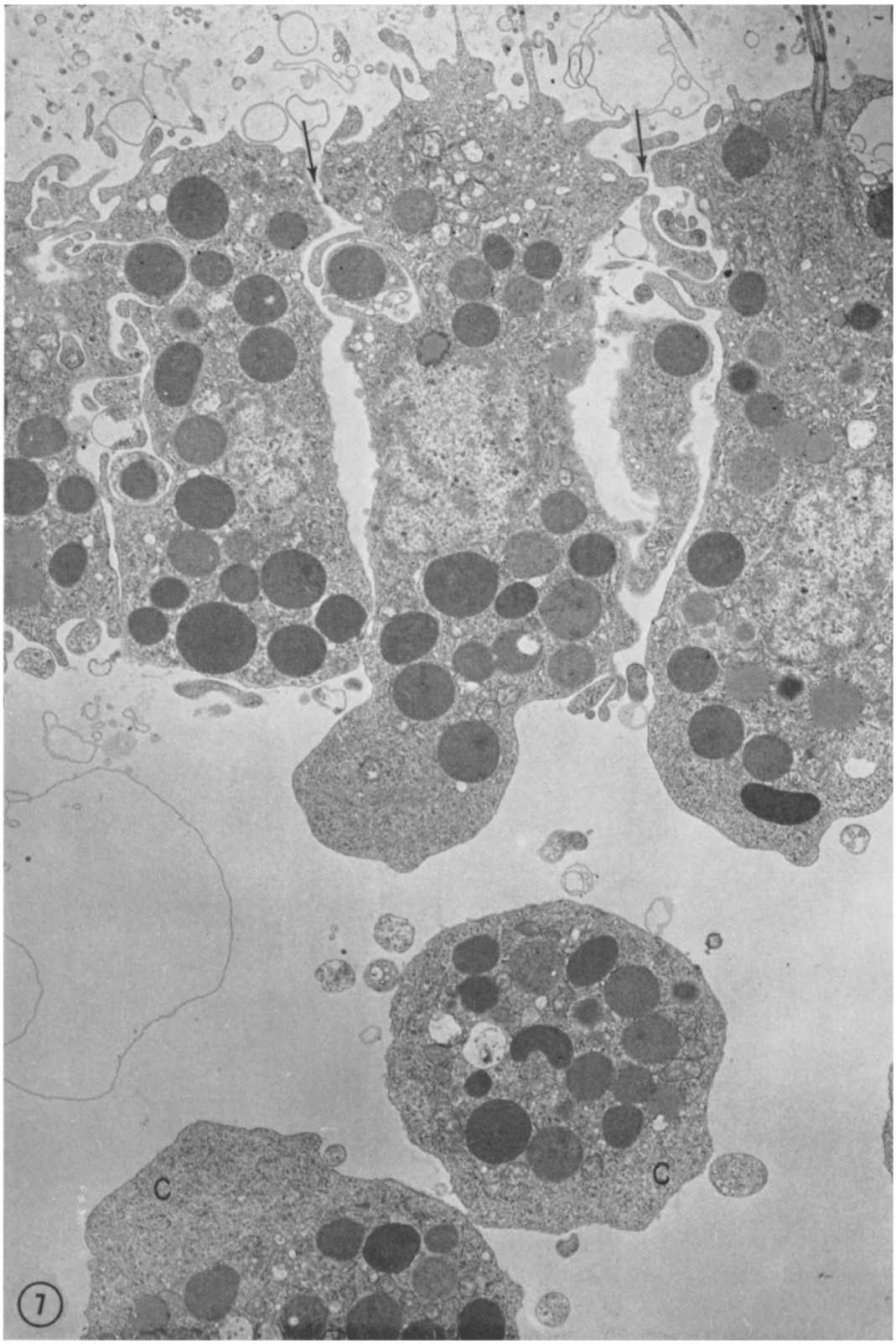
The vesicle population in the pseudopodia should be reported in greater detail, for they may relate to subsequent events in skeletal deposition. These vesicles consist of the following three distinct types: a smooth surfaced single vesicle varying in diameter from 100 to 400 mμ (e.g. see Fig. 11 *b*), a coated vesicle generally about 100 mμ in diameter (Fig. 12), and a so-called "double" vesicle (Fig. 12). The latter is made up of two or more closely adherent vesicles which appear to share in common a portion of the membrane between them.

Primary Mesenchyme Cells During and Following Cable Formation

Gustafson and Wolpert (1961) and Okazaki (1960) observed that at the level of resolution achievable with the light microscope the pseudopodia of the primary mesenchyme cells "fuse" to form cables. The cell bodies, however, remain distinct. As the embryo grows, the pseudopodial cables take precise orientations within the blastocoel, and their form appears to anticipate the deposition of the skeleton in such a way that angularities in the skeleton appear at the exact location of changes in the orientation of cable cytoplasm.

Our observations demonstrate that there is an actual cell fusion so that the cable is a true syncytium made up of cytoplasm from the pseudo-

FIGURE 7 Section cut through the vegetal pole of a late blastula. Projecting from the basal surfaces of the ectodermal cells are some lobes. These lobes correspond to the basal lobes seen by light microscopy (Fig. 6) and are undoubtedly related to the pulsatory pseudopodia of Gustafson and Wolpert (1961). They appear on the basal surfaces of the ectodermal cells which are in the process of migration into the blastocoel to become the primary mesenchyme. The lobes are rich in ribosomes and cisternae of rough-surfaced ER, and they lack, or contain very few of, the larger formed elements of the cytoplasm. The outer or apical surfaces of these ectodermal cells is thrown up into numerous, irregular projections which extend into the hyaline later. The septate desmosomes, normally encountered on the apicolateral margins of the ectodermal cells, have disappeared or are in the process of disappearing (see arrows), but the cells interdigitate with each other as if embracing each other. The ectodermal cells of an early blastula (Fig. 1) and the ectodermal cells at the animal pole region of a later blastula or gastrula do not interdigitate in this fashion. In the blastocoel one can distinguish portions of two newly-formed primary mesenchyme cells. Most interesting here is the cap (*C*) situated on one side of these cells; its contents are identical with that present in the basal lobes and thus can be distinguished from the rest of the cytoplasm. We have concluded that when the ectodermal cells migrate into the blastocoel, the contents of the basal lobe remain segregated as a ribosome-rich cap on one margin of the now spherical newly-formed primary mesenchyme cell. × 11,000.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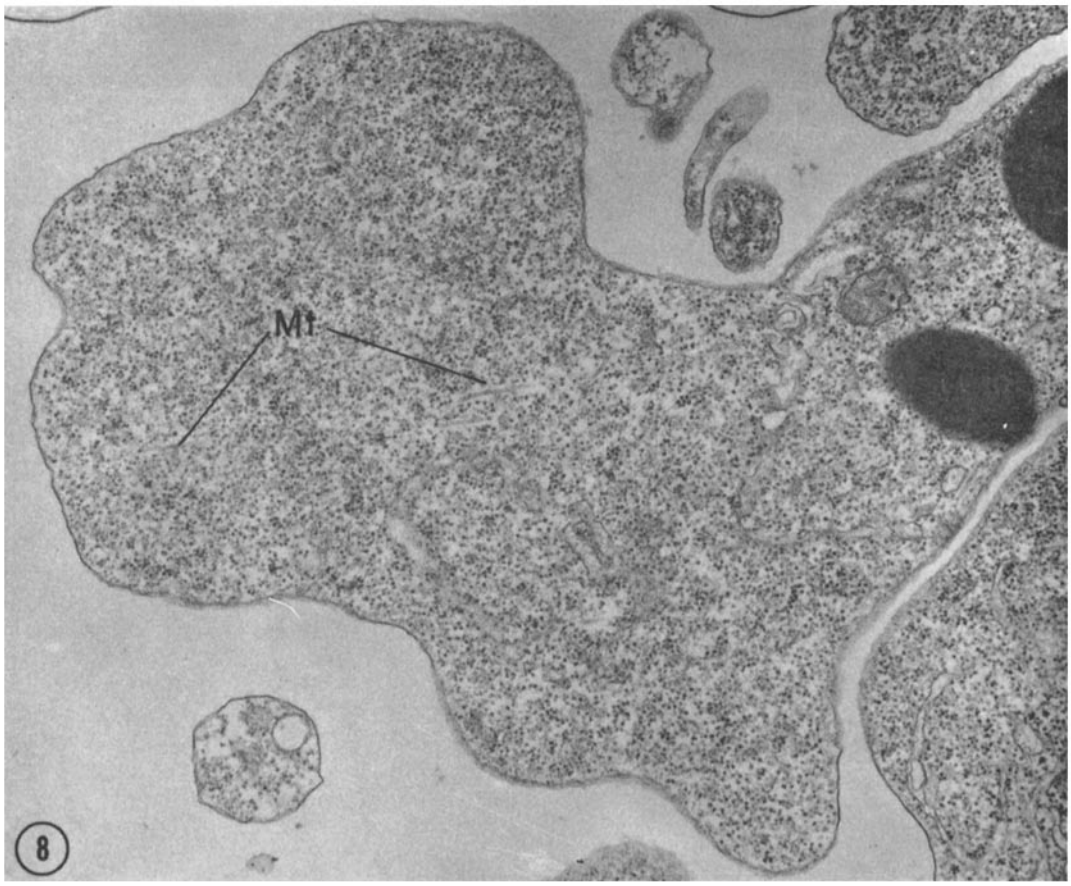


FIGURE 8 A basal lobe at higher magnification. Within this structure one can make out numerous ribosomes, a few short cisternae of rough-surfaced ER, and some microtubules (*Mt*). × 28,000.

podia of a number of cells. As indicated above, the portion of the cell containing the nucleus and much of the cytoplasm which we will refer to here as the cell body remains distinct from the cell bodies of adjacent cells whose pseudopodia have fused to form the cable. The cell bodies are connected to the cable by a number of thin stalks about 0.5 μ in diameter and 1–2 μ in length (Fig. 13). Deposition of skeletal spicules (Figs. 18 and 19) within the cable cytoplasm follows cable formation.

The Cell Body

CELL CONTENTS: The Golgi complex appears as units of stacked lamellae which lie adjacent to the side of the nucleus nearest the cable (Figs. 13 and 15). Most of the yolk granules and short segments of ER occupy the opposite pole. In one instance, after extensive searching, we found a pair of centrioles, but unlike the situation in the ectodermal cells (Fig. 5), these bodies were not oriented with their axes normal to each other. Instead they lay nearly parallel to each other near the nuclear envelope in the region of a Golgi complex.

DISTRIBUTION OF MICROTUBULES: The microtubules appear randomly arranged in the cell body (Fig. 16). None of them make contact with either centriole but seem rather to terminate on small densities ("satellites") located near the nuclear envelope. Other than this, the points of origin or insertion for the microtubules in the cell body have not been located

The Stalks

We have thus far been unable to determine the precise number of stalks which connect the cyto-

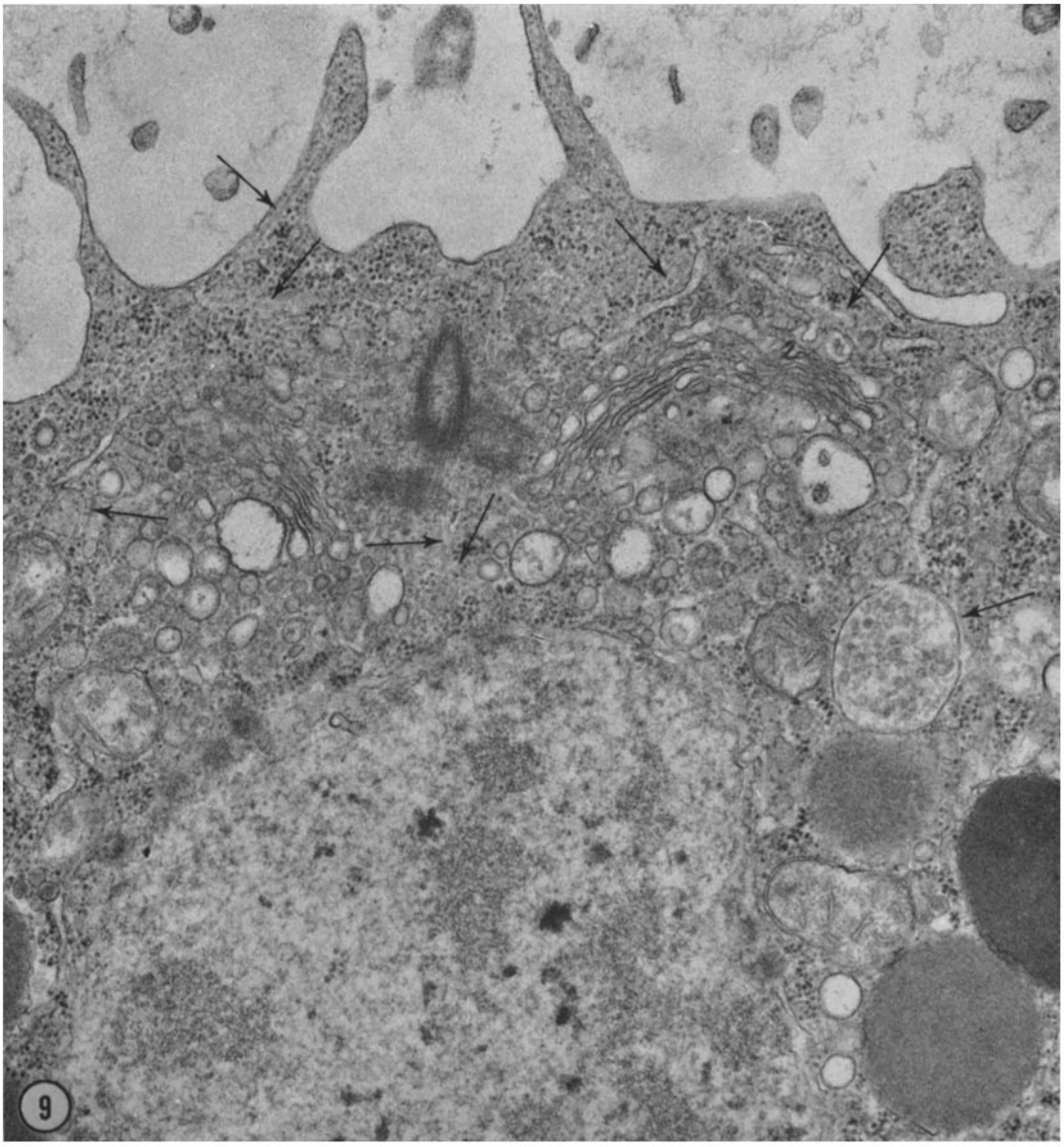


FIGURE 9 The apical end of an ectodermal cell at the vegetal pole region. This cell, if allowed to continue development would presumably have migrated into the blastocoel as a primary mesenchyme cell. The Golgi zone consists of stacks of flattened cisternae and is situated around the basal body which is seen here in oblique section. The cisternae are nearly parallel to the free surface; they are not organized in the form of a cone as they are in the ectodermal cells of the early blastula. The microtobules (arrows) no longer focus on the basal body as they did in Fig. 2. × 35,000.

plasm of the cell body to the cytoplasm of the cable or whether indeed a precise number exists. Generally two such connections are present within each section cut parallel to the cable (Fig. 13).

Of greatest interest in the cytoplasm of the stalk is the high concentration of microtubules which parallel the long axis of the stalk (Fig. 14 *a*); they extend into the cable cytoplasm.

The Cable

DISTRIBUTION OF ORGANELLES IN THE CABLE: The cable cytoplasm contains a large number of mitochondria, microtubules, and vesicles both of the double and coated variety. Yolk granules, oil droplets, the Golgi complex, and nuclei were never encountered. Two types of processes extend from the cable. One type, illustrated in Fig. 17, is characterized by its slender dimensions (0.1 μ in diameter) and its content which includes a bundle of filaments each about 50 A in diameter. The second type of process is much longer and contains mitochondria, ribosomes, vesicles, and microtubules. When these larger processes make contact with the basement membrane underlying the ectoderm, they generally do so at the point of junction of three or more ectodermal cells (Fig. 13).

DISTRIBUTION OF MICROTUBULES: Almost all the microtubules present in the cable cytoplasm are parallel to its long axis (Fig. 14 *b*). Some of the microtubules have been seen to extend obliquely from the stalk into the cable cytoplasm (Figs. 14 *a* and *b*).

SKELETAL DEPOSITION: The skeleton, illustrated in a micrograph of a living pluteus (see Fig. 18), is not preserved by routine procedures for electron microscopy. Most of this loss occurs in the final stage when sections are cut. Apparently the skeleton dissolves out of the sections either during or following the cutting process, and this has not been prevented as hoped by highly alkaline solutions in the knife-trough.

Several observations on skeletal deposition can be made, however. The first is that the skeleton forms within a vacuole in the cable cytoplasm (Fig. 19). Secondly, an electron-opaque material is nearly always present near the limiting membrane of the vacuole. Similar dense material is found in coated vesicles near the skeletal vacuole (Fig. 19). The center of the skeletal vacuole, which most likely contains almost pure calcium carbonate, invariably disappears from the section, leaving a hole in the Epon.

EXTRACELLULAR FIBRRILS: Small extracellular fibrils approximately 150 A in diameter are present in the blastocoel (Figs. 15 and 17). Frequently these structures extend from the basement membrane to the cable, acting perhaps as a primitive type of connective tissue.

DISCUSSION

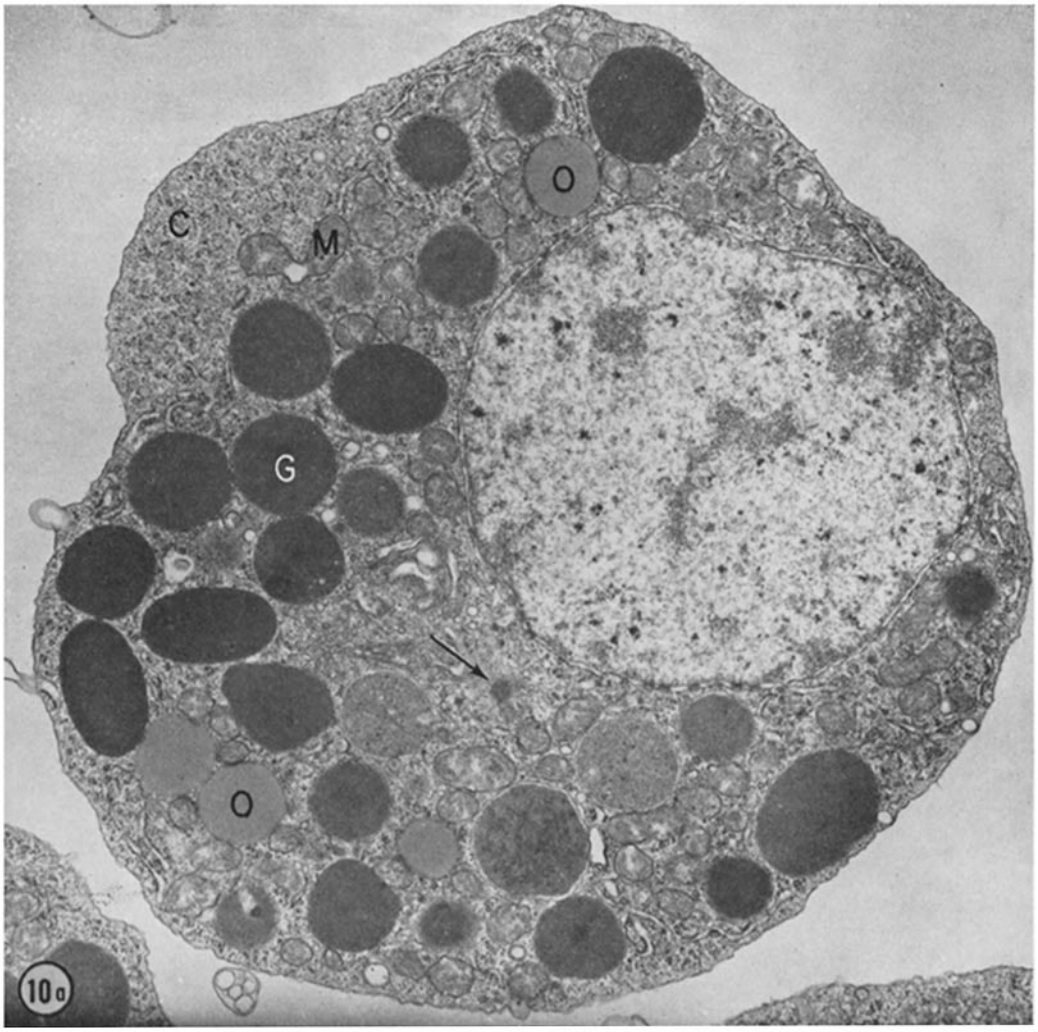
Microtubules and the Production of Cell Shape

In the observations presented above we have demonstrated that during the formation and development of the primary mesenchyme, individual cells undergo at least five distinct changes in cell shape. These changes are diagrammatically depicted in Fig. 20. At each stage the cytoplasmic microtubules are distributed in such a way that they could influence the shape of the cell at that particular stage (see Fig. 21). If, as seems indicated by available observations, these elements anticipate in their growth and distribution the develop-

FIGURE 10 *a* Low-power electron micrograph of a section cut through an entire primary mesenchyme cell. This cell was found in a section adjacent to that illustrated in Fig. 6. The nucleus, surrounded by yolk granules (*G*), oil droplets (*O*), and mitochondria (*M*), is off to one side of the cytocentrum. The latter, near the exact center of the cell, contains a centriole (see arrow). The Golgi apparatus consisting of two stacks of flattened cisternae lies to one side of the centriole. Throughout the remainder of the cytoplasm there are ribosomes and short segments of rough-surfaced ER. In the upper left-hand portion of the cell is a cap (*C*) easily identifiable by the complete absence from this region of yolk granules, oil droplets, and mitochondria; instead of larger structures this cap contains an almost pure population of ribosomes, many of which have aggregated into polysomes. This region corresponds to the homogeneous region pointed out by the arrows in Fig. 6. × 16,000.

FIGURE 10 *b* Higher magnification of the centrosphere. Microtubules in large numbers converge on the centriole. Their arrangement is reminiscent of the spokes of a wheel. × 70,000.

FIGURE 10 *c* The centrosphere of an adjacent cell. In this micrograph the normal structure of the centriole can be seen with microtubules converging on it. × 70,000.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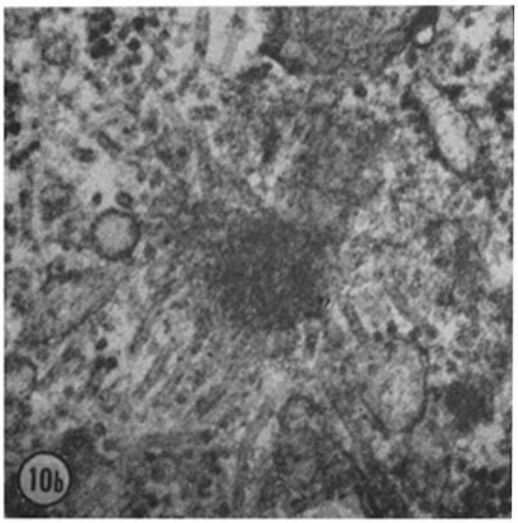
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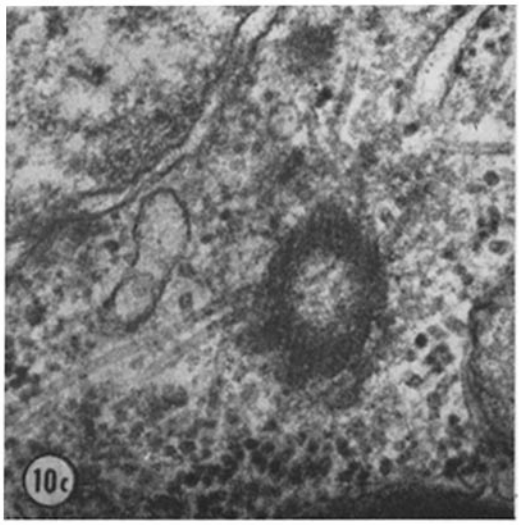
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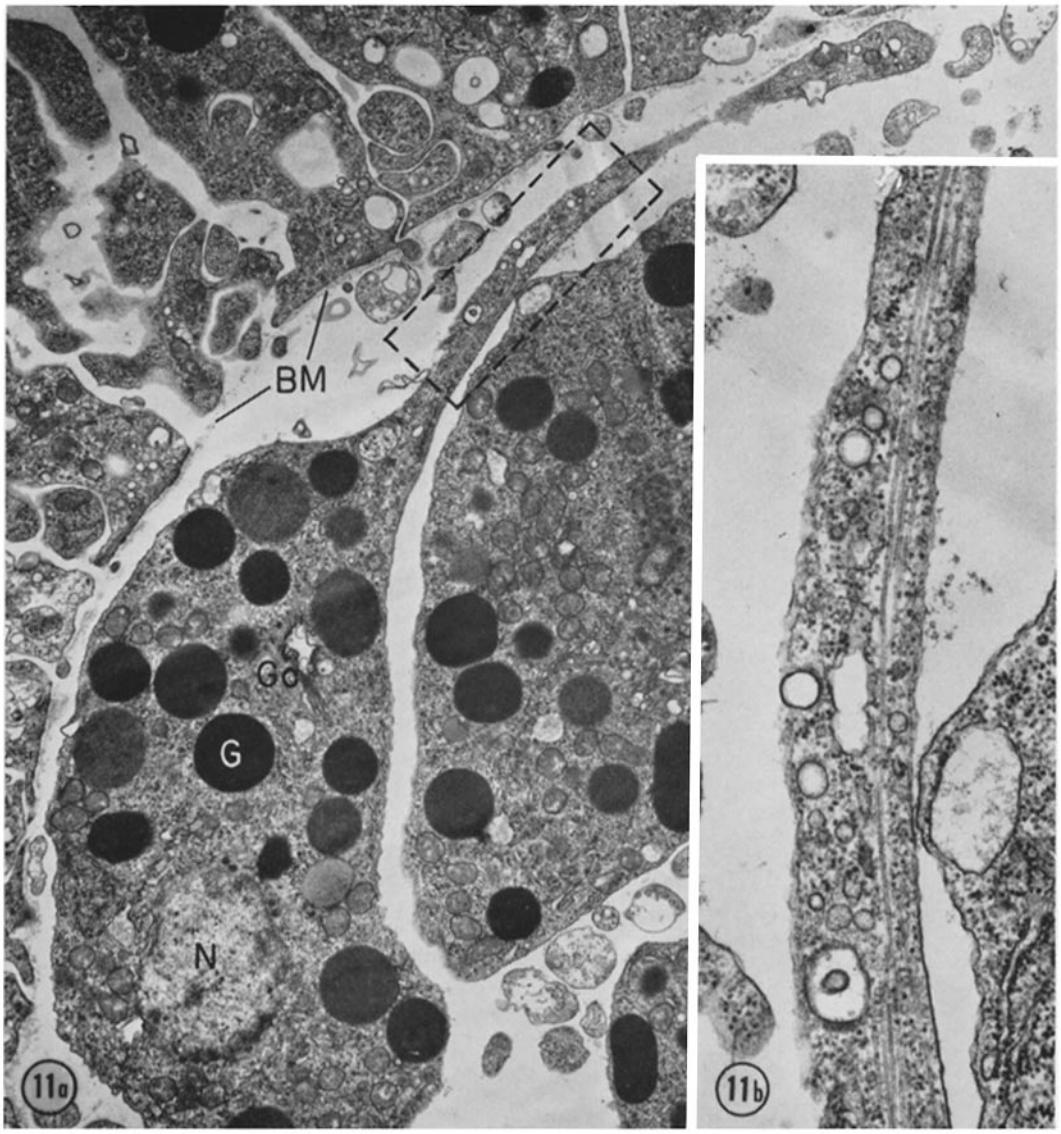


FIGURE 11 *a* Electron micrograph of a primary mesenchyme cell fixed during its exploratory movements in the blastocoel. Extending from this cell is a long pseudopodium which has been implicated in the movements of these cells (Gustafson and Wolpert, 1963). This cell lies in the blastocoel near the ectoderm. It is separated from the ectoderm by a tenuous basement membrane (*BM*). A portion of the ectoderm can be seen on the left of this micrograph. Within the main body of the cell one can easily distinguish the nucleus (*N*), yolk granules (*G*), the Golgi apparatus (*Go*), numerous mitochondria, and rough-surfaced ER. With the exception of a few mitochondria none of the above mentioned elements are visible in the pseudopodium. × 8,200.

FIGURE 11 *b* Higher magnification of a portion (within rectangle) of the pseudopodium illustrated in Fig. 11 *a*. In this process there are numerous ribosomes, small vesicles, and most prominently, a large number of microtubules. The latter lie parallel to the long axis of the pseudopodium and cluster together. × 85,000.

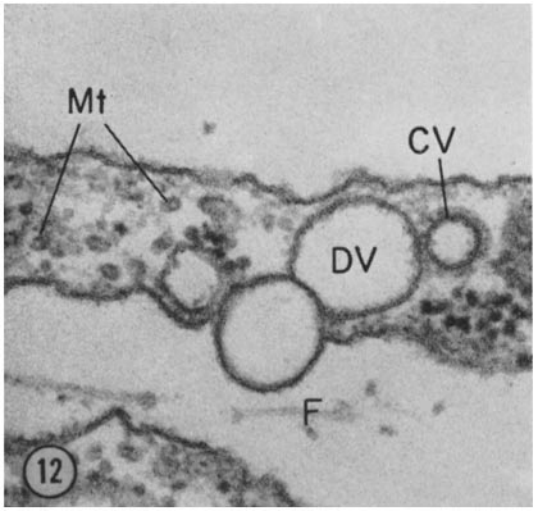


FIGURE 12 Cross-section through a portion of a pseudopodium of a primary mesenchyme cell. Prominent in this micrograph are the microtubules (*Mt*) and the vesicles. The latter appear as two distinct types, a coated vesicle (*CV*) and a double vesicle (*DV*). The double vesicle consists of two closely apposed vesicles which share at the point of contact a common membrane as well as a small patch of the plasma membrane which limits the pseudopodium. These paired vesicles are common in the primary mesenchyme cells. Present near this pseudopodium is an extracellular fibril (*F*). These fibrils are commonly found within the blastocoel. × 100, 000.

ment of cell form, then they may be assigned a responsible role in form differentiation. (Evidence that the microtubules play such a role is presented in the second paper in this series, Tilney and Gibbins, 1969).

Evidence in support of the concept of a temporal relationship between the asymmetry developed and the presence of microtubules has been gradually accumulating. The elongation of the developing cells of the lens (Byers and Porter, 1964; Arnold, 1966), the growth of cilia (Renaud and Swift, 1964), the differentiation of scales of insects (Overton, 1966), the growth of nerve processes (Peters and Vaughn, 1967), and the reextension of the axopodia of *Actinosphaerium* after retraction caused by low temperature (Tilney and Porter, 1967), hydrostatic pressure (Tilney et al., 1966), and colchicine (Tilney, 1968) all support this concept. The most convincing evidence that the microtubules may in fact control cell asymmetry comes from the recovery of the axopodia of *Actinosphaerium*, following low-temperature treatment (Tilney and Porter, 1967). Here it was demonstrated that many of the axonemes, known to be composed in large part of a bundle of microtubules, reform in the cell body of *Actinosphaerium* prior to the actual reextension of the axopodia. The regrowth of the axopodia is concomitant with the elongation of these axonemes which, in turn, when examined with the electron microscope, consist of a double-coiled array of microtubules.

There are striking similarities between the axopodia of *Actinosphaerium* and the long pseudopodia which extend in specified directions from the primary mesenchyme cells; the latter have frequently been described as "bristle-like" (Gustafson and Wolpert, 1963). In both systems one finds stiff processes; in both systems one observes oriented microtubules; and in both cases particles stream to and fro.

Since the microtubules in all these instances, and in particular in *Actinosphaerium*, seem directly related to the expression of a specific cell asymmetry, it is reasonable to conclude that cytoplasmic microtubules may play a similar role in the determination of the form of cells at each stage in the developmental sequence in *Arbacia* embryos (see Fig. 20). The most likely means by which they could perform this role is by giving form and orientation to the plasmagel. It is envisioned that in the particular case of the primary mesenchyme of *Arbacia* these gels and their distribution change with each successive form change. Eventually one of the forms is perpetuated in the skeleton.

Wolpert and Gustafson (1961 *a*) and Okazaki (1960) have shown that the $CaCO_3$ skeleton of the pluteus is deposited in the cable cytoplasm and that irregularities in the contour of the cable are faithfully reproduced in the skeleton. In the past, most investigators have felt that the position of the cable is determined by specific interactions between the ectodermal cells and processes which extend from the cable cytoplasm (Gustafson and Wolpert, 1961; Wolpert and Gustafson, 1961 *a*). However, in light of the transplantation studies of von Ubisch (1939, 1955), there is reason to ques-

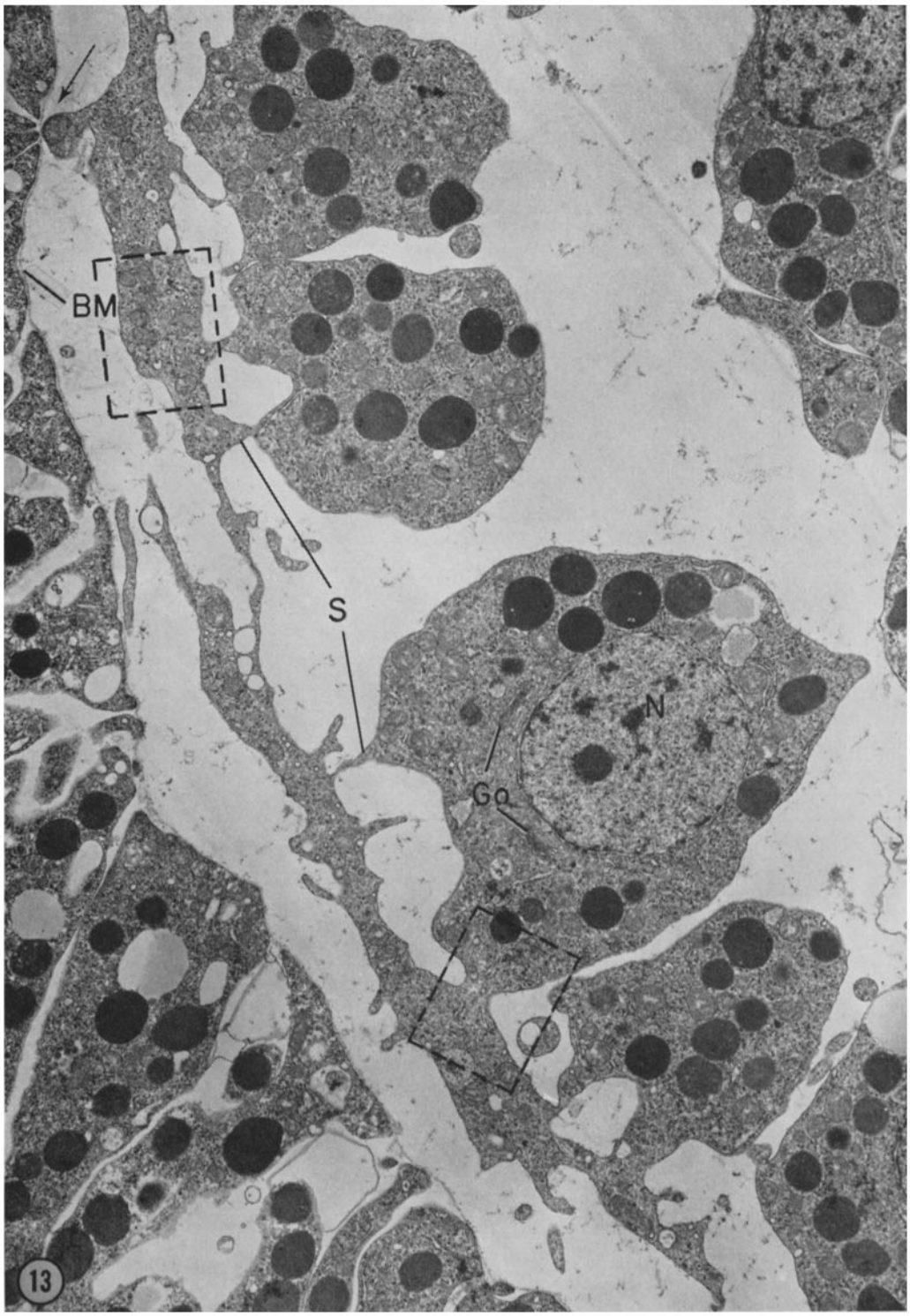
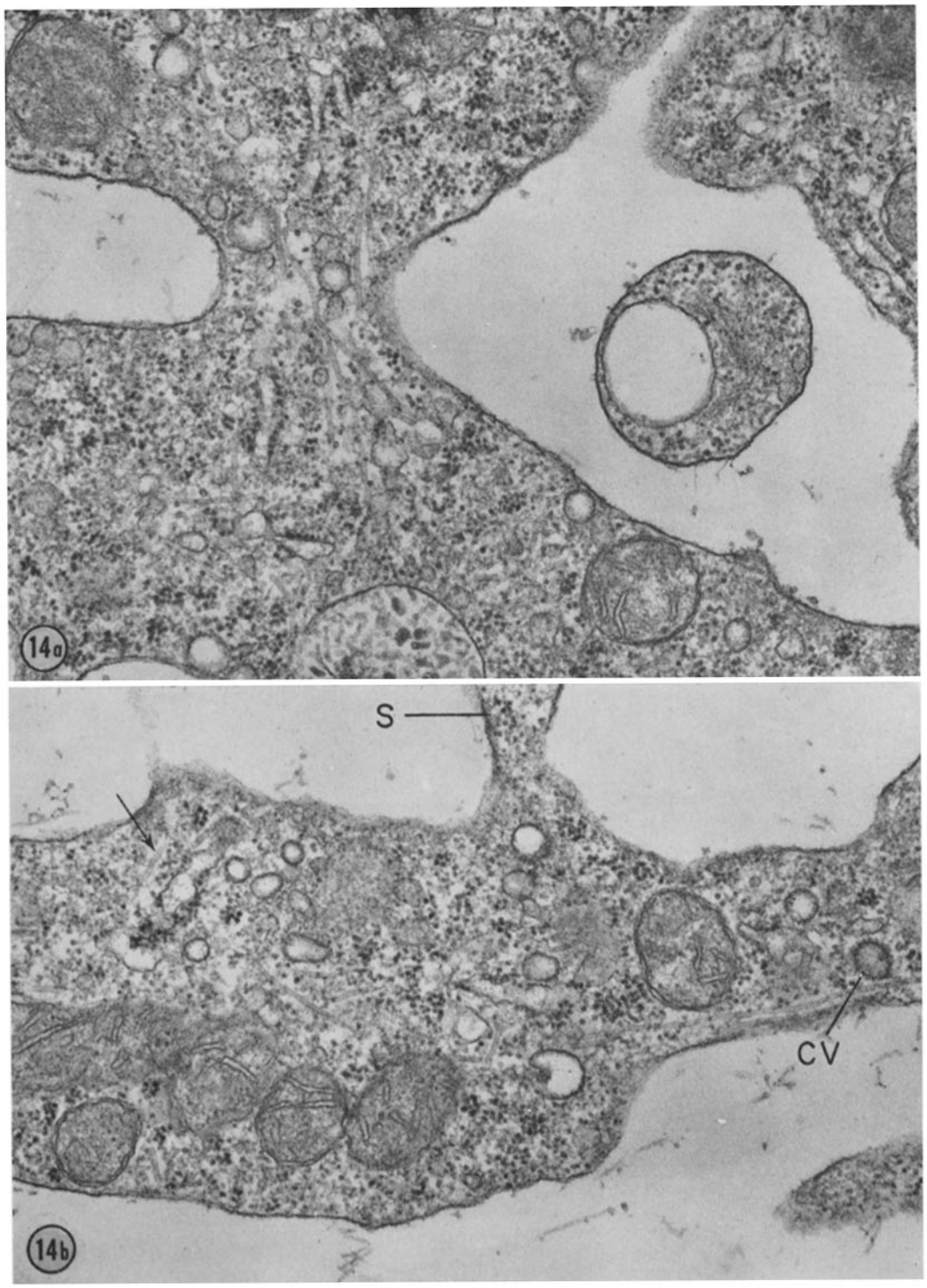


FIGURE 13 This low-magnification electron micrograph illustrates the form and arrangement of the primary mesenchyme, once the pseudopodia of several primary mesenchyme cells have fused to form a cable syncytium. The relation of the cell bodies to the cable cytoplasm and the relation in turn of the cable cytoplasm to the ectoderm can be easily observed. Each cell body is connected to the cable by one or more stalks (*S*), the cell bodies being without exception on the side of the cable opposite the basement membrane (*BM*) which underlies the ectoderm. Short processes extend from the cable cytoplasm towards the ectoderm (see arrow), but these have not been seen to penetrate the basement membrane. Within the cell body the nucleus (*N*) is in a central position. The Golgi zone (*Go*) lies on one side of the nucleus, and the other formed elements occupy the remainder of the cytoplasm of the cell body. Fine extracellular fibrils are present throughout the blastocoel. Squared-off areas are shown in Figs.



14 *a* and *b*. × 10,500.

Figs. 14 *a* and 14 *b* are micrographs of portions of Fig. 13, enlarged to show detail. These regions are indicated by the dotted lines on Fig. 13.

FIGURE 14 *a* This illustrates the distribution of microtubules in the narrow stalks which connect the cell bodies to the cable. These tubular elements are common in this region and lie parallel to the long axis of the stalk. × 42,000.

FIGURE 14 *b* This illustrates the distribution of microtubules within the cable cytoplasm. These elements generally lie parallel to the long axis of the cable. The microtubules in the stalk (*S*) extend into the cytoplasm of the cable, so some appear at oblique angles to the long axis of the cable (note arrow). Present also within the cytoplasm of the cable are mitochondria, ribosomes, and vesicles. The latter appear as smooth vesicles. coated vesicles (*CV*), and double vesicles. × 42,000.

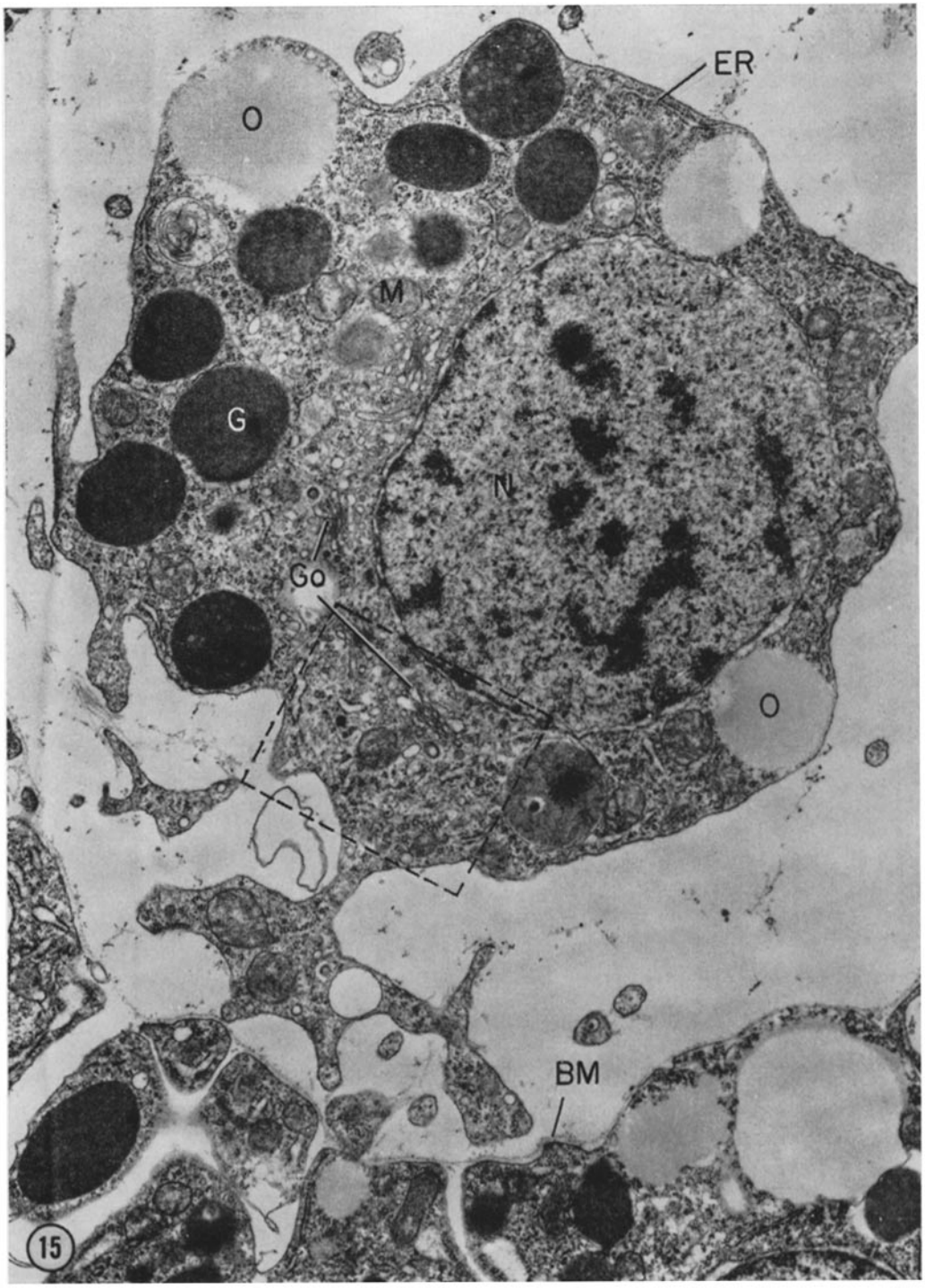


FIGURE 15 Low-magnification electron micrograph of a section cut approximately normal to that illustrated in Fig. 13. Thus the cable appears almost in cross-section. Illustrated in this micrograph are a cell body of a primary mesenchyme cell, a small portion of the cable cytoplasm, and the stalk connecting these two parts of the cell. Portions of the basal region of several ectodermal cells can be seen on the bottom of this micrograph on the opposite side of the basement membrane. Most prominent in the cell body is, of course, the nearly spherical nucleus (*N*). Along one margin of the nucleus is the Golgi apparatus (*Go*) appearing as a series of stacks of flattened cisternae. Peripheral to this complex are yolk granules (*G*), mitochondria (*M*), oil droplets (*O*), and rough-surfaced ER (*ER*). Small projections extend from the cable cytoplasm towards the ectoderm, a portion of which is seen in the lower portion of the micrograph. These processes make contact with the basement membrane (*BM*) but seem not to penetrate it. × 19,000.

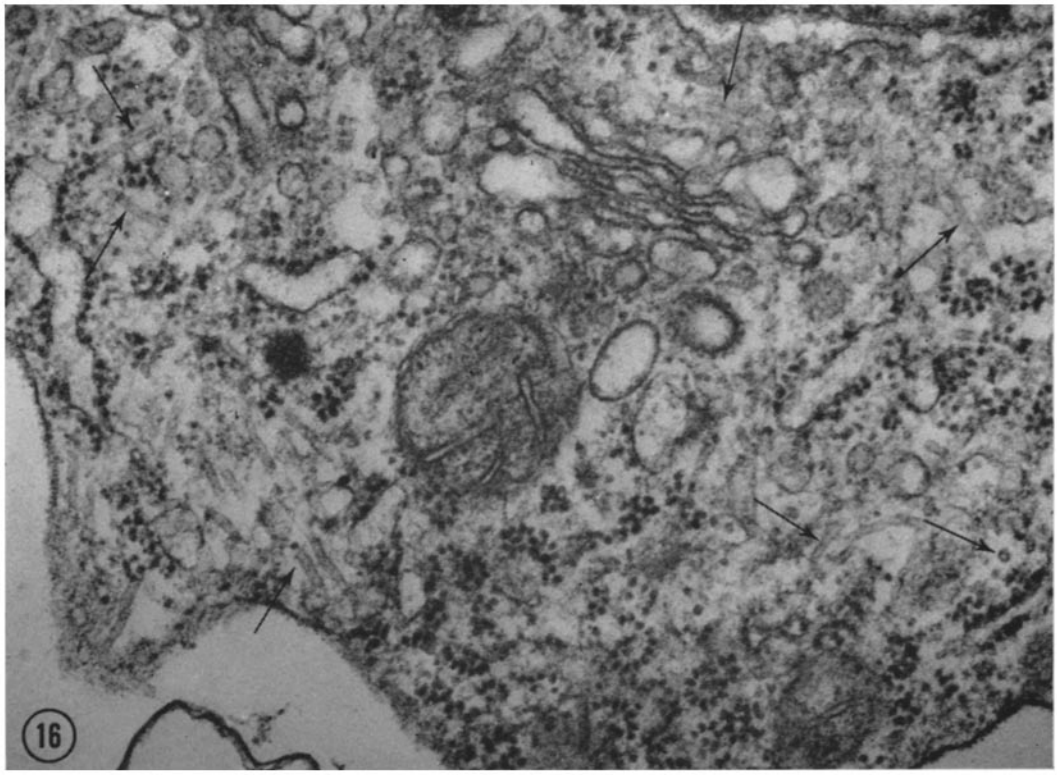


FIGURE 16 A higher magnification of a portion of the cell body illustrated in Fig. 15 (the region is indicated by the dotted lines in Fig. 15). This micrograph has been included to illustrate the distribution of microtubules in this region. Arrows have been placed on the micrograph to show that although there are numerous microtubules, they are randomly oriented and do not converge on any one point as they did in the newly-formed mesenchyme cells (see Fig. 10 *b*). × 70,000.

tion this interpretation. These latter studies indicate that the control of the skeletal pattern, which is species specific, does not reside in the ectodermal cells but rather resides in the mesenchyme. The micromeres (mesenchyme precursors) from one species were substituted for those of another, and the resultant skeletal pattern in the early stages appeared identical with the source of the mesenchyme and independent of the source of the ectoderm. From the preceding considerations the microtubules appear to be the components of the mesenchyme cells which possess the appropriate characteristics needed for the expression of this information.

To summarize then, we have demonstrated that during the development of the primary mesenchyme the microtubules undergo sequential changes in distribution and orientation which are reasonably related to changes in cell shape. The climax of these changes is the development of a $CaCO_3$ skeleton which is deposited within, and which corresponds exactly with the orientation of the cytoplasm of the cable. We have demonstrated that the microtubules are set out in patterns which we propose influence the development of cell form at each stage in the morphogenesis of the primary mesenchyme and finally the organization of the skeleton. If this contention is correct (see second paper in series, Tilney and Gibbins, 1969), one is stimulated to inquire what factor or factors determine the patterned distribution and orientation of microtubules in these cells. Whatever this component is, it must control the development of cell form as well. More will be said about this below.

Skeletal Deposition

In a detailed study of spicule formation in sea urchin larvae, Okazaki (1960) demonstrated that

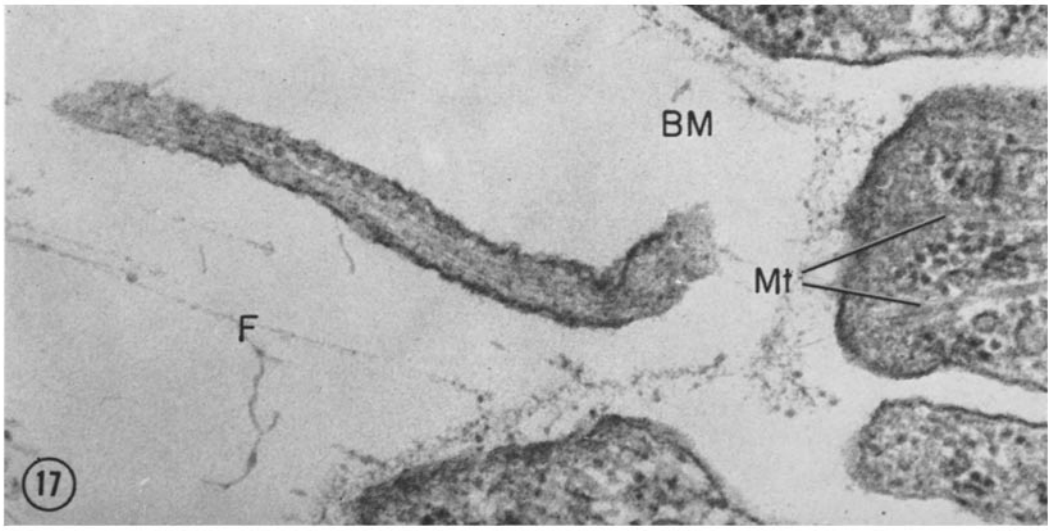


FIGURE 17 The slender process illustrated in this micrograph extends from the cable cytoplasm to the ectoderm. Within it are a number of fibrils each about 50 A in diameter. Just beneath the basal surfaces of the ectodermal cells, present on the right of this micrograph, is the basement membrane (*BM*). Extracellular fibrils (*F*) are frequently seen connected to this tenouus structure; they extend out into the blastocoel. Within the ectodermal cells, one can see a number of microtubules (*Mt*). These elements appear to terminate on an amorphous material which lies just beneath the basal plasma membrane. × 77,000.

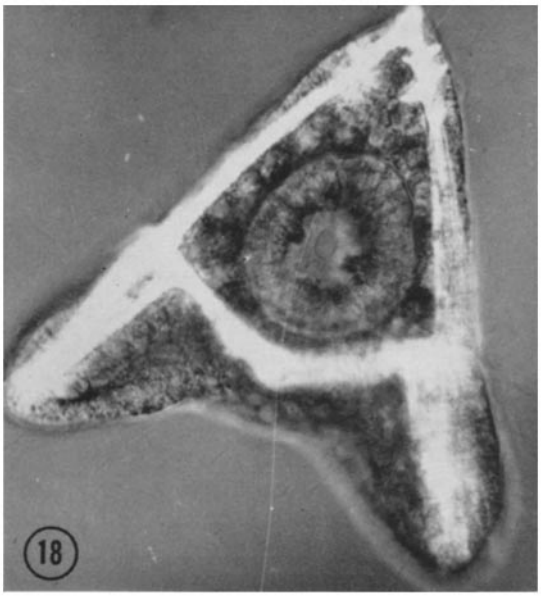


FIGURE 18 Micrograph taken with a polarizing microscope of an early pluteus larva at the prism stage. The skeleton is highly birefringent and can be easily recognized. × 170.

the skeleton of the pluteus is deposited in an organic matrix formed by the primary mesenchyme cells. By interfering with the development of the spicule or by dissolving it away after it had been formed, she showed that the organic matrix was part of the cell chains referred to in our studies as the cable. The exact relationship of the spicule to the cable itself, however, was not clear from her

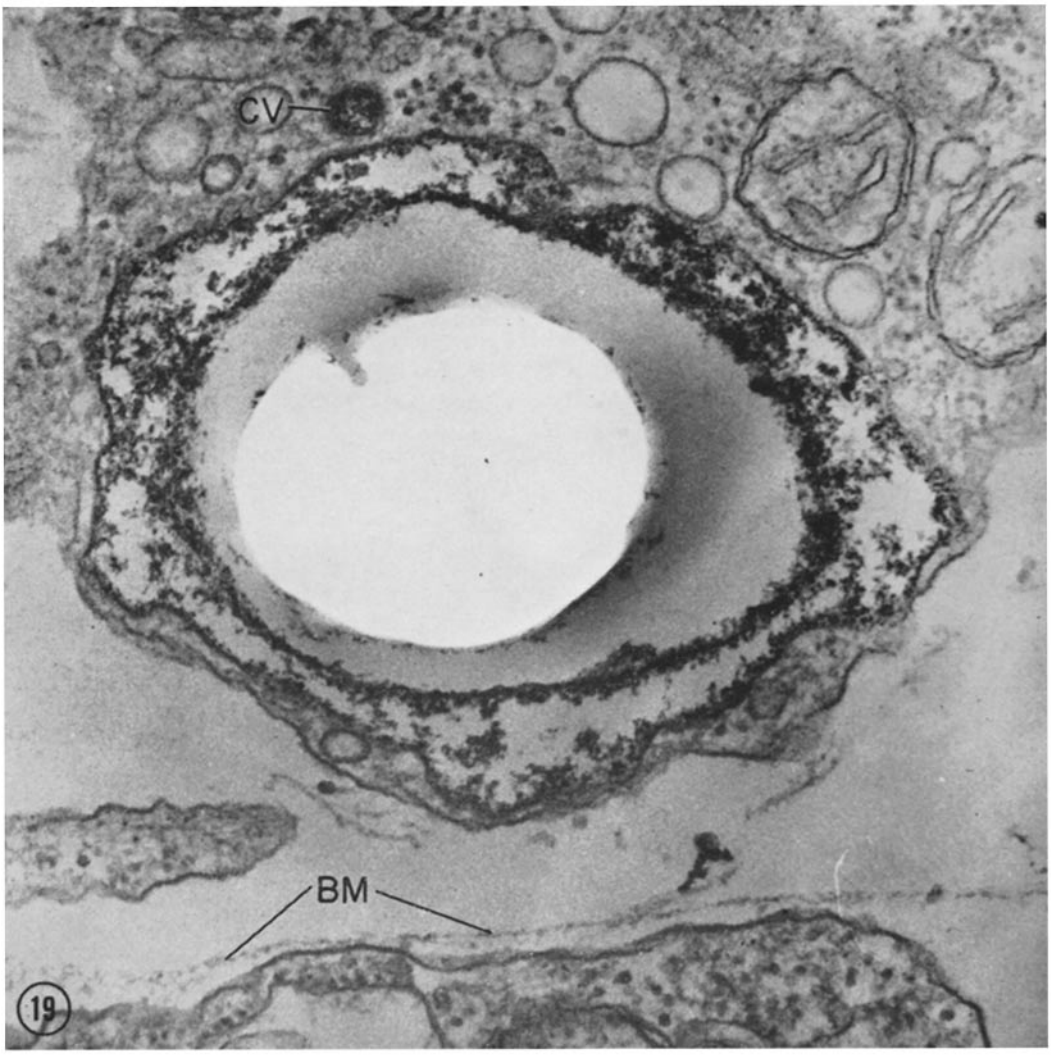


FIGURE 19 Transverse section through the cytoplasm of a cable which is in the process of forming the skeleton. The skeleton forms within a membrane-limited vacuole in the cytoplasm of the cable. Within this vacuole is some electron-opaque material. The center of the vacuole, containing the skeleton, disappears from the section, leaving a hole in the Epon. A small coated vesicle (*CV*) containing the same dense material as that in the skeletal vacuole is present near the cable. The basement membrane (*BM*) and a portion of an ectodermal cell lie at the lower edge of the micrograph. × 60,000.

studies. Her work also demonstrated that the skeleton consisted of two components, an organic component of unknown composition which resisted solution in acidified seawater and an easily-dissolved inorganic component recognized by earlier workers as $CaCO_3$ (see Bevelander and Nakahara, 1960, for references).

Our study, with the greater resolution provided by the electron microscope, demonstrates that both organic and inorganic components of the skeleton are deposited within a membrane-limited space which is surrounded by the cytoplasm of the cable. Neither the skeleton nor any part of it was ever found to be continuous with the cable cytoplasm or outside the limits of the cable.

In the sea urchin skeleton the nature of the

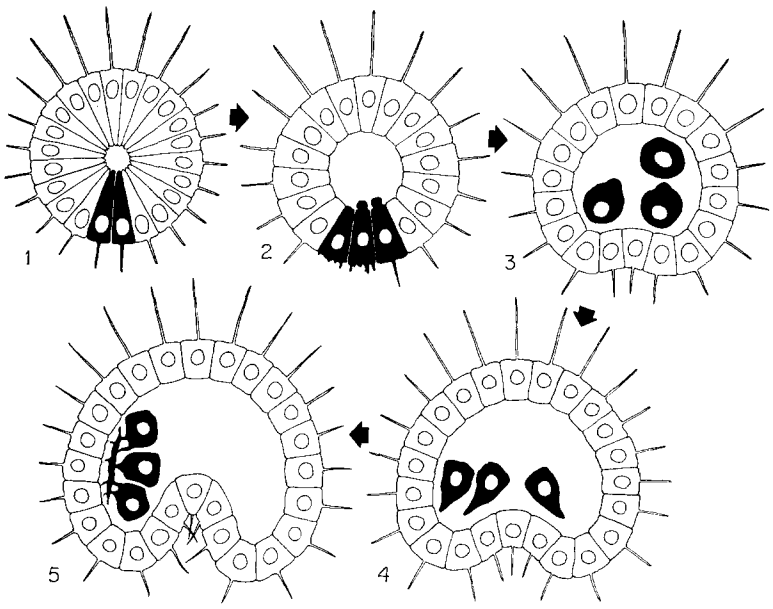


FIGURE 20 This drawing diagrammatically depicts stages in the formation and differentiation of the primary mesenchyme beginning at early blastulation. From stage *2* onwards mitosis does not occur in the cells in black. The changes in cell shape which each primary mesenchyme cell must undergo during its formation and differentiation are illustrated. In the early blastula (stage *1*) the cells have a long conical shape. As blastulation proceeds these cells shorten (stage *2*), and certain cells at the vegetal pole region undergo pulsatory movements producing on the basal surfaces small, often irregular lobes. The cilium is resorbed, and the apical surface is thrown up into folds. Shortly thereafter these cells migrate into the blastocoel where they take on a nearly spherical shape (stage *3*). These cells, now called the primary mesenchyme, send out pseudopodia (stage *4*) with which they are thought to migrate around the blastocoel. At this time the archenteron begins to indent. The pseudopodia of the primary mesenchyme cells come into contact and fuse, thus forming a common cable of cytoplasm which is specifically oriented within the blastocoel (stage *5*). It is within this cable that the calcareous skeleton of the pluteus is deposited. The cell bodies of the primary mesenchyme cells remain distinct from each other and from the cable cytoplasm, being connected to the latter only by short stalks.

organic matrix is not known, but our electron micrographs do not reveal any fibrous structure which might be expected if the process of spicule formation was similar to bone formation in vertebrates (Glimcher, 1960). It is highly probable that the matrix material is a protein or mucoprotein which is produced locally in the cable cytoplasm. It may be homologous to the basement lamella of other cells. It is also probable that this matrix material is represented by the electron-opaque material present around the periphery of the vacuole, and that the fully formed $CaCO_3$ spicule is represented by the hole in the embedding plastic where this crystalline, largely inorganic material has "fallen out" of the section.

The abundance of two types of vesicles in the cable cytoplasm at this stage, the coated variety (similar to those described by Roth and Porter, 1964, and Bowers, 1965) and the double variety, suggests that these may be involved in the process of skeleton formation, but their exact role can only be speculated upon at the present moment.

As already mentioned in some detail earlier in this discussion, the shape and orientation of the skeleton is determined by the shape and orientation of the pseudopodial cable, and the orientation of the cable appears to be influenced by the microtubules. It is conceivable that vacuoles containing the inorganic and organic components of the skeleton could become aligned within the protoplasmic mass of the cable, where in fusing they would come to adopt the form of the pseudopodium in which they reside. It is probable also that the organic component of the skeleton is laid

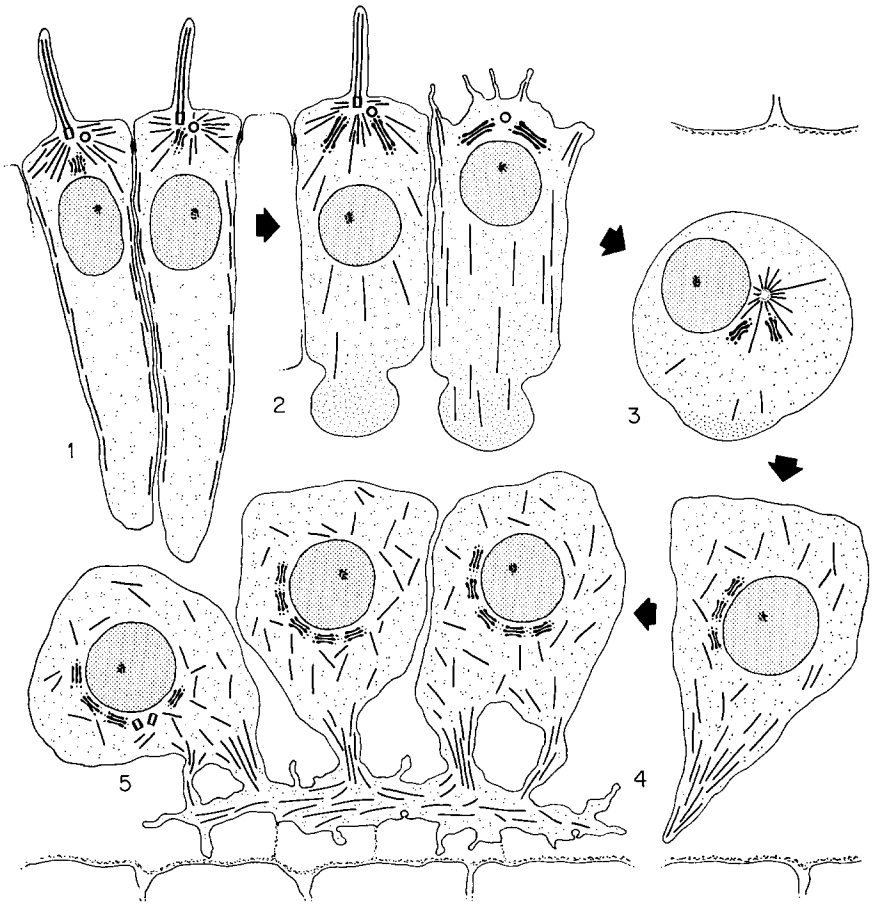


FIGURE 21 The distribution of microtubules is depicted at each stage in formation and differentiation of the primary mesenchyme. The cells seen here correspond to the cells indicated in black in Fig. 20. The arrows separate stages *1–5*. In every case, microtubules are oriented parallel to the direction of the cell asymmetry at that stage. This distribution has stimulated us to suggest that these elements may be related to the development of cell form at each stage in this developmental sequence.

down first within the cable and that the deposition of the inorganic component follows thereafter in an orderly fashion. An analogy exists, therefore, between skeletal deposition in sea urchin embryos and bone formation in vertebrates where collagen and ground substance are laid down and organized in patterns by osteoblasts, and the calcium salts are deposited relative to this preformed pattern.

Possible Significance of the Basal Lobe

This unique lobe, which is described here for the first time, makes its appearance on the blastomeres of the vegetal pole region towards the end of blastulation. It is from these cells that the primary mesenchyme will form. It cannot be without significance that within these lobes numerous ribosomes and short segments of rough-surfaced ER are segregated from the larger formed elements of the cytoplasm, namely mitochondria and yolk granules. This remarkable segregation of RNA-rich material suggests that this material may be set aside ultimately to appear in discrete regions of the mesenchymal cytoplasm.

The newly-formed mesenchyme cells, which have arisen from the blastomeres, present on one side of the cell a ribosome-rich "cap." Pseudopodia soon grow out of these newly-formed mesenchyme cells. Since the cap disappears during pseudopodia formation and since each pseudopodium contains large numbers of ribosomes and a few mitochondria, but no yolk particles or Golgi cisternae, we have suggested that the pseudopodia arise from

the cap and thus include the cap cytoplasm. Since the pseudopodia of adjacent mesenchymal cells later fuse to form the cable syncytium, the cytoplasm of the cable must be derived from the combined pseudopodial cytoplasm. Thus it appears that ribosomes which were initially included in the basal lobe could have been segregated there to find their way ultimately into the cytoplasm of the cable.

There are other instances in invertebrate embryology where cytoplasmic components are segregated. One is the well-known case of polar lobe formation during the mosaic development of *Dentalium* (see Wilson, 1904). This lack of equality in the distribution of cytoplasmic constituents results in the formation of the 4d cell, the cell which gives rise to the mesoderm. It had been shown many years ago that removal of this lobe results in an incomplete embryo; in fact most of the mesodermal organs do not form (Wilson, 1925). This observation has led several investigators to try to determine what substances, first segregated in the polar lobe, give the 4d cell the potentiality for forming mesenchyme. Recent studies (Berg and Kato, 1959; Collier, 1961, 1965) have shown that in the polar lobe there is a large pool of nucleic acid precursors and, according to Davidson et al., (1965) this material appears to be instrumental in gene activation.

One wonders then what might be the specific purpose for this segregation of ribosomes for the cable cytoplasm. Although the information we present cannot settle the question, it is reasonable to suppose that the ribosomes segregated in the basal lobe of the ectoderm may be set aside for some role in skeletal deposition. The mechanism of the segregation is not clear but does not appear to involve the microtubules.

Control of the Distribution of the Microtubules

On the basis of observations presented in this paper, we pointed out that the microtubules were distributed in such a way that they might be held responsible for each of the forms that the primary mesenchyme cells adopt during their formation and development. In order to achieve this at any one stage the microtubules would have to be distributed according to some recognizable plan. These conclusions lead one to analyze carefully the cytoplasm of the cell for any clue as to what might control the distribution of microtubules. An obvious place to look for control mechanisms is at the ends of the microtubules. Although our observations are still preliminary, we have presented evidence in this report for the existence of at least two apparently different sites which may be important in exerting some control over the distribution of the microtubules. One of these sites is present in the ectodermal cells of the early blastula and consists of satellites situated around the basal body of the cilium. Another is in the newly-formed mesenchyme cells (stage 3, Fig. 21) and consists of the centriole. Interestingly enough the microtubules do not converge or diverge from either of these sites in the stages which immediately follow or precede stage 3. Thus in this developmental sequence in which mitosis seems not to occur, microtubules at one stage converge on satellites around the basal body, at the next do not focus, at the next stage appear to make direct contact with the centriole, and at the final stage appear unfocused again. One possible interpretation of these data is that during development, sites from which the assembly of microtubules could begin are sequentially activated and inactivated, as well as redistributed. We would suggest, therefore, that an orderly progression of shape changes during development may be controlled by the sequential activation and inactivation of sites which in turn affect the distribution of the microtubules.

There is evidence for the concept of activation of microtubule-controlling centers in other situations. Much of this evidence has been reviewed by Inoué (1964), Inoué and Sato (1967), Roth (1964), and Porter (1966). Suffice it to say that centers such as the kinetochore, and in animal cells the centriole, are related to the appearance of birefringent fibers (Campbell and Inoué, 1965; Wilson, 1925; Dirksen, 1961). As other possible centers which appear to be involved in the regulation of microtubule polymerization, one should include the midbody of animal cells and the cell plate of plant cells.

In interphase cells other than those described in this communication there appear to be sites which regulate the distribution of microtubules and by so doing appear to control the form of the interphase cell and the distribution of some of the bodies within the cell. Clearly our ability to recognize the

sites which control microtubule distribution is at the moment rudimentary, and our knowledge of their composition is meagre. It is highly likely that before they can be accurately characterized improvements in the techniques of preservation and visualization may be required similar to those which were necessary to reveal cytoplasmic microtubules. Nevertheless, even though the evidence presented in this paper in support of their existence is meagre, it is adequate to indicate their potential significance in embryonic development.

For Figs. 20 and 21 our thanks go to Mrs. Helen Lyman.

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