Cleavage Patterns, Cell Lineages, and Development of a Cytoplasmic Bridge System in Volvox Embryos

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ABSTRACT We report an extensive scanning electron microscope (SEM) study of cleavage planes, cell shape changes, and cell lineages during cleavage of the asexual embryo of Volvox carteri f. nagariensis. Although our data generally confirm the basic description of cleavage developed by others using light microscopy, there is one important exception. We observed that the fourth cleavage plane is much more oblique than had previously been recognized. We show that, as a result, the four tiers of cells in the 16-cell embryo overlap extensively, and the new generation of asexual reproductive cells, or gonidia, are derived from three of these tiers (rather than two, as previously believed).

Our study focused on the development of the highly organized system of cytoplasmic bridges that appears during cleavage. Hundreds of cytoplasmic bridges are formed in each division cycle as a result of incomplete cytokinesis. Existing bridges are conserved and divided between daughter cells while new bridges are formed at each division. Hence, the number of bridges per embryo increases regularly even though the number per cell declines from the fourth cleavage on. The bridges are organized into bands that girdle the cells at a predictable level and exhibit a regular 500-nm interbridge spacing; bridge bands of adjacent cells are in register and form a structural continuum throughout the embryo which we term “the cytoplasmic bridge system.” The only place where bridges are not present is along a pair of intersecting slits, called the phialopore. We describe in detail the development of this bridge-free region.

The potential of Volvox carteri as a model system for analyzing the genetic control of development has been stated frequently in recent years (3, 10, 11, 14, 23, 25, 28, 29). We in this laboratory have been particularly interested in the potential this system provides for analyzing the genetic control of morphogenesis. Having described the cellular basis for inversion (the dramatic morphogenetic process in which the embryo turns inside out) and having discerned the central importance of a network of cytoplasmic bridges in the inversion process (28, 29), we turned our attention to the study of a collection of >50 morphological mutants that had been isolated as putative “inversion mutants.” In case after case, we observed that the gross defects that became apparent during inversion were a consequence of more subtle aberrations of cell division and/or cytoplasmic bridge formation that occurred during early cleavage. This focused our attention on the need for a more detailed analysis of cleavage and the development of cytoplasmic bridges in wild-type embryos and provided the primary impetus for this study. While the study was in progress, however, a new dimension was brought to our attention by the publication of a model that attempted to explain the interesting and important phenomenon of somatic vs. reproductive cell determination (which occurs during cleavage) on the basis of cell lineages and the patterns in which previously established contact zones are inherited by sister blastomeres at successive cleavage divisions (27).

Members of the genus Volvox are simple, multicellular, eucaryotic organisms, and each individual is composed of only two cell types: somatic and reproductive. Depending on specific conditions (24), reproductive cells are able to enter either sexual or asexual developmental pathways. In V. carteri f. nagariensis (the species we are examining) asexual reproduction takes place through the rapid cleavage of the reproductive cells, called gonidia. A miniature individual, containing the full complement of somatic and reproductive cells that will be found in the adult, is formed in the course of 11 or 12 cleavage divisions that occur in the absence of growth. All cells of the embryo are joined together by an intricate system of cytoplasmic bridges elaborated during cleavage. Because embryos at the end of cleavage are inside-out with respect to the adult configuration...
(i.e., flagellar ends of cells point inward and gonidia protrude from the outer surface), normal adult morphology requires that inversion follow cleavage.

The patterns of embryonic cleavage in various species of *Volvox* have been described in varying amounts of detail by Janet in 1923 (13), Pocock in 1933 (22), Darden in 1966 (5), Kochert in 1968 (16), and Starr in 1969 and 1970 (24, 25). Although the presence of cytoplasmic bridges during cleavage was detected by Janet and later described by Pocock and Kochert, their studies were all limited by the resolving power of the light microscope; as a consequence, the nature, numbers, locations, arrangements, and mechanism of formation of cytoplasmic bridges could not be clearly discerned. On the other hand, several investigators have examined cytoplasmic bridges of embryos of *Volvox* and other related genera of Volvocaceans in the transmission electron microscope (TEM) (1, 2, 6, 7, 8, 12, 18, 21, 28, 29). But because none of those examinations were coupled to systematic studies of cleavage and none involved the tedious reconstruction from serial sections that would be required to fully describe the bridge system by use of the TEM, they have also left us without a thorough description of the ontogeny of the bridge system.

Our study uses scanning electron microscopy (SEM) to study in detail cell division planes, cellular shape changes, the cellular lineages of gonidial cells, the nature of the differentiative division that sets off gonidal from somatic initials, and the mechanism of formation of the phialopore during cleavage in *V. carteri* f. nagariensis strain HK10 (the parental strain from which all our morphological mutants have been derived). In addition we have described in detail the numbers, locations, and arrangements of cytoplasmic bridges throughout this cleavage process and have generated the concept of a novel morphological entity—a "cytoplasmic bridge system"—which our companion paper (9) will show plays a crucial structural role in the morphogenesis of this species.

**MATERIALS AND METHODS**

Axenic cultures of *V. carteri* f. nagariensis, strain HK-10, were obtained from the Indiana University Culture Collection of Algae (now the University of Texas Culture Collection of Algae), maintained in axenic culture in standard *Volvox* medium (SVM) (24). and used in the studies reported here. Under culture conditions which have been previously described (15), individuals undergo rapid, synchronous development, completing the asexual life cycle in 48 h.

In initial studies, some of which are presented here, preparation of specimens for SEM was made as previously reported (28, 29). Subsequently, it was determined that improved preservation for SEM could be obtained with the following modification, which was then used in all other studies. Cleaving embryos of selected stages were fixed in 0.5 ml of 0.025% glutaraldehyde in SVM (pH 7.0) for 15 min; 0.5 ml of 2% OsO₄ in H₂O was then added to the above, resulting in final concentrations of 1% OsO₄ and 0.0125% glutaraldehyde. After an additional 30 min, specimens were washed for 10 min in SVM and then transferred to 2.5% glutaraldehyde in SVM (pH 7.0) where they were fixed overnight at 4°C. Specimens were then rinsed in H₂O and mounted on cover slips which had been coated with 1 mg/ml polylysine-HBr in H₂O. The embryos were removed from their glycoprotein vesicles with sharpened tungsten needles and the resulting free embryos, either intact or broken open with the tungsten needles, were positioned in the desired orientation at a nearby location on the same cover slip. Specimens were then dehydrated in ethanol, critical-point-dried, coated with 50 nm of gold in a Hummer II or a Polaron E5000 sputter coater, and viewed with either a Cambridge Stereoscan Mark 2A or a Hitachi S-450 SEM.

**RESULTS**

**Patterns of Cell Division from Early Cleavage through the Differentiative Division**

Many aspects of cleavage in this strain of *Volvox* have been well described previously by Starr based on light microscope examination of living embryos (24, 25). Similar observations will be recounted here along with certain additional details revealed by SEM visualization (Figs. 1 and 2), to provide a complete account of the cleavage process as we now understand it without continual referral to Starr's papers.

The onset of cleavage is preceded by a condensation of the previously vacuolated, spherical gonidium and results in a marked flattening of its anterior end (that end facing the external surface of the parental spheroid) and a less pronounced flattening of its posterior end. On the anterior surface, the first division (I) begins with the development of a deep rhomboidal pit (Fig. 1a); then a cleavage furrow is initiated at two opposing corners of the pit, and extends toward the periphery of the gonidium (Fig. 1b). Often this furrow is not completed before the onset or completion of the second division. The second division (II), like the first, is initiated on the anterior surface as a smooth furrow that emanates from two corners of the rhomboidal pit and extends toward the periphery (Fig. 1b). On the posterior surface, however, a very different appearance is observed at the initiation of both divisions I and II: instead of a smoothly progressing furrow one sees a series of irregular, puckered, punctate depressions (Fig. 1c). This difference in the appearance of the invaginations on opposing sides of the cells continues to be observed throughout cleavage. At each division, a smooth, continuous furrow is formed at the nuclear ends of the cells (which are derived from the anterior portion of the gonidium), while an irregular, punctate "furrow" is formed at their chloroplast ends.

During the second division, the interface resulting from the first cleavage bends twice, forming three facets: two lateral facets and one smaller central facet (this bending has been initiated, but not completed, in the specimen shown in Fig. 1c). Although the second division may appear to be perpendicular to the first on the anterior face of the embryo (Figs. 1d and 3a), a posterior view of a 4-cell embryo shows the inter-

**Figure 1.** All embryos are oriented so that the first division interface runs from the upper left to lower right corner, as represented in Fig. 3. (a) Gonidium undergoing formation of rhomboidal pit during first cleavage division (anterior view). (b) Extension of first cleavage furrow toward periphery of dividing gonidium (anterior view). (c) Completed interface resulting from the first division beginning to bend from three facets: one small central facet and two lateral facets. Note the punctate indentations which presage formation of the second division interface (posterior view). (d) Early 4-cell embryo exhibiting cruciform slits at the anterior pole separating the bridge-free surfaces which will become the margins of the phialopore (anterior view). (e) 4-cell embryo illustrating the fact that the second division interfaces do not meet at the anterior pole and are oblique with respect to the first division plane (posterior view). (f) Late 4-cell embryo exhibiting the anteriorward extension and clockwise curling of one lobe of each blastomere (anterior view). (g) Early cup-shaped 8-cell embryo which consists of a tier of four slightly anterior cells and four posterior cells (anterior view). (h) Late 8-cell embryo exhibiting a decrease in the size of the phialopore as a result of centripetal extension of the anterior blastomer (anterior view). Note filopodial extensions extending from cell boundaries toward neighboring cells. a-h: bar, 10 μm. X 1,000.
faces resulting from the second division to be oblique with respect to those resulting from the first (Figs. 1e and 3c). Furthermore, the two second division interfaces do not meet at the posterior pole; rather, each abuts the first interface at one of the two "bends" described above. As a result, the 4-cell embryo is bilaterally, not radially, symmetrical: two opposing blastomeres, which we will term the "B" blastomeres, are in contact with one another along the central facet resulting from the first division while the other two opposing (or "A") blastomeres share interfaces only with adjacent B cells (Fig. 3c). From the descendents of these four cells, the four quadrants of the embryo (two A and two B quadrants) will be formed in
subsequent cleavages.

Before the third division, a lobe of each 4-cell blastomere extends slightly anteriorly and curls in a clockwise direction (Fig. 1f). The third division (III) is radial and oblique, cutting off these anterior lobes to form a shallow, cuplike 8-cell embryo consisting of four slightly anterior cells (which we term the A1,3 and B1,3 cells) that overlap extensively with, but lie in a clockwise orientation relative to, their more posterior sister cells (the A2,4 and B2,4 cells) (Figs. 1g and 3d). The relationship of the newly formed interfaces with those formed in the first and second divisions is shown in Fig. 3d-f.

The space between the blastomeres at the anterior pole of the embryo is called the phialopore; it is this opening through which the embryo will turn inside out ("invert") shortly after the completion of cleavage. Between the third and fourth divisions, the anterior four cells of the 8-cell embryo extend slightly anteriorly, but primarily toward one another, diminishing the size of the phialopore (Figs. 1h and 3g and h). Thus begins the transition from the shallow cup-shaped form of the early embryo to the spherical form that it will achieve by late cleavage. During their extension the cells become progressively more crescent-shaped (Fig. 1h).

The anteriormost cells continue their centripetal extension, decreasing further the size of phialopore, as the fourth division (IV) occurs (Fig. 2a and 3j). The fourth cleavage planes are again radial and oblique, and once again anterior cells are cut off in a clockwise direction relative to their posterior sister cells. The result is that each quadrant consists of four overlapping cells that curve in a clockwise direction from posterior to anterior. The entire 16-cell embryo thus consists of four extensively overlapping tiers containing four cells each (two A1 and two B1 cells in the most anterior tier, two A2 and two B2 cells in the second tier, and so on) (Figs. 2b and 3j-l).

The fifth division (V) yields a total of 32 cells (Figs. 2c and 3m). The fifth cleavage planes are only slightly oblique; they more closely approximate equatorial cleavages than meridional cleavages, whereas the reverse was true in the first four divisions. Note that the embryo retains most of its flattened appearance but that the position of the anterior tier of cells has shifted relative to that of the posterior tier. (i) Posterior pole of late 8-cell embryo is much the same as that of early 8-cell embryo. (j) Anterior pole of 16-cell embryo showing further diminishing of phialopore size and the swastika-shaped appearance of the slits IV, fourth division plane. The embryo consists of four overlapping tiers of four cells each numbered, by convention, 1 through 4, from anterior to posterior. (The tier 4 cells are visible only in side and posterior views.) (k) Side view of 16-cell embryo showing extensive overlap of tiers of cells. (l) Posterior view of 16-cell embryo, again the obliqueness of division IV interfaces is evident. (m) Anterior view of 32-cell embryo. a, anterior; p, posterior members of sister cell pairs formed by division V. The fifth division (which was approximately equatorial) resulted in the partitioning of cells with goniidium-forming potential into the anterior hemisphere. The 16 anterior cells visible here—four each of 1a, 1p, 2a, and 3a cells—are all competent to form gonidial initials at the next division. (n) Side view of 32-cell embryo showing division of the 1a, 1p, 2a, and 3a cells and the 2p, 3p, 4a, and 4p cells into anterior and posterior hemispheres, respectively. (Note that the 2p cell is posterior to the 3a cell). (o) Posterior view of 32-cell embryo. (p) Anterior view of 64-cell embryo following division VI (differentiative division). The 1a, 1p, 2a, and 3a cells have each divided unequally to yield a gonidial initial (G) and a somatic cell initial. (q) Side view of 64-cell embryo showing that the gonidal initials are restricted to the anterior hemisphere. (r) Posterior view of 64-cell embryo.
sions. Therefore, the newly produced sister cells have a simple anterior-posterior (rather than a clockwise) relationship to one another. We term the anterior cell of each pair an "a" cell and the posterior member of each pair a "p" cell (Figs. 3m-o).

A differentiative cleavage occurs in one half of the cells at the sixth division (VI) (Figs. 2d and 3p-r): the 16 cells of the anterior hemisphere—four la, four lb, four 2a, and four 3a cells—divide unequally to yield 16 small cells (somatic initials) and 16 larger cells (the gonidial initials). It is the latter cells which will eventually give rise to the next generation. The 16 cells of the posterior hemisphere meanwhile divide equally, giving a total of 48 somatic cell initials at this stage. At division VI the furrows of the la cells lie nearly parallel to the circumference of the phialopore. All other cells divide somewhat more obliquely so that anterior cells are cut off in a somewhat counterclockwise direction.

In favorable specimens it can be clearly seen that at the seventh division the gonidial initials once more cleave unequally, each throwing off another somatic initial, thus yielding a total of 128 cells (112 somatic initials and 16 gonidial initials) at the end of cleavage VII (Fig. 2e). Determining whether such redivision of gonidial initials to yield additional somatic initials occurs regularly at the seventh (and subsequent) divisions required the indirect approach of calculating how many divisions of how many progenitor cells would have been required to produce the number of somatic cells characteristically observed in adults. Somatic cells of randomly selected adults were counted using a camera lucida. Two cohorts, differing in somatic cell number by very nearly a factor of two were observed. The smaller spheroids possessed 1,983 ± 17 somatic cells (mean ± standard error). This corresponds closely to the figure of 1,984 expected if each gonidial initial set off at division VI divides asymmetrically three more times, and all somatic initials go through a total of eleven divisions. The expected numbers if gonidia divided two or four times after cleavage VI would be 1,920 and 2,016, respectively. The larger spheroids, having twice as many somatic cells, obviously had gone through a twelfth cleavage cycle. The fraction of embryos going through a twelfth cleavage is small and variable under our present culture conditions and may be density-dependent.

The last four or five cleavages of the somatic initials are all radial and probably exhibit the alternating clockwise and counterclockwise, oblique orientations previously described for late stages of cleavage in V. aureus (13).

Organization of Cytoplasmic Bridges within Bridge Bands

Numerous cytoplasmic bridges are formed in the interface between sister blastomeres at every cleavage from the first division (Fig. 4a) to the last. The locations of these bridges, and the relationships among them, can be determined by mechanically separating the cells of fixed embryos and viewing their surfaces with the SEM; broken bridges appear as bands of stubs (or occasionally shallow holes) on the surfaces of the separated blastomeres (Figs. 4 and 5). Thus developmental changes in bridge numbers and arrangements during cleavage can be readily followed.

Throughout cleavage, the cytoplasmic bridges are arranged in discrete bands consisting of three to five staggered rows. In some early cleavages, most notably the first, the bridges are frequently arranged somewhat irregularly in certain regions, giving the bridge bands overall a rather ragged appearance in very young embryos (Fig. 4b). From the beginning, however, there are regions of each bridge band in which the arrangement and spacing of the bridges is much more regular; consequently the bridge band is smoother in outline in those regions. Such regularity of bridge arrangement and spacing becomes the rule in later cleavages (Figs. 4d and f, and 5a-f), so that by the 8-cell stage most bridge bands have a very regular appearance and the bridges approximate a hexagonal close-pack arrangement with a mean center-to-center spacing of 0.5 µm between adjacent bridges.

Bridge Number as a Function of Cleavage Stage

Bridges exposed on broken embryos were counted on two different kinds of specimens to obtain two different sets of values. When fragments were large enough that the stage of the embryo and the identity of each cell in the fragment (according to the system of identification used in Fig. 3) could be deduced from the SEM image, most exposed cellular facets could be identified as having been derived from the furrows of particular cleavage divisions. Such specimens were used to determine the number of bridges formed in particular cleavage and how those bridges were subdivided among sister cells during subsequent divisions. On the other hand, isolated cells from embryos of known stages that were favorably positioned on the SEM stub could frequently be used to determine total number of bridges per cell even if the original location of the cell (and hence the identity of its various facets) could not be determined.

For a variety of technical reasons, this type of analysis became progressively more difficult at later cleavage stages and reliable data were not obtained between the sixth and final cleavage divisions. Hence the data for these later stages were estimated by extrapolation, using the following assumptions:

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**Figure 4** (a) Formation of cytoplasmic bridges during the first cleavage division. This is a closeup view of the floor of the rhomboidal pit of the embryo shown in Fig. 1 b. Bar, 1 µm × 6,800. (b) Cracked 2-cell embryo showing bridge stubs on surface of one blastomere (small arrows). Because division I is rarely completed before the onset of division II, large areas of connecting cytoplasm in the chloroplast region were broken when this blastomere was separated from its sister (large arrows). Bar, 5 µm × 900. (c) Cracked 4-cell embryo showing one A and one B blastomere. Bridge bands seen here resulted from the second cleavage division and are concentric with the inner surface of the embryo. Bar, 5 µm × 1,000. (d) Bridge band resulting from second division. Note regularity of bridge spacing and hexagonal packing pattern. Bar, 5 µm × 4,000. (e) Half of an embryo which is undergoing the third cleavage division. (Note that proximal furrows appear smooth and continuous.) Exposed bridge band resulted from the first division. Bar, 5 µm × 850. (f) Isolated A cell which is undergoing the third division. Exposed bridges resulted from divisions I and II. Bar, 5 µm × 2,650.
(a) Cell number varies as described earlier. (b) After division IV, the average length of the furrows in each division is \(1/\sqrt{2}\), or 0.7071, the length of the furrows in the preceding division.\(^2\) (c) The density of cytoplasmic bridges in all cleavage furrows is constant and hence the number of bridges formed in successive furrows varies by a factor of 0.7071. (d) At each division, pre-existing bridges are preserved and divided equally between daughter cells, while new bridges are formed between them. Selected specimens used to obtain bridge counts are presented in Figs. 4 and 5 and the major results of this analysis are summarized graphically in Fig. 6.

The validity of the extrapolations used in calculating bridge numbers in late cleavage appears to be supported by two facts: (a) the extrapolation fits the observed data, within the limits of experimental error, for divisions IV through VI; and (b) the number of bridges per average cell at the end of cleavage (for an embryo exhibiting only eleven divisions) extrapolates to 26, which is virtually identical with our measured mean value of 25.

The number of bridges in the entire embryo rises most rapidly during the first four divisions and the number of bridges per cell changes little during this phase. After division IV, the rate of increase in bridge number per embryo levels out to a simple exponential increase and the number of bridges per average cell declines monotonically. These observations are consistent with our earlier observation that the first four cleavage planes are all approximately meridional (and hence the furrows are long) and that the fifth is the first that approximates an equatorial cleavage.

**Cellular Localization of Bridge Bands and Formation of the Phialopore**

The location of the bridge bands on the lateral surfaces of the blastomeres is highly regular. From the first cleavage to the last, each bridge band defines an arc that is virtually concentric with the inner surface of the embryo (Figs. 4 and 5). In early cleavage these bridge band arcs lie distinctly proximal to the cellular equators (Figs. 4b–f and 5). But as cleavage progresses and the average (proximal-distal) length of the cells decreases (from \(\approx 20\ \mu m\) at the 4-cell stage to \(\approx 4\ \mu m\) at the end of cleavage), the bridge bands appear to lie closer to the cellular equators. It would appear that this relative displacement can be attributed primarily to a diminution in volume of the distal (chloroplast) end relative to the nuclear end of the cells.

\[^2\) This constant represents the ratio of chord lengths exhibited by two sets of regular hexagons whose areas differ such that it would require n of the larger and 2n of the smaller to pave a given surface; its use is justified by the observation that after the first few cleavages Volvox blastomeres generally exhibit hexagonal packing (13).

![FIGURE 5](https://example.com/figure5.png) **FIGURE 5** (a) Two blastomeres isolated from an 8-cell embryo. Bridges exposed on the anterior cell (arrows) resulted from the third division. Bar, 5 \(\mu m\) \(\times\) 2,600. (b) Portions of three cells isolated from an 8-cell embryo. Note regular spacing of bridge stubs. Also note continuity of bridge band stubs with unbroken bridges in interior (arrowheads). Bar, 5 \(\mu m\) \(\times\) 3,000. (c) Isolated posterior 8-cell blastomere. The bridge band almost completely rings the cell at the subnuclear level. Bar, 5 \(\mu m\) \(\times\) 3,000. (d) Half of the 32-cell embryo illustrating near perfect registration of bridge band from cell to cell and showing that the bridge system is concentric with the inner surface of the embryo. Note total absence of bridges on the exposed surface of the 1a cells and abrupt termination of the bridge band on the anterior surface of the 2a cell. Bridges that are visible on the 2a cell connected it to its neighboring 1a cell. Bridge-free surfaces define the phialopore. Bar, 10 \(\mu m\) \(\times\) 1,600. (e) Higher magnification of 32-cell bridge band. Bar, 1 \(\mu m\) \(\times\) 4,400. (f) Cracked 64-cell embryo which was treated with high initial solute concentration resulting in shrinkage of cells, thus allowing visualization of unbroken bridges between cells that remain in contact. Note continuity of broken bridge band with intact bridge band. Bar, 1 \(\mu m\) \(\times\) 6,500.

![FIGURE 6](https://example.com/figure6.png) **FIGURE 6** Graphic representation of cell numbers and bridge numbers during cleavage. On the solid line indicating \(\log_2\) cell number, open circles represent total cell number (before the differentiative cleavage) or number of somatic cell initials (after the differentiative cleavage) and filled circles represent number of gonidal initials. Points to the left of the vertical line in the middle of the graph were derived from actual counts; points to the right of that line were obtained by extrapolation.

(a) Two blastomeres isolated from an 8-cell embryo. Bridges exposed on the anterior cell (arrows) resulted from the third division. Bar, 5 \(\mu m\) \(\times\) 2,600. (b) Portions of three cells isolated from an 8-cell embryo. Note regular spacing of bridge stubs. Also note continuity of bridge band stubs with unbroken bridges in interior (arrowheads). Bar, 5 \(\mu m\) \(\times\) 3,000. (c) Isolated posterior 8-cell blastomere. The bridge band almost completely rings the cell at the subnuclear level. Bar, 5 \(\mu m\) \(\times\) 3,000. (d) Half of the 32-cell embryo illustrating near perfect registration of bridge band from cell to cell and showing that the bridge system is concentric with the inner surface of the embryo. Note total absence of bridges on the exposed surface of the 1a cells and abrupt termination of the bridge band on the anterior surface of the 2a cell. Bridges that are visible on the 2a cell connected it to its neighboring 1a cell. Bridge-free surfaces define the phialopore. Bar, 10 \(\mu m\) \(\times\) 1,600. (e) Higher magnification of 32-cell bridge band. Bar, 1 \(\mu m\) \(\times\) 4,400. (f) Cracked 64-cell embryo which was treated with high initial solute concentration resulting in shrinkage of cells, thus allowing visualization of unbroken bridges between cells that remain in contact. Note continuity of broken bridge band with intact bridge band. Bar, 1 \(\mu m\) \(\times\) 6,500.
exceptions, of course, are the cells bordering the phialopore; as these cells cleave, each retains a bridge-free surface on one face. Thus, by the end of cleavage each phialopore lip is lined by ~18 somatic initials that, although linked to cells on other sides, retain one bridge-free surface each. The phialopore of the fully cleaved embryo retains the shape it had at the 16-cell stage (Fig. 2f).

DISCUSSION

Cleavage Patterns and the Derivation of Gonidial Initials

In 1923 Janet (13) published the results of a painstaking analysis of cleavage in "Janetosphaera aurea" (now known as Volvox aureus). We concur with Starr (25) that the pattern of cleavage divisions that Janet described for V. aureus is applicable in a general way to cleavage in V. carteri up to the differentiative division (which is a hallmark of V. carteri development but is not observed in V. aureus). However, the SEM analysis reported here reveals at least two differences between the orientations of early cleavage planes in V. carteri and those reported by Janet for V. aureus. These differences had not been detected by previous workers who examined V. carteri cleavage with light microscopy only (16, 24, 25).

The first of these differences is probably of minor importance: whereas Janet reports that the relationship between cleavage furrows I and II in V. aureus is such that each A blastomere (using the nomenclature we introduce here) lies to the right of its sister B blastomere when the 4-cell embryo is viewed from the posterior pole, we observe the reverse relationship in V. carteri (Figs. 1 e and 3 c). This difference, originally detected in static SEM images, has been confirmed by examination of time-lapse movies of cleaving embryos (data not shown). Although recognition of this difference was important in our efforts to identify individual cleavage furrows in broken embryos for the purpose of counting the bridges formed at each division, it appears not to have any greater significance.

The second difference we observed is important, however, because it relates to the cell lineage of the gonidal initials and must be taken into account in any model which attempts to involve cell lineage as a factor in the gonidal determination process. Janet describes the fourth cleavage plane of V. aureus as being nearly equatorial (13). In describing cleavage in the strain of V. carteri used in our studies, Starr (25) reproduced Janet's figures, showing a nearly equatorial fourth cleavage plane and concluded that, as a result of the orientation of this cleavage, the entire anterior half of the embryo would be derived from tiers 1 and 2 of the 16-cell embryo and that in the sixth cleavage gonidal initials would be derived from those cells of the 32-cell embryo that we have designated 1a, 1p, 2a, and 2p in Figures 3 m-o (25). Because the plane of the fourth cleavage is difficult to discern clearly by light microscopic examination of living V. carteri embryos, we found no reason to question Starr's interpretation from our initial light microscope studies. However, SEM analysis clearly reveals that the fourth cleavage, like the first three, is much more nearly meridional than equatorial in V. carteri (Figs. 2 b and 3 k) and that, as a consequence, the four tiers of cells in the 16-cell embryo overlap much more extensively than Janet's drawings suggest for V. aureus. In V. carteri, the fifth cleavage plane is the first that approximates an equatorial direction (Fig. 3 n); hence it is the fifth, not the fourth, division that delineates the anterior and posterior hemispheres in this species. The result of this is that it is not the 1a, 1p, and 2 cells as previously supposed, but the 1a, 1p, 2a, and 3a cells that normally give rise to gonidal initials by an unequal cleavage at division VI (Figs. 2 c and d, and 3 m-r).

A provocative model for gonidial determination based on cell lineages has recently been published by Sumper (27). This model proposes that it is the contact faces inherited by sister blastomeres at each division that provides both the counting and the spacing mechanism that regulates appearance of reproductive cells. Unfortunately, the cell lineages this model predicts for gonidal initials do not correspond with the lineages actually observed, either according to Starr's earlier interpretation or according to our reinterpretation.

A second attempt to relate gonidal determination to cell lineages has been made in our laboratory. Based on experiments that suggest that gonidial vs. somatic determination may involve DNA rearrangements, one of our colleagues has attempted to determine whether the normal locations of gonidia in V. carteri could be accounted for by a regularized pattern of changes at the DNA level analogous to the "cassette model" for mating-type switches in homothallic yeast strains. Furthermore, no regularized gene switching mechanism can readily account for the fact (which any explanation of gonidal determination must do to be fully satisfactory) that in a clonal population of V. carteri grown under conditions where the modal gonidal number is 16, occasional individuals are seen with greater or lesser numbers of gonidia. (Although we have not attempted to make a systematic examination of cleavage in these exceptional cases with the SEM, we suspect that they may result from occasional asymmetric cleavage divisions in 2p cells or occasional failure of 3a cells to cleave asymmetrically at division VI.)

Pall (20) has proposed a model, based on analysis of certain mutants, which suggests that gonidial determination is based on cell size: large cells become gonidia and small ones become somatic cells. Even if valid, however, this model fails to deal with the question of why the cells that first divide asymmetrically and then cease dividing prematurely (thereby becoming gonidal initials) are regularly located where they are.

It appears now that of the hypotheses proposed to date to explain gonidial determination the one most compatible with all existing data (including the cell lineages described in this paper) is that of Kochert (17). His data suggest that it is an ultraviolet sensitive, particulate inclusion asymmetrically distributed in uncleaved gonidia and cleaving embryos that determines the location of the next generation of gonidia in wild-type V. carteri embryos. This hypothesis is clearly worthy of more examination from a cytological and molecular point of view.

Further studies of "pattern mutants" in which reproductive cell numbers and/or locations are distributed differently from

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3 A preliminary account of this work has been published in abstract form by N. Zagris, and D. L. Kirk (30). A more complete account is in preparation.
those of wild type in a heritable, predictable fashion (3, 11, 23)
are known to be underway (R. J. Huskey, personal communi-
cation) and may be expected to shed more light on the question
of gonidial determination. Of particular interest are the mulA
mutants which possess clusters of two to four gonidia in the
locations where a single gonidium normally occurs, indicating
that in this strain gonidial initials may divide one or two times
to produce multiple daughter cells with gonidal potential (3).
Because our present study clearly shows that gonidial initials
normally divide about three times to produce additional soma-
cell initials (as Starr [25] had anticipated they might), it
will be most interesting to learn how the cleavages of gonidial
initials in the mulA strains differ from those of the gonidial
initials of wild type.

Cytoplasmic Bridge System

Janet (13) stated that after each cleavage division of the V.
aureus embryo, the two sister cells remain linked by a single
“plasmodesma of incomplete cytokinesis” (to quote the original
French: “Chaque facette laterale comporte un plasmodesme
d’irachecvement de division cellulaire”). He also asserted that
these “plasmodesmata” contain remnants of the mitotic spindle.
This description clearly left the impression that the con-
nections formed between sister cells during Volvox cleavage
are homologous to the “midbodies” that have since been seen
frequently in dividing animal cells and described in consider-
able detail (e.g., reference 19). Bisalputra and Stein later reex-
amined the connections in cleaving V. aureus embryos (2), and
although they suggested on the basis of ultrastructure that
“cytoplasmic bridges” would be a more apt term than plas-
modesmata for the connections, their unamplified statement
that the bridges form as a result of incomplete cytokinesis did
not clearly dispel the notion that these bridges resembled
midbodies in origin or numbers. Neither have the reports of
subsequent investigators who have detected such bridges and
described their ultrastructure in a number of other Volvocacean
species (1, 2, 6, 7, 8, 12, 18, 21, 28). This is not surprising, since
obtaining a complete picture of a bridge system like that of V.
carteri via TEM analysis would require a tedious process of
serial sectioning and reconstruction that has apparently not yet
been undertaken.

However, SEM analysis makes it immediately apparent that
any analogy between the cytoplasmic bridges of V. carteri and
the midbodies of animal cells is wholly inappropriate. This is
most clearly seen in an SEM view of a 2-cell embryo (Figs. 4a
and 4b), where it is clear that instead of the one bridge that
would be predicted by a midbody analogy, over 200 bridges
are formed in the first cleavage furrow.

Our analysis of bridge numbers, spacings and locations
during and after successive stages of division provide data
(Figs. 4, 5, and 6) to support the following sequence of gen-
eralizations: (a) At all divisions, numerous bridges are formed in
each cleavage furrow; the number of new bridges formed at
each division is, at least as a first approximation, proportional
to the lengths of the cleavage furrows formed at that division.
(b) Once formed, bridges are conserved and subdivided be-
tween sister cells at subsequent divisions at the same time
that new bridges are being formed between them. As a conse-
quence, the total number of bridges in the embryo rises through-
out cleavage, but the number of bridges per average cell falls
monotonically from the fifth cleavage on. (c) Bridges are
always restricted to a more-or-less well-defined band on the
lateral face of each cell (Figs. 4b-f and 5a-f), a location which
the TEM analysis reported in our companion paper (9) shows
to be between the nuclear and chloroplast levels of the cells.
(d) In most cases, particularly after the first one or two cleav-
ages, bridges within each band exhibit a rather regular 500-nm
center-to-center spacing, and the relationships among adjacent
bridges within a band approximates that which would result
from hexagonal close-packing (Fig. 4d). (e) The bridge bands
of all neighboring cells are always in nearly perfect register
with one another throughout cleavage (Figs. 4c and e, 5a, b,
and d-f).

The integrity of the bridge bands within each cell, and the
fact that bridge bands of adjacent cells are completely in
register, lead us to suggest that beyond being linked to its
immediate neighbors by a number of bridges, each cell of a
Volvox embryo can be thought of as being linked to all others
by a single, coherent network, or continuum, of cytoplasmic
bridges which we propose to call the “cytoplasmic bridge
system” of the Volvox embryo. The function of this cytoplasmic
bridge system is to link the cells into an embryo of defined
shape. Our companion paper (9) will deal with the mechanism
of bridge formation, the structural basis for the integrity of the
bridge system, and how the integrity of the bridge system
provides the basis for the morphogenetic feat of inversion.

The fact that a continuous band of cytoplasmic bridges is
formed in each cleavage furrow, and that such bands are
subdivided (essentially equally) among daughter cells at sub-
sequent divisions, would appear to pose a fundamental diffi-
culty for a model such as that published by Sumper (27), which
postulates an asymmetric distribution of previously formed
contact zones among sister cells as an important determinant
of cell fates. It is worth noting in this regard that as far as we
are aware, no form of specialized cell junction other than
cytoplasmic bridges has yet been described in the family
Volvocaceae, to which Volvox belongs.

Previous authors have described the existence of two sets of
cytoplasmic bridges (one “medial” and one “basal” set) in the
embryos of various Volvocacean species, such as Pandorina
morum (7), Eudorina elegans (8, 18), and Volvox tertius (21).
The only time we have seen bridgelike structures at the basal,
chloroplast, ends of V. carteri embryonic cells is when a
cleavage division is in progress. The chloroplast end of each
cell is always the last portion to complete division. Further-
more, the appearance of numerous points of invagination along
the presumptive cleavage plane at the chloroplast end of the
cell (Fig. 1c) contrasts sharply with the single smooth furrow
typically seen at the apical end (Figs. 1b, and 4e, f) and suggests
that the mechanism of cytokinesis may differ between these
two regions. In any case, cells which have completed
 cytokinesis at their apical ends frequently possess one or more
broad regions of undivided cytoplasm at their distal ends. These
undoubtedly would bear some resemblance to cytoplasmic brid-
ges if viewed in TEM sections. However, SEM analysis
makes it quite clear that in specimens where cytoplasmic
connections are clearly visible at the chloroplast ends of all (or
nearly all) furrows of the most recent division, similar structures
are not seen along earlier division furrows. From these obser-
vations we conclude that the connections at the basal (chlo-
roplast) ends of V. carteri cleavage furrows are transient structures
indicative of the fact that division was still in progress at the
time the specimens were fixed; they bear little structural or
functional resemblance to the medial bridges which are com-
ponents of the cytoplasmic bridge system described above.
Formation of the Central Cavity and the Phialopore

Despite superficial similarities (a layer of cells surrounding a central cavity), there are fundamental differences between a fully cleaved Volvox embryo and the blastula stage of an animal embryo. In an animal embryo the blastocoele typically develops on the interior of a ball of cells that remains topologically "closed" throughout cleavage. In Volvox, in contrast, the central cavity originates on the exterior of a flattened embryo; and even though the cavity becomes almost completely surrounded by cells as a result of subsequent events, the embryo remains topologically "open" as a consequence of the persistence of the phialopore slits. The significance of this difference becomes grossly apparent right after cleavage: when cells at equivalent points in the two types of embryos undergo fundamentally similar shape changes, the topologically closed animal embryo invaginates (gastrulates), whereas the topologically open Volvox embryo evaginates (inverts) through its phialopore (28, 29).

The central cavity of the Volvox embryo begins as a rhomboidal pit on the surface of the flattened gonidium before the first cleavage (Fig. 1a). The phialopore lips are derived from the bridge-free regions left on the anterior surfaces of the first four blastomeres following cleavages I and II (Figs. 1d and 3a). The surrounding of the central cavity and the formation of the definitive phialopore both occur as lobes of these four blastomeres—and eventually cells derived from these lobes—extend anteriorly and centripetally toward one another between the second and fifth cleavage stages. During early stages of this cellular extension it might appear that the phialopore slits are destined to lie along the boundaries between the four quadrants of the embryo—in the planes defined by cleavage furrows I and II (Figs. 1d and 3a). But, surprisingly perhaps, the detailed nature of the extensions that occur subsequent to cleavage III are such that the phialopore comes to bisect each quadrant: during this period the four anterior cells extend in such a manner that the bridge bands that were formed in the first and second furrows (and now link each anterior cell to the posterior cell of the next quadrant to the right) are extended forward, while the bridge bands that were formed in cleavage III are not similarly extended. This interpretation is confirmed by SEM analysis of cracked embryos, which reveals that at the 16- and 32-cell stages of the first two cleavage stages the bridge-free regions—the phialopore slits—are displaced clockwise from the planes defined by cleavage furrows I and II (Figs. 5d). The result of this is that by the 16-cell stage the bridge-free regions—the phialopore slits—are displaced clockwise from the planes defined by cleavages I and II and the phialopore takes on its characteristic swastika appearance (which is then retained throughout subsequent cleavage stages) (Figs. 2a-f, 3j, m, and p).

The mechanism by which these controlled cellular extensions occur to produce the hollow spherical shape of the embryo is the subject of continuing study in our laboratory. Preliminary studies suggest that the process can be selectively inhibited by cytochalasin D and that interference with it causes defects in adult morphology that mimic those seen in certain morphological mutants.

We consider it noteworthy that embryos between the 4-cell and the 32-cell stages of cleavage regularly exhibit extensions resembling filopodia in the region of the cleavage furrows (Figs. 1h and 2b). Two considerations suggest that these structures are genuine features of the embryo and not fixation artifacts: (a) such projections can be seen (but not clearly resolved) in time-lapse movies of live, cleaving embryos; (b) when embryos of different stages are fixed in the same vessel, such extensions are only seen in embryos of the stages specified above. We postulate that they are somehow involved in the cellular extension process; if so, the details of this relationship will await further study.

Concluding Remarks

In the next paper we report an analysis of the mechanism by which the cytoplasmic bridge system arises during cleavage, how its structure may be stabilized, and how it functions in subsequent morphogenetic events. Combined with the detailed description of the cleavage patterns, cell lineages, organization of the bridge system and formation of the phialopore reported in this companion paper, these observations should provide a firm basis for future analysis of the specific defects present in particular strains of morphogenetic mutants, an analysis which is now going on in our laboratory.

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