Mechanism of Formation, Ultrastructure, and Function of the Cytoplasmic Bridge System during Morphogenesis in *Volvox*

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**ABSTRACT** The cytoplasmic bridge system that links all cells of a *Volvox* embryo and plays a crucial role in morphogenesis is shown to form as a result of localized incomplete cytokinesis; sometimes bridge formation occurs before other regions of the cell have begun to divide. Vesicles, believed to be derived from the cell interior, align along the presumptive cleavage furrow in the bridge-forming region. Apparently it is where these vesicles fail to fuse that bridges are formed.

Conventional and high voltage transmission electron microscopy analyses confirm that bridges are regularly spaced; they possess a constant, highly ordered structure throughout cleavage and inversion. Concentric cortical striations (similar to those observed previously in related species) ring each bridge throughout its length and continue out under the plasma-lemma of the cell body to abut the striations of neighboring bridges. These striations are closely associated with an electron-dense material that coats the inner face of the membrane throughout the bridge region and appears to be thickest near the equator of each bridge. In addition to the parallel longitudinal arrays of cortical microtubules that traverse the cells, we observe microtubules that angle into and through the bridges during cleavage; however, the latter are not seen once inversion movements have begun. During inversion, bridge bands undergo relocation relative to the cell bodies without any loss of integrity or change in bridge spacing. Observation of isolated cell clusters reveals that it is the sequential movement of individual cells with respect to a stationary bridge system, and not actual movement of the bridges, that gives rise to the observed relocation.

The formation and rearrangement of cell-to-cell attachments at specific times and places during development is one of the most important and least understood aspects of morphogenesis in multicellular eucaryotes (19). More than three decades ago, Holtfreter (9) pointed out the importance of “selective adhesion” in the invagination of cell sheets. Although subsequent work has amply confirmed his assessment, most of the systems which have been traditionally studied have not allowed detailed analysis of developmental changes in attachment points between cells or of the particular roles played by these attachments in generating form. This paucity of information has made it impossible to approach experimentally the question of how such changes are controlled at the gene level (as they must be, at least in part, since they play a crucial role in the development of species specific form). Analysis of simpler, more accessible model systems may provide the necessary conceptual groundwork upon which the study of such processes in more complex organisms can be based. Our previous work (7, 21, 22) has shown that *Volvox* is a promising model organism with which to analyze these problems.

Cells of a *Volvox* embryo are linked to one another, not by cell junctions of the sort observed in animals, but by an extensive network of cytoplasmic bridges. These bridges form in predictable numbers and locations during cleavage (7) and they later undergo a dramatic relocation relative to the cell bodies—a relocation that has been shown to be an essential component of the movements of inversion, the process by which an embryo turns completely inside out (21, 22). Preliminary analysis of numerous mutant *Volvox* strains, isolated because of heritable defects in adult morphology, suggests that a prime cause of such defects may be some aberration in the numbers, structures, or organizational relationships of the bridges formed during early embryonic development.

The potential exists in this system, therefore, for elucidating how specific genes regulate steps in the elaboration of an organelle that plays a central role in the development of the normal adult form of a multicellular organism. Exploiting that potential requires, however, a rather detailed understanding of
the normal structure, development, and function of the organelle.

Cytoplasmic bridges have been found in all members of the family Volvocaceae thus far studied, and certain aspects of their ultrastructure have been described in several members of the group, including *Eudorina* (6, 15), *Pandorina* (5), and several species of *Volvox* (1, 2, 10, 16, 21, 22). Although several of these investigators (2, 3, 5, 16) have suggested (explicitly or implicitly) that bridges are formed as a result of incomplete cytokinesis, little direct evidence has been presented to support this contention. In fact, there has not even been a great deal of detailed examination of cytokinetic mechanisms in the family. Cell division in the Volvocaceans and their relatives has frequently been shown to involve formation of a constricting furrow (reminiscent of the furrows characteristic of animal cells) that begins at one or more points on the cell and progresses inward (1, 3, 5, 11, 15). However, several investigators have noted the existence of vesicles in the region of the furrow, and Bisalputra and Stein (2) have raised, but left unresolved, the possibility that fusion of vesicles may contribute to completion of the furrow.

We report here the result of our examinations of cleaving *Volvox* embryos (by a variety of microscopy methods) which leave little doubt that cytokinesis does, indeed, involve vesicle alignment (as well as furrowing) and that failure of certain vesicles to fuse completely in one particular zone appears to be the mechanism by which the cytoplasmic bridges are formed. In addition, we will describe certain aspects of cytoplasmic bridge ultrastructure and associations with cytoskeletal elements. Finally we report the outcome of experiments designed to determine how relative relocation of these bridges takes place during the subsequent morphogenetic process of inversion.

**MATERIALS AND METHODS**

*Volvox carteri f. nagariensis*, strain HK-10, was cultured as previously described (13, 18) and embryos were isolated and prepared for light microscopy (LM), scanning electron microscopy (SEM), and conventional transmission electron microscopy (TEM) by previously described methods (7, 21, 22). Lanthanum staining for TEM was carried out as follows. Embryos of desired stages were fixed for 10 s in 2.5% glutaraldehyde and 1% lanthanum nitrate in standard *Volvox* medium (SVM) that had been buffered to pH 7.2 with 0.2 M cacodylate (C-SVM buffer). An equal volume of 2% OsO₄ in H₂O was then added. Specimens remained in the resulting mixture 1 h before being transferred sequentially through the following solutions: 2% lanthanum nitrate in C-SVM buffer for 1 h, C-SVM buffer for 10 min, 2.5% tannic acid (Mallinkrodt) in H₂O for 15 min, C-SVM buffer for a brief rinse, a 1:1 mixture of 2% lanthanum nitrate in C-SVM buffer and 2% OsO₄ in H₂O for 30 min. Subsequent processing for TEM examination was as previously described (21, 22). Preparation of samples for high voltage electron microscopy (HVEM) was as previously described for conventional TEM (21, 22), except that sections were cut 0.25-0.5 μm thick and stained longer: 15 min in 50% ethanol saturated with uranyl magnesium acetate and 3 min in Venable and Coggeshall’s lead citrate (20). Such thick sections were examined either with the JEM 1000 electron microscope at the University of Colorado (Boulder) or the A.E.I. EM7 electron microscope at the University of Wisconsin (Madison).

**RESULTS**

**Ultrastructure of the Cytoplasmic Bridge System**

SEM studies reported in our preceding paper (7) demonstrated that throughout cleavage the cells of a *Volvox* embryo are girdled by numerous regularly spaced cytoplasmic bridges in a single well defined band. TEM examination reveals that throughout cleavage and early inversion these bridge bands occur at a level occupied on the cell interior by a curved zone of cytoplasm (usually Golgi-rich) which lies between the nucleus and the chloroplast (Fig. 1). TEM studies also reveal the average diameter of the bridges to be ~200 nm and confirm the 500-nm center-to-center spacing of nearest neighbor bridges throughout cleavage and inversion (Figs. 2, 3, 4, 5, and 7).

Two types of membrane specializations have been observed previously in the bridge region of other Volvocacean embryos after the onset of inversion (1, 15, 16). These are a layer of electron-dense material coating the inner surface of the plasmalemma throughout the bridge region, first reported by Marchant for inverting *Eudorina elegans* embryos (15), and a series of parallel membrane striations, first described by Pickett-Heaps for *V. tertius* (16) and later described by Marchant as appearing to be embedded in the electron-dense membrane.

![Figure 1](image-url) Thin section of 16-cell embryo showing portions of two blastomeres. Note that the cells are connected by cytoplasmic bridges (arrowhead) at the level of a curved zone of cytoplasm—which is occupied predominantly by Golgi complex (G) and vesicles—between the nucleus (N) and the chloroplast. Bar, 1 μm. × 8,250.
coating of *E. elegans* (15). In *V. carteri* we find these two types of membrane specializations present in the bridge region throughout cleavage as well as inversion.

Circular (or possibly spiral) striations, with a mean center-to-center spacing of 25 nm, girdle each bridge throughout its length and continue out under the adjacent plasmalemma of the cell body as a series of concentric rings that abut similar rings which encircle neighboring bridges; as a result, the entire plasmalemma of the bridge region has a striated appearance in favorable sections (Figs. 2 and 3a-c). In oblique sections through a bridge the striations are truncated as the plane of section passes out of the membrane region and therefore appear as short, regularly spaced line segments (Fig. 3a, large arrows). In regions where the plane of section is normal to the membrane, the striations are sectioned transversely and appear as a granularity with a 25-nm repeat spacing on the inner aspect of the membrane (Fig. 3a, small arrows).

The electron-dense coating of the inner surface of the plasmalemma (like the striations which appear to be closely associated with it) extends throughout the entire bridge region, but takes on a different appearance depending on the plane of section (Figs. 2, 3, 4, 5, and 7). In randomly oriented sections through a cytoplasmic bridge region, the most frequently observed and characteristic features are a series of striking, electron-dense, elliptical to circular rings (Figs. 2, 3d, 4a-e, 5, and 7). When thick sections containing such rings are viewed at various tilt angles, it is apparent that such a ringlike profile may result simply from the optical reinforcement obtained when the electron-dense coating of a bridge is viewed from any angle not perpendicular to the bridge axis (Figs. 4b-e, and 5a and b). Closer examination suggests, however, that such an interpretation may be incomplete. In a thin longitudinal section that appears to include the central axis of the bridge, the electron-dense cortical material appears to be thickest at the bridge equator (Fig. 3a, arrow x). Where a similar section passes above or below the central axis of a bridge, however, a band of less electron-dense, vaguely fibrillar material is seen traversing the bridge at its equator (Fig. 3a, arrow y). These images suggest that the cortex is further specialized at the center of each bridge to form an annulet (see also Fig. 5a and b).

SEM observation of cells that have sustained a modest amount of shrinkage artifact during fixation or dehydration suggests that the membrane specializations described above have the consequence of reinforcing the membrane in this region and giving it greater dimensional stability than the rest of the plasmalemma. In such shrunken specimens, shrinkage in the bridge region is not random; rather, it occurs as a series of wrinkles midway between bridge centers, leaving the bridge stubs at the centers of a series of hexagonally packed hillocks.

**Figure 2** An 0.15–0.19 µm thick section of 32-cell embryo, cut tangential to the cell surface through the bridge band region; the bridges have been sectioned transversely and appear as ringlike profiles. Note concentric striations which extend outward from each bridge and abut striations from neighboring bridges. Bar, 0.25 µm. × 56,600.
FIGURE 3  (a) Thin section of 8-cell embryo showing longitudinal section through two bridges. Note the electron-dense coating visible on the inner face of the plasmalemma throughout the bridge region; in the immediate vicinity of a bridge, this coating possesses a distinct granularity with a 25-nm periodicity (small arrowheads). Note also truncated striations where the plane of section is oblique to plasmalemma (large arrowheads). Arrow X points to the cortical density at its thickest point—the bridge equator. Arrow Y points out vaguely fibrillar material just beneath the plasmalemma at the bridge equator. x 52,000.  (b) Microtubule (arrowhead) apparently traversing a cytoplasmic bridge of a preinversion cell. Also note striations. x45,500.  (c) Glancing section along the length of a bridge of a fully cleaved embryo revealing clearly the pattern of cortical striations. x 64,000.  (d) Oblique section through the bridge region of a preinversion cell. Note band of microtubules closely associated with the bridge. x 73,500. Bars, 0.25 μm.

Bridge-Cytoskeletal Relationships

Microtubules comprise the most obvious cytoskeletal feature in Volvox embryos throughout cleavage and inversion. During cleavage, there is a large population of microtubules which originate near the basal bodies of sister cells and course distally within the cortical regions of each cell past the bridge region (Figs. 4 and 5). These microtubules resemble the “cleavage microtubules” which were first described in Chlamydomonas (11); they are believed to arise before cleavage and demarcate the plane in which cleavage occurs. Although the majority of the microtubules in this area traverse the bridge region parallel to one another and to the nuclear-chloroplast axis of the cell, a smaller number of microtubules, interdigitated with the former, are seen to angle toward the bridges from a variety of directions (Figs. 3b and d, and 5c). Because these latter microtubules have not yet been studied systematically in serial sections, their termini cannot be identified with certainty; occasionally, one appears to traverse a bridge between sister cells (Fig. 3b). Although such microtubules angling toward or into the bridges are seen regularly during cleavage and the preliminary stages of inversion, it should be noted that we have never yet detected them in cells in which the movements of inversion have been initiated. (Parallel, longitudinal arrays of cortical microtubules are still numerous throughout inversion, however.)

A suggestion of a second kind of cytoskeletal feature has been detected in inverting embryos: thick sections of mid-inversion cells cut parallel to the surface, produce images in the HVEM suggestive of the possible presence of a cortical filamentous network emanating from, and perhaps linking, the bridges (Fig. 7b and c).

Mechanisms of Cytokinesis and Cytoplasmic Bridge Formation

When embryos that are believed to be about to initiate a new round of cytokinesis are fixed, broken open, and prepared for SEM examination, a most provocative feature is frequently observed: in the region of a preexisting bridge band where the next cleavage furrow is to be expected, a series of regularly spaced, deep indentations is seen (Fig. 8). These indentations are in register with the fully formed bridge band on which they are located, and they possess what clearly appear to be completed cytoplasmic bridges, although there frequently is no other indication on the cell surface that cytokinesis has been initiated (Fig. 8). For completeness, two additional observations should be noted. First, in some instances, similar regular invaginations have been observed in locations where no future bridge band is to be expected. The significance of these “ectopic” indentations remains obscure. Second, such indentations were not invariably seen on cells that had already initiated furrowing at the basal body end, hence, the point of initiation of cleavage may be variable.
FIGURE 4  (a) Thick section through sister blastomeres. Cortex is densely stained as a result of microtubular arrays extending from basal bodies (bb) to bridge region and beyond. Note electron-dense rings in bridge region at the subnuclear level. Bar, 1 μm, x 11,300. (b–e) Higher magnification tilt views of bridge region showing changing profiles of electron-dense bridge membrane: (b) azimuth (A) = 45°, tilt (T) = 25°. (c) A = 90°, T = 20°. (d) A = 180°, T = 20°. (e) A = 270°, T = 20°. Bar, 0.5 μm, x 36,200.
Based on the above observations we developed the following working hypothesis: (a) that cytokinesis in Volvox may be initiated, at least in some instances, in the presumptive bridge band region; (b) that bridge bands are formed by the alignment and partial fusion of vesicles formed on the cell interior; and (c) that the regularly spaced locations in which fusion of such vesicles does not occur are the sites where bridges remain in the completed cleavage furrow. This hypothesis led to the following prediction: if specimens at stages equivalent to those shown in Fig. 8 were prepared for TEM instead of SEM, vesicles should be observed lined-up along the presumptive plane of cleavage, and peripheral vesicles should be observed to communicate with the extracellular space before more central ones do. As a first test of that prediction, embryos were fixed in the presence of lanthanum nitrate to stain the surface of membranes in communication with the extracellular space (8). These embryos were subsequently sectioned in a plane perpendicular to the next presumptive furrow until the level of the nuclear-chloroplast interface (the region where the next bridge band should be formed) was reached. The resulting specimens confirmed the prediction: in embryos that appeared to be at an early stage of cytokinesis a continuous row of

**FIGURE 5** (a–b) Stereopair of bridge band region showing bridge membrane densities and microtubular arrays running parallel to and angling into the bridges. (a) T = 30°; (b) T = 0°. Bar, 0.5 μm. × 20,600. (c) Thick section showing microtubules which angle into bridges (arrowheads). Bar, 0.25 μm. × 37,500.

**FIGURE 6** Surface view (SEM) of a cell which was separated from its sister blastomere after being fixed under conditions that generated mild shrinkage. Note hexagonally shaped, raised hillocks with a bridge stub at the center of each. (The stubs themselves have two different appearances depending on which side of the bridge equator the fracture occurred on.) Bar, 0.5 μm. × 24,500.
vesicles was frequently seen midway between sister nuclei; whereas the most peripheral of these vesicles were stained with lanthanum, the central ones were not (Fig. 9a and b). Serial sections of these embryos revealed that such aligned vesicles (with only the most peripheral ones lanthanum-stained) turned out not to be restricted to the presumptive bridge band region but were also seen in more distal portions of the presumptive furrow, between sister chloroplasts (Fig. 9c). Within the same set of preparations, embryos could be found in which cytokinesis appeared to be closer to completion. Sectioning such embryos in a plane equivalent to that shown in Fig. 9c revealed that by this stage all vesicles along a presumptive furrow are lanthanum stained (Fig. 9d). In all of these embryos, fully formed bridges of completed furrows were observed to be completely coated with lanthanum (data not shown). Taken together, the latter two observations suggest that the failure of lanthanum to stain centrally located vesicles in specimens such as those depicted in Fig. 9a–c is not merely a trivial consequence of the fact that the colloid is unable to penetrate to the center of a narrow, labyrinthine furrow.

**Analysis of Cytoplasmic Bridge Relocation during Inversion**

SEM examination of fragmented embryos of selected stages reveals that the number of bridges per cell (20–30 with a mean of ~25) remains constant from the end of cleavage through the end of inversion. As previously reported (21, 22), and in contrast with the situation during cleavage, bridge bands undergo a marked change in cellular location during inversion: before inversion, bridges are located near the cellular equators as they are during cleavage, but by the end of inversion they are located at the chloroplasm ends of all cells (Figs. 10a–e). Cells approaching the zone of maximum curvature in an inverting embryo possess bridge bands at intermediate locations (Fig. 10c) (22).

Despite extensive examination by SEM and TEM, we have failed to detect any evidence of breakage of preexisting bridges or formation of new bridges during the bridge relocation process. Nor have we ever detected a cell in which a portion of the bridge band (on the side toward the region of maximum curvature, for example) has undergone relocation while the rest of the bridge band remains at its original location. There is, however, a change in the width of the bridge bands during inversion (from two staggered rows before inversion to as many as five or six staggered rows by the end of inversion). This increase in width of the bridge band is accompanied by a decrease in cell circumference in the region girdled by the bridges (from ~8 μm at the equator of a preinversion cell to ~3 μm at the chloroplasm end of a postinversion cell), thus accounting for the fact that the change occurs in the absence of any change in bridge numbers or spacings.

The fact that cells and cell clusters isolated from preinversion embryos go through a sequence of shape changes equivalent to those seen in intact inverting embryos (21) suggested the possibility that observation of the bridge relocation process might be feasible. To that end, live preinversion embryos were fragmented by passage through a fine capillary pipet, and pairs or small clusters of cells, in which the location of the intercellular attachment sites (bridges) could be clearly inferred, were observed continuously and photographed at intervals, using Nomarski optics. It was observed that instead of bridges moving along the length of neighboring cells, while the cells remained essentially stationary, the cells moved—one at a time—relative to the shared bridges. A typical isolated cell pair is shown in Fig. 11. At the time of isolation, these cells, like all the cells in the embryo, were spindle-shaped and joined at their midpoints. By the time the first photograph was taken, one cell, labeled a, had undergone the spindle-to-flask shape transformation (a characteristic of cells entering the region of maximum curvature in an intact embryo), and in the process had undergone translation relative to its neighboring cell, b, such that it had come to be joined by its chloroplast end to the midpoint of b (Fig. 11a). Subsequently, as cell b underwent the spindle-to-flask transformation, it also moved relative to the attachment point (shared bridges), until the two cells had come to be joined at their tips. (Meanwhile cell a had undergone the flask-to-columnar shape change characteristic of cells in the intact embryo that have passed through the region of maximum curvature.) In short, it is the cells that move, one by one, relative to shared bridge bands and not the bridge bands that move relative to otherwise stationary neighboring cells.

**DISCUSSION**

*The Functioning of the Cytoplasmic Bridge System in Inversion*

The cytoplasmic bridge system in Volvox (and other Volvocacean) embryos is a functionally unique array of intercell-
FIGURE 8  (a) Blastomere from a 4-cell embryo. Large arrowheads indicate bridge stubs resulting from previous division. Small arrowheads point out regularly spaced indentations between newly forming bridges. Bar, 5 µm. X 15,200. (b) Higher magnification of bridge-forming region of a. Note that a second row of bridges (arrowhead) is partially visible. Bar, 1 µm. X 15,200. (c) Bridge formation at surface of 4-cell blastomere in location where third cleavage furrow is expected. Arrowheads indicate extent to which the third division furrow has progressed on either side of newly formed bridges. Bridge stubs on previously formed band resulted from the first division. The second division plane is visible in the upper left corner. Bar, 1 µm. X 4,800. (d) Higher magnification of the bridge-forming region of c. Bar, 0.5 µm. X 30,700.
Figure 9  (a) Section through two sister cells in a cleaving embryo that was lanthanum stained during and after fixation. The relationship between the sister nuclei (n) and the chloroplasts (c) indicates that the presumptive cleavage furrow has been cut at the level of the (curved) zone of cytoplasm that separates nucleus from chloroplast, i.e., the region where the next bridge band should be formed. X 6,800. (b) Higher magnification of the furrow region shown in a. Note vesicles lined up along the presumptive cleavage furrow (arrows); only the more peripheral vesicles (I) contain lanthanum stain, whereas the central ones do not. X 17,700. (c) Section through the chloroplast end of the same cells seen in a. Sister chloroplasts with numerous starch granules (s) are separated by a line of vesicles (arrows) of irregular size and shape; only the external surface (E) and the most peripheral vesicle (I), has been lined with lanthanum. X 10,200. (d) Section through the chloroplast end of sister cells at a more advanced stage of cytokinesis than that shown above. Note that virtually all the vesicles aligned along the presumptive furrow (arrows) are lined with lanthanum, indicating they are in communication with the external surface (E). X 6,600. Bars, 1 µm.
ular connections. We concur with several previous investigators (2, 14, 16) who have pointed out that the cytoplasmic bridges of Volvox are fundamentally different from the plasmodesmata of higher plants in size, ultrastructure, and distribution. But their most distinguishing feature is functional. Although Volvox bridges undoubtedly do serve, like plasmodesmata and the cytoplasmic bridges of developing gametes of animals (4, 12), as channels for communication, they have an additional function in the generation of adult form. Most investigators who have examined development of Volvocacean embryos have invoked some role for the cytoplasmic bridges in inversion even though several different ideas have been expressed about what that role might be (5, 14, 16, 21, 22). Our previous studies suggest they play a mechanical role analogous to that played by specialized cell junctions in animal embryos.

We have previously shown that during inversion of V. carteri embryos the cytoplasmic bridges undergo a relocation (in a relative sense at least) from the midpoint to the chloroplast ends of the cells, that this relocation accompanies a cellular transition from spindle-shape to flask-shape in the cells and that bridge relocation together with the cell shape changes are sufficient to account for the geometry of the inverting embryo (22). We have further shown that when embryos are treated with cytochalasin D, the relocation of the bridges—but not the transition to the flask cell shape—is selectively and reversibly blocked and that when cytochalasin is removed from the medium, bridge relocation and inversion resume (22). From these observations we concluded that relocation of the cytoplasmic bridges relative to the cell bodies plays a crucial role in generating the movements of inversion (22).

A priori we were able imagine three fundamentally different mechanisms by which relocation of cytoplasmic bridges relative to the cell bodies might proceed. These three mechanisms are represented schematically in Fig. 12. (a) “Relocation” might occur by a “zippering” process in which preexisting bridges at the midpoint of each cell are broken as new bridges are produced at more distal locations. (b) “Relocation” might occur by the bridges being actively moved down the length of the neighboring cells they link. (c) “Relocation” might be a relative process in which cytoplasmic bridges remain essentially fixed while the cells move with respect to the bridges that link them to their neighbors. Our data clearly favor the latter mechanism (c).

The first piece of evidence in support of mechanism c is indirect. Because bridge relocation is a sequential process occurring first in cells bordering the phialopore and then in cells progressively further from it (21, 22), if either mechanism a or b were operative one would predict that some cells in the region where bridge relocation is taking place, should have undergone bridge relocation on one side, but not on the other (as diagrammed in Fig. 12 a and b). Such cells have never been seen. Instead SEM examination reveals that cells in which bridges are undergoing relocation relative to the cell body always possess a single, coherent band of bridges girdling the cell at an intermediate level (Fig. 10). This is what would be

2 It has been suggested that two sets of bridges exist in Pandorina, one “medial” and one “basal” set and that a selective breaking of the medial set accompanies inversion (5). Because the TEM analyses reported here, like SEM studies previously reported (7, 21), provide no evidence for a set of “basal” cytoplasmic bridges in V. carteri (other than lobes of undivided cytoplasm existing transiently at the chloroplast ends of cells during cytokinesis [7]), we have discounted this possibility as having any relevance for V. carteri.
expected only if it were the cells that moved sequentially relative to a stationary cytoplasmic bridge system, as illustrated in Fig. 12c.

More convincing is the direct evidence provided by microscopic examination of isolated cell clusters undergoing the movements of inversion after being mechanically released from preinversion embryos (Fig. 11). Here it is quite clear that the cells move individually and sequentially relative to the bridge band that joins them to their neighbors. In isolated cell clusters, as in the region of maximum curvature of an intact inverting embryo, movement relative to the bridge system is coupled with development of the flask cell configuration.

Fig. 13 summarizes diagrammatically the way in which we believe these processes of sequential movement and change in shape are integrated in the embryo to account for the phenomenon of inversion. In this process, the cytoplasmic bridge system (shown in Fig. 13 as a heavy black line running throughout the embryo) plays a key role as the coherent, yet flexible structural framework against which the cells work to execute inversion. As cells near the phialopore move relative to the bridge system and become flask-shaped, they come to be linked at their narrowest points; this automatically generates the curling of the cell sheet that swings cells to the exterior. As cells progressively further from the phialopore sequentially execute similar movements and shape changes (while cells that have reached the exterior assume simple columnar shapes), the ring of negative curvature moves as a wave toward the opposite pole of the embryo until the latter has turned completely inside out.

The unique functional role of the cytoplasmic bridge system during inversion—which is to provide a coherent, flexible framework against which cells can exert force and move—implies the existence of structural features that will account for the integrity of the bridge system under stress, its flexibility, and its ability to relocate in a sequential manner. These features may include the presence of specialized attachment points on the cells and the bridge itself, as well as the existence of flexible linkages that allow for movement relative to the bridge system without breaking or forming new connections.

If it were not for the bridge-free regions on cells lining the phialopore slits, however, the same changes would lead to invagination, or gastrulation, rather than inversion.
membrane coating may also participate in such a stabilizing role. This view is consistent with our observation that the membrane immediately surrounding each bridge appears to be more resistant to shrinkage artifact (over areas roughly coextensive with the concentric ring systems) than the remainder of the cell surface (Fig. 6). Furthermore, we speculate that it may be the extent of the concentric striations around each bridge that defines minimum interbridge spacing and accounts for the hexagonal packing pattern of bridges that is observed in each bridge band (7).

Although so far we have had difficulty obtaining clear evidence in support of the idea, we postulate that there may be a second function for the cortical specializations in the bridge region: to provide attachment sites for filaments that may be involved in linking cytoskeletal elements to the bridge-region plasmalemma (Fig. 3) and linking adjacent bridges to one another (Figs. 2 and 7). We have had difficulty, however, in resolving such filaments with sufficient clarity and regularity to describe them in detail. Because such a network of filaments, together with the membrane specializations described above, could do much to explain how the bridges provide a coherent framework against which the cells exert force during inversion, we are continuing in our efforts to visualize them in a more satisfactory manner.

We have been equally unsuccessful to date in clearly elucidating the cytological basis for the movement of the cells relative to the bridge system during inversion. Several observations made in our laboratory, but not reported in detail here, suggest that an actin-myosin based contractile system exists in these embryos. Embryos examined by time-lapse cinemicrography clearly exhibit the capacity for contraction. One wave of contraction passes over the embryo in the interval between cleavage and inversion and another occurs at the beginning of inversion. The ionophore A21387 induces (in the presence but not in the absence of ionic calcium) a violent, premature wave of contraction that causes the phialopore to open widely (22). Embryos treated with a combination of saponin (to permeabilize the cells) and phalloidin (to promote actin polymerization) exhibit thick bundles of filaments that decorate in a classical fashion with the S1 fragment of heavy meromyosin (K. J. Green and P. Detmers, unpublished observations). Unfortunately, however, we have not yet been able to determine where the nucleation sites for such actin filaments may be located in the cells before treatment.) All these facts, combined with the observation that cytochalasin D selectively and reversibly inhibits movement of the cells relative to the cytoplasmic bridge system (22), led us to postulate that the movements of inversion involve an actin-myosin based system. Investigations designed to elucidate the details of this system are ongoing.

Cortical microtubules clearly play at least two, but probably more, roles in *Volvox* morphogenesis. First, there is little doubt that in *Volvox*, as in *Chlamydomonas* (11), longitudinal bundles of “cleavage microtubules” (which originate near the basal body region and which are part of the “phycoplast” system characteristic of the dividing cells of many green algae (17)) delineate the channel in which cytokinesis will occur. Second, these parallel arrays of cortical microtubules persist throughout embryogenesis, and it appears to be their elongation which mediates the cellular elongation and shape changes that accompany inversion (21, 22). The fact that before the onset of inversion we also see numerous microtubules angling obliquely from the longitudinal arrays into the bridge region (Figs. 3b and 5a-c), but that no such oblique microtubules are seen in cells engaged in inversion movements, leads us to suggest that
the latter microtubules provide a strutwork whereby cells are held in place relative to the bridge system before inversion. Removal of the oblique microtubules would appear to be required to permit inversion movements.

There is evidence, however, that microtubules also play a more active role in the movement of cells relative to the bridge system during inversion. Treatments that cause disassembly of the longitudinal microtubules cause immediate collapse of cellular projections as well as cessation of bridge movement, and when microtubules are allowed to reassemble, inversion movements resume (22). Putting these observations together with the earlier suggestion that an actin-myosin contractile system may be involved, we have derived the following working hypothesis to account for the cell movements of inversion: once the strutwork of angling microtubules has been removed, an actin-myosin system functions to slide the longitudinal microtubular framework in one direction past the bridges. Because the longitudinal microtubules appear to be firmly attached at the basal body end of the cell, such movement would have the effect of moving the entire cell body with reference to the bridges. Much additional work will be required to test this hypothesis.

Mechanisms of Bridge Formation and Cytokinesis

Although previous investigators have stated that cytoplasmic bridges in the Volvocales arise as a result of incomplete cytokinesis (2, 3, 5, 16), no direct evidence to support this statement—and to rule out de novo formation of bridges after completion of a cleavage furrow—has been presented until now. The SEM study of cleavage in our preceding paper left no doubt that bridges are formed before a cleavage furrow has been completed (7). SEM images in the present paper show, in at least some cases, that formation of what appears to be a new extension of the cytoplasmic bridge system is the first externally visible sign that a new round of cytokinesis has begun (Fig. 8). Furthermore, lanthanum-stained specimens viewed in thin section in the TEM support the concept that formation of such an extension of the bridge system involves fusion of vesicles arising from the cell interior (Fig. 9) because within a single file of vesicles aligned in the presumptive cleavage plane, the most peripheral ones are lanthanum stained (and hence have apparently achieved continuity with the extracellular space) while the central ones are not. We presume that these vesicles may arise from the Golgi complexes which are abundant in the bridge-forming zone and may be aligned by the phycoplast microtubules as in certain filamentous algae (17), but as yet we have no evidence that either of these presumptions is valid.

Sometimes bridge formation is visible before any other external signs of cleavage are present and sometimes it is not (Fig. 8, and Fig. 4b of reference 7). We suspect that alignment of bridge-forming vesicles regularly begins on the cell interior before other aspects of cytokinesis commence, but that variability in the time at which peripheral vesicles fuse with the surface accounts for the variability in the stage of cytokinesis at which bridge formation becomes externally visible. Furthermore, it appears that when fusion of such bridge-forming vesicles with the surface is an early event, the locus at which this has occurred may serve as the site of initiation of a smoothly progressing cleavage furrow (Fig. 8a and c).

Taken together with the results in the preceding paper (7), our observations here on the mechanism of bridge formation indicate that cell division in _V. carteri_ occurs by a combination of different cytokinetic mechanisms operating in a regionally specific manner. Several previous investigators have concluded that cytokinesis in various members of the order Volvocales involves an ingressive furrow (analogous, if not homologous, to that of animal cells) that begins at one or more points on the surface, and progresses inward (1, 3, 5, 11, 15). Bisalputra and Stein have also raised the possibility that fusion of vesicles might be involved (2). Our observations indicate that both are involved. Division of the nuclear ends of the cells appears to occur by a smoothly ingressing furrow (Fig. 4b, e, and f of reference 7). So far we have never seen signs of vesicle alignment at the nuclear ends of the cells. Division in the central, bridge-forming region of the cell, in contrast, appears to occur predominantly by vesicle fusion. Division of the chloroplast end of the cell is always the last to be completed and may involve both kinds of mechanisms—or a third mechanism. Division of the chloroplast end is foreshadowed by a series of irregular, punctate depressions on the surface (7). To us these appear to resemble localized cortical contractions rather than vesicles fusing with the plasma membrane. Meanwhile, however, vesicles—much less regular in size and shape than those seen in the bridge-forming region—can be seen lined-up in the plane between sister chloroplasts (Fig. 9c). Further work will be required to fully understand how these cytokinetic events are integrated at the chloroplast end of the cells.

Another important unsolved question is why vesicle fusion in the bridge-forming region is incomplete and results in the formation of the regularly spaced bridges that play such an important role in morphogenesis. Are the vesicles in this region structurally specialized relative to those at the chloroplast end? Before alignment and fusion, do they bear the electron-dense coating and concentric striations that will characterize mature bridges? Is alignment of the vesicles in this region and/or determination of bridge locations mediated by a specialized cytoskeletal framework? Is complete fusion of vesicles in the bridge-forming region prevented by cytoplasmic inclusions that eventually come to form the specialized region—the "bridge annulet"—at the center of each bridge (Figs. 3a and 5a, and b)? Answers to all these questions will require painstaking ultrastructural analysis of cells fixed during the brief intervals between successive rounds of cytokinesis.

Concluding Remarks

Although many key questions remain unanswered, results presented in this paper and the preceding one (7) provide a substantial background for analysis of the primary cytoplasmic defects in the numerous mutant strains of _Volvox_ in our collection that exhibit defects of cleavage and/or inversion. Those studies in turn should provide important insights into the mechanisms whereby specific genes regulate the membrane-cytoskeletal interactions that are essential for the generation of species-specific adult form.

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4 Obviously, whatever the mechanism of cell movement is, a substantial flow of plasma membrane past the bridges must accompany the relocation.
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