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Differentiation of Reproductive Cells in *Volvox carteri**

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SYNOPSIS. The life cycle of *Volvox carteri* was studied in axenic culture using the NB-3 and the NB-7 strains isolated from Nebraska. Vegetative colonies of both strains contain 8-12 asexual reproductive cells (gonidia) which divide to form daughter colonies. During daughter colony formation, the reproductive cells of the daughters are delimited at an early stage of cleavage. Gonidia are delimited at the division from 16 to 32 cells, but eggs and male initial cells are not differentiated until the division of the 32-celled stage. In all instances the reproductive cells are the products of unequal cleavages.

Male and female colonies are formed in separate clones. Female colonies contain approximately 20 eggs. Male colonies have approximately 50 male initial cells, each of which forms a sperm bundle containing 64 or 128 sperm. Sperm bundles penetrate female colonies and fertilize the eggs. Zygote formation, zygote germination, and the development of gone colonies is described. Sexual type was inherited in a 1:1 ratio.

Male colonies appear spontaneously in the male strain, but female colonies were formed in the female strain only in the presence of a substance produced by colonies from male cultures. This female inducing substance is produced in male cultures primarily, if not exclusively, by male colonies rather than by vegetative colonies. The female inducing substance is heat labile and non-dialyzable. Activity is destroyed by Pronase, but not by trypsin, chymotrypsin or ribonuclease.

Gonidia appear to be most susceptible to female induction during the early stages of their expansion prior to cleavage.

THE colonial green flagellates of the family Volvocaceae present an interesting study in communal association of cells. The more primitive members of the family are scarcely more than loose groupings of structurally and physiologically similar cells, while the more advanced forms are complex associations of cells structurally and physiologically specialized for different functions. In *Gonium, Pandorina* and *Volvulina* all cells of the colony have the potential to function both as asexual reproductive cells and as isogamous gametes. Colonies of certain species of *Eudorina* have the 1st tendencies toward differentiation into somatic and reproductive cells. In these species, cells composing one or more of the anterior tiers of cells are much reduced in size. These cells are much delayed in cleavage to form daughter colonies and, under certain conditions, may not divide at all. True division of labor is encountered in the genus *Volvox,* where colonies are differentiated into a large number of somatic cells with no potential for division and a much smaller number of reproductive cells.

The merits of using *Volvox* as an experimental organism in studies of cellular differentiation have been discussed by Darden (10) in an investigation of the sexual differentiation of a strain of *Volvox aureus*. Darden emphasized the lack of knowledge of factors controlling developmental processes in other species of the genus.

Since its discovery by Carter (8) in 1859, *Volvox carteri* has been studied by many investigators. Powers (26), Playfair (23), Shaw (38), Iyengar (16), and Apte (1) described the more obvious details of the structure of *V. carteri* from natural populations. Metzner (20, 21) described the structure of a variety of *V. carteri* from populations growing in tanks used to raise aquatic plants. Metzner was able to maintain *V. carteri* in Uspenski solution (44) and in source water to which Uspenski's solution had been added. Cultures were successful, however, only when large numbers of all types of colonies were used as inoculum and even then they could not be maintained indefinitely.

Altho many investigators have described the structure and life cycle of *V. carteri,* it had never been studied axenically in clonal culture, and detailed studies of the embryonic development of colonies had not been published. The isolation of several strains of *V. carteri* provided material for a study of the factors controlling differentiation of reproductive cells in this species of *Volvox.*

TAXONOMIC CONSIDERATIONS

In 1859, Carter described a form of *Volvox* which he erroneously identified as *Volvox globator* from collections made in India. Stein (42) realized that this was a previously undescribed species and named it *V. carteri.* In 1908, Powers (26) described *Volvox weismannia* from collections made near Rocheport, Missouri. Shaw (38), recognizing the similarities between *V. carteri* and *V. weismannia,* reduced *V. weismannia* to a variety of *V. carteri.* Printz (27), Iyengar (16), and Metzner (21) followed Shaw in including *V. weismannia* as a variety of *V. carteri,* while Smith (39) considered them separate species. Pascher (22) doubted the validity of considering *V. weismannia* as a separate species, but stressed the need for comparative studies.

The strains used in this study are of the type described by Powers as *V. weismannia,* but I have followed Shaw in including them as a variety of *V. carteri.* A comparative study of the varieties of *V. carteri* was beyond the scope of this investigation, but I am at present collecting isolates

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of *V. carteri* from various parts of the world. Perhaps these can be used to clarify taxonomic relationships in this species.

MATERIALS AND METHODS

Two strains of *V. carteri* were used in this study. NB-3 is the male strain and NB-7 is the female strain. These were isolated in September, 1965 from natural populations occurring in Seward County, Nebraska. Colonies were isolated under a dissecting microscope using a fine glass pipette, washed thru several changes of sterile distilled water, and inoculated into tubes of Pringsheim's soil-water medium containing $CaCO_3$ (41). After a clonal population had become established, colonies with mature daughter colonies were selected for preparation of axenic cultures. These colonies were ruptured by forcing them thru a pipette which had been drawn out to a diameter slightly smaller than that of the colony. Daughter colonies thus released were washed thru several successive changes of volvox medium (28) and inoculated into tubes containing approximately 20 ml of volvox medium. Cultures were checked for bacterial contamination with nutrient broth, and by microscopic examination.

Unless otherwise noted, both stock and experimental cultures were maintained in a lighted constant temperature room. Illumination with an intensity of 350-400 ft-c was provided by banks of cool white fluorescent tubes. The lights were controlled by a clock device which automatically provided 16 hours of light and 8 hours of darkness per 24 hour period. The temperature was maintained at 20 ± 1 C. Non-axenic stock cultures were grown in soil-water tubes and transferred monthly. Axenic stocks were maintained in 250 ml Delong flasks and transferred every 2 weeks.

CELL AND COLONIAL STRUCTURE

Colonies of the NB-3 and NB-7 strains of *V. carteri* are oval to spherical with 2,000-6,000 cells arranged in a single layer (Figs. 1, 2). Each colony contains 2 types of cells: a small number of reproductive cells and a relatively larger number of somatic cells.

The protoplast of each somatic cell is typically chlamydomonad and has a single cup-shaped chloroplast with basal pyrenoid, a central nucleus, 2 contractile vacuoles, and 2 flagella. A thick hyaline sheath surrounds each somatic protoplast. The lateral edges of adjacent somatic cells are angular by mutual compression. In side view the cells form polygonal prisms with the cell protoplast located in the outer portion of the prism. Somatic cells are thickest in the posterior portion of the colony and become thinner toward the anterior portion and over the reproductive cells. Developing daughter colonies are enclosed in vesicles which expand into the interior of the colony. A watery gelatinous material fills the remaining space inside the colony.

The component of the colonial matrix responsible for the structural integrity of the colony is apparently proteinaceous since treatment with the proteolytic enzymes trypsin, chymotrypsin or Pronase completely dissociates colonies of *Volvox* to single cells. Pronase at a concentration of 50-100 μg/ml was routinely used for dissociation experiments. Somatic cells freed from the colony by Pronase continue active motion for some time, but eventually die even if washed free of Pronase. Reproductive cells are not harmed by Pronase isolation and develop normally if care is taken to remove all Pronase by washing.

Vegetative, female and male colonies of *V. carteri* have similar somatic cell structure, but each contains a different type of reproductive cell. Vegetative colonies contain asexual reproductive cells (gonidia) which give rise to daughter colonies by repeated division; female colonies contain eggs, which form zygotes after fertilization; and male colonies contain male initial cells, which divide to form sperm bundles.

Mature vegetative colonies of both the NB-3 and the NB-7 strains average 600-700 × 700-800 μ (Figs. 1, 2). Eight to 12 gonidia are usually present and are characteristically arranged in alternating tiers of 4 (Figs. 2, 20, 21). In colonies with 8 gonidia, division of the gonidia to form daughter colonies is nearly simultaneous, altho there is often some tendency for the anteriormost tier of gonidia to divide 1st. In colonies with more than 8 gonidia the posteriormost tier is composed of smaller gonidia which are delayed in cleavage. They may be present as undivided gonidia in colonies which contain nearly mature daughter colonies in the first 2 tiers (Fig. 1).

Gonidia are large (80-100 μ), spherical, highly vacuolate cells containing numerous pyrenoids (Fig. 7). The nucleus is suspended in the center of the cell by thick cytoplasmic strands extending from the peripheral layer of cytoplasm. Mature gonidia are not flagellated, but 2 highly refractile structures similar to the basal bodies of the somatic cells can often be seen in the anterior end of the gonidium. Faint surface striations radiate from a small clear area at the anterior end of each gonidium (Fig. 8).

Female colonies produced by the NB-7 strain are slightly smaller than vegetative colonies. They contain 8-25 eggs (most frequently 16-20) which are not arranged in the regular tiers so characteristic of gonidia in vegetative colonies, but are scattered over the posterior 2/3 of the colony (Fig. 4). Mature eggs are smaller (30-50 μ) and less vacuolate than gonidia and are biflagellate. The flagella are most easily seen on eggs released from the parent female colony by treatment with Pronase or by breaking open the parent colony under the weight of a coverslip on a glass slide.

The NB-3 strain normally produces dwarf male colonies characteristic of the species (Figs. 3, 5). These are the same size or slightly larger than vegetative colonies at birth, but expand only slightly after release from the parent colony. Mature dwarf male colonies are 250-600 μ in diameter and contain 200-500 cells, approximately 50 of which are male initial cells. Male initial cells are similar to gonidia in appearance, but are smaller and less vacuolate. Division of male initial cells to form sperm bundles occurs before the male colony is released from the parent colony. Each male colony contains both 64- and 128-celled sperm bundles. Occasionally sperm bundles are released inside the parent colony before birth of the dwarf male.

In addition to dwarf male colonies, strain NB-3 produces occasional male colonies which are the same size as vegetative colonies and which contain only 8 or 10 sperm bundles (Fig. 6). Moreover, these sperm bundles are arranged in tiers of 4 in the manner of gonidia in vegetative colonies and contain a larger number of sperm (approx-

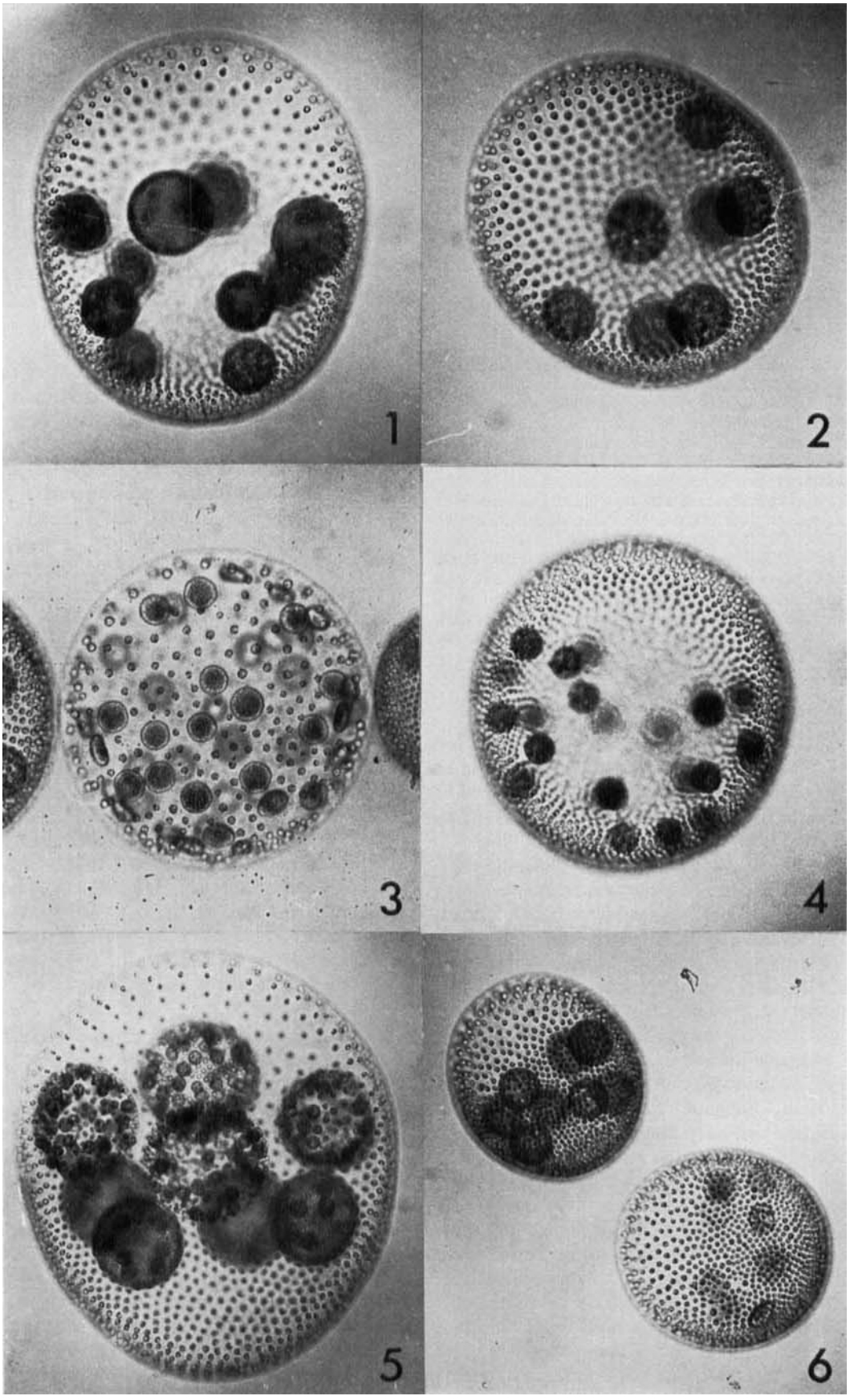


Fig. 1. Vegetative colony. Note anterior 2 tiers contain post-inversion daughter colonies while the 2 gonidia in the posteriormost tier are still undivided. × 125. Fig. 2. Vegetative colony with mature gonidia. Note ranking of gonidia in 2 tiers of 4. × 140. Fig. 3. Surface view of dwarf male colony with mature sperm bundles. × 200. Fig. 4. Female colony. Note eggs scattered thru the posterior ⅔ of the colony. × 150. Fig. 5. Vegetative colony containing 4 vegetative and 4 dwarf male colonies. × 90. Fig. 6. Comparison of a young vegetative colony (upper left) and a non-dwarf male colony. × 120.

imately 256) than do sperm bundles in dwarf male colonies. It appears, then, that gonidial initials can in some cases divide to form sperm bundles rather than daughter colonies.

ASEXUAL REPRODUCTION

In the genus *Volvox* asexual reproduction is accomplished by the cleavage of asexual reproductive cells (gonidia) to form daughter colonies. In *V. carteri* gonidia may cleave to form vegetative, female or male daughter colonies. Early cleavage stages in the formation of all 3 types of colonies are similar to those reported for *V. aureus* by Janet (17) and Darden (10). In *V. carteri*, however, differentiation of reproductive cells occurs much earlier in embryonic development than in *V. aureus*.

Development of Vegetative Colonies. Gonidia which are about to begin division show a pronounced flattening in a plane perpendicular to the 1st division plane. Further flattening and curvature give the 2-celled embryo (Fig. 9) a bowl-shaped appearance in side view. The concave side, which represents the anterior (phialopore) pole of the embryo, is directed toward the exterior of the parent colony. The plane of the 2nd division is frequently oblique to that of the 1st division so that at the 4-celled stage the embryo consists of a pair of cells which are in contact with each other and a pair of cells which are not in mutual contact. The 8-celled embryo (Fig. 10) is made up of 2 alternating tiers of 4 cells and is shaped like a shallow bowl. At this stage, the bowl has a wide opening (phialopore) which becomes progressively smaller as division proceeds. The closed bottom of the bowl represents the posterior pole of the embryo. Further radial divisions form the 16-celled embryo (Fig. 11). Cells of the 16-celled embryo are nearly uniform in size and are arranged in 4 overlapping tiers of 4 cells each.

In developing vegetative daughter colonies of *V. carteri*, the 1st evidence of differentiation into somatic and reproductive cells is seen at the division from 16 to 32 cells. Each cell in the anteriormost 2 tiers (*a* and *b* in Fig. 12) of the 16-celled embryo divides unequally to form a large gonidial initial cell (a_1 and b_1 in Fig. 14) and a smaller cell (a_2 and b_2 in Fig. 14) which is usually somatic in nature. Thus 8 gonidial initials are differentiated at the division from 16 to 32 cells. These form the anterior 2 tiers of gonidia seen in the mature colony. Under certain conditions, however, more than 8 gonidia are present in colonies of *V. carteri*. These additional gonidia form tiers or partial tiers in the posterior portion of the mature colony and characteristically lag in division to form daughter colonies. These gonidia are derived from gonidial initials formed in subsequent divisions of cells designated a_2 in the 32-celled embryo (Figs. 13 and 14). In the division from 32 to 64 cells, each a_2 cell can divide unequally to produce a large gonidial initial cell and a smaller somatic initial cell (Fig. 15). Thus gonidia which form the third (posteriormost) tier in mature colonies are formed 1 division later than the 8 gonidia which constitute the anterior 2 tiers. This probably accounts for their smaller size and characteristic lag in division.

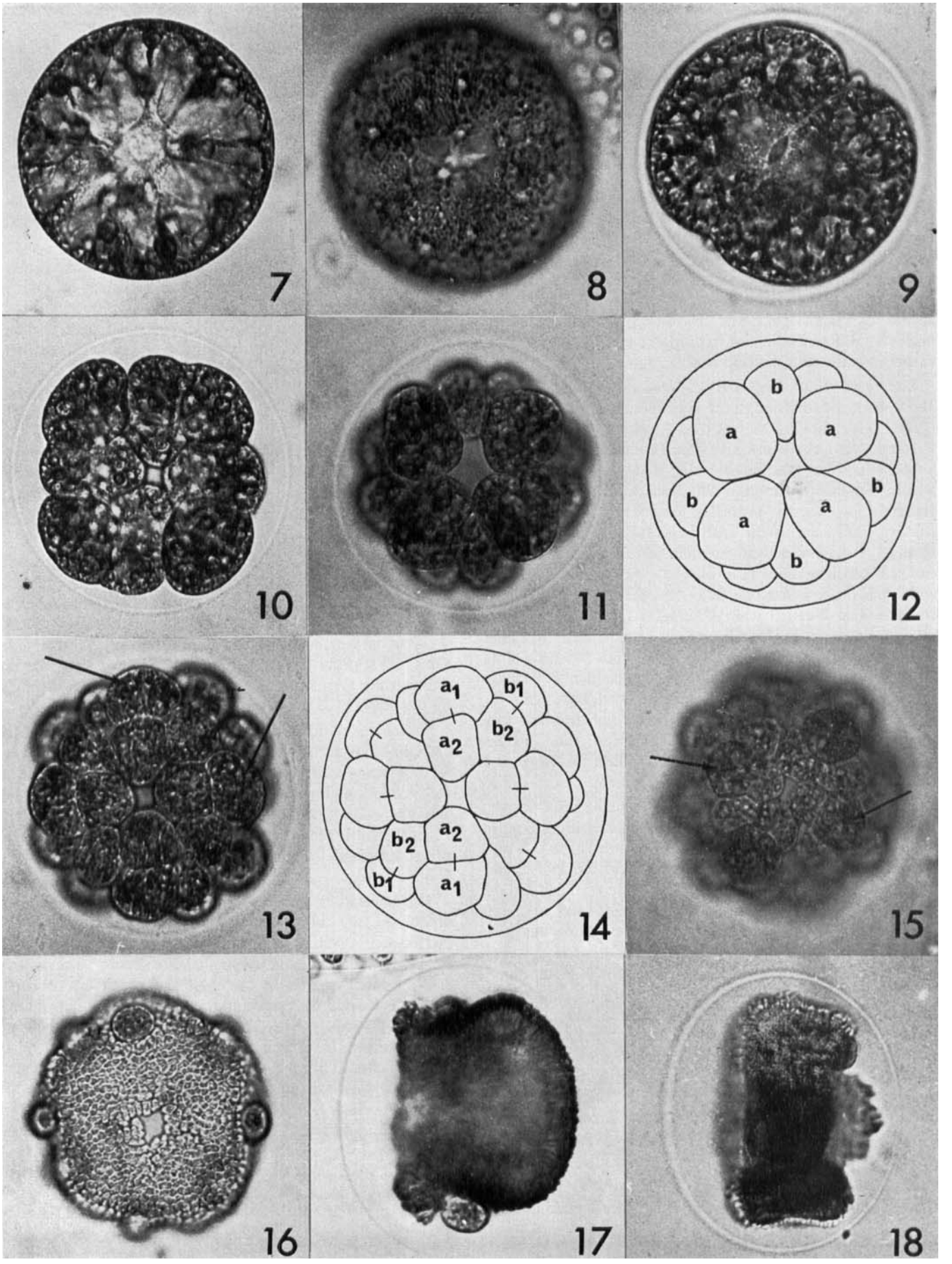
Gonidial initials formed at the division from 16 to 32 cells bud off a somatic initial cell at the division from 32 to 64 cells. Gonidial initials formed at the 32-64 celled division (which form the posterior tier of smaller gonidia) bud off a somatic initial cell at the 64-128 celled division. Beyond this stage cell lineages are difficult to follow, and it was not determined at what stage in subsequent cleavage gonidial initials cease unequal division.

Subsequent divisions result in the formation of a hollow sphere of cells with the gonidia conspicuously placed on the outer surface and the anterior ends of the cells directed toward the interior (Fig. 16). The phialopore becomes inconspicuous or completely absent at this stage. After cleavage is complete the embryo enters a period of structural differentiation. This post-cleavage period is characterized by dents which appear at various places on the surface of the embryo. Individual somatic cells of the embryo also undergo structural differentiation. They become elongated, and colorless, pseudopod-like processes may extend from the outer ends of cells in the posterior portion of the embryo.

Inversion begins with the formation of 4 or more lobes around the newly reappeared phialopore (Fig. 16). As these lobes begin to separate one can often see cytoplasmic strands connecting some of the lobes. These strands can also be seen at earlier stages of cleavage if embryos are smashed under the weight of a coverslip on a glass slide. Thus it appears that cells of developing embryos are connected by cytoplasmic strands as noted by Pocock in *Volvox tertius* (25).

As inversion proceeds, the lobes around the phialopore bend back over the surface of the embryo (Figs. 17, 18). At this stage each cell has 2 short flagella which are in active motion. In the terminal stages of inversion the phialopore lobes come together in what is now the posterior portion of the young daughter colony and close, in zipper fashion, leaving little or no trace of an opening (Figs. 19, 20, 21).

The developing daughter colony now enters a period of expansion and differentiation. Formation of the gelatinous matrix of the colony begins and the cells become separated from one another. Increase in daughter colony size in this post-inversion period is accomplished both by enlargement of individual cells and by the secretion of matrix material between the cells. Eyespots are formed during this stage, and the somatic cells assume the typical adult shape. Gonidia begin to expand and become more vacuolate, reaching a diameter of approximately 25 μ before the daughter colony is released from the parent colony. During this maturation period only one flagellum can be seen protruding from the surface of the daughter colony matrix above each somatic cell. Since 2 short flagella were present at inversion, one of these must have elongated so that it became of sufficient length to protrude from the colony matrix, while the other remained short and embedded in the matrix. The shorter flagellum later elongates so that at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release from the parent colony the somatic cells have the typical biflagellate condition. This process of differential flagellar elongation is similar to that described for *Eudorina, Pandorina,* and *Astrephomene* by Goldstein (15).

Mature daughter colonies rotate slowly in their vesicles before release from the parent colony. They are released thru individual pores which form in the parent matrix over each mature daughter colony. It was suggested by Pocock (24) that mature daughter colonies release a "ferment" which digests the pore in the parent colony thru which *Volvox* daughter colonies escape. When Pronase is applied to colonies of *V. carteri,* one of the 1st manifestations of its action is the birth of daughter colonies in all stages of cleavage. Even undivided gonidia are released in a process reminiscent of the natural release of daughter colonies. This suggests the involvement of a proteolytic enzyme in natural daughter colony release, but further experiments are needed before definite conclusions can be drawn.

Newly released daughter colonies may be as large as 200 μ. They swim actively and undergo further expansion before cleavage of their gonidia begins.

Development of Female Colonies. Cleavage of gonidia to form female daughter colonies is similar to that of vegetative daughter colony formation up to the 16-celled stage. The 16-32 celled division in formation of female daughter colonies does not involve unequal divisions, however, so all the cells of the 32-celled female embryo are about the same size (Fig. 22). Differentiation of egg initial cells takes place at the 32-64 celled division by unequal division of some of the cells of the embryo (Fig. 23). The cells which divide unequally to form egg initials are not homologous to those which produce gonidial initials in developing vegetative daughter colonies. Egg initials are not formed in the regular tiers characteristic of gonidial initials, but are scattered over the anterior 2/3 of the embryo. This is reflected in the random distribution of eggs seen in the mature female colony. Egg initials divide unequally in the 64-128 divison with the smaller products becoming somatic initials. It was not determined at what stage egg initials cease dividing.

The later stages of cleavage, inversion, and maturation of female daughter colonies are similar to those described above for vegetative daughter colony formation (Fig. 24). Eggs are flagellated at maturity, but the flagella are not of sufficient length to protrude from the matrix of the female colony.

Development of Dwarf Male Colonies. The early stages in cleavage of gonidia to form dwarf male colonies are similar to the corresponding stages in female daughter colony formation. At the 32-celled stage all the cells of the developing male embryo are approximately the same size. Male initial cells are formed by unequal division of cells in the 32-64 celled division. More male initial cells are differentiated in the same manner at the 64-128 celled division. Male initial cells are formed at random over the embryo, and only a small area at the posterior portion of the embryo is free of reproductive cells (Fig. 25). Fifty or more male initial cells are typically formed. After inversion (Figs. 26, 27) the dwarf male colony expands to its full adult size while still enclosed in the parent colony (Figs. 5, 28).

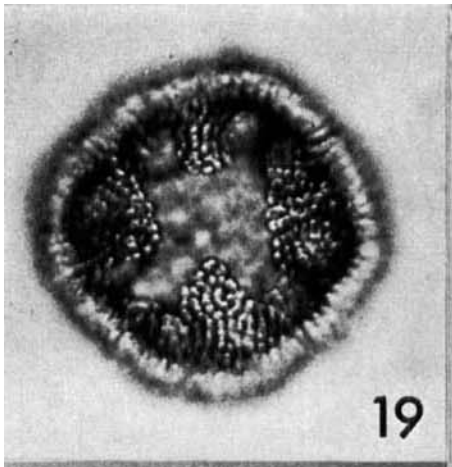
Division of male initial cells to form sperm bundles takes place before release of the dwarf male from the parent colony. Early stages of sperm bundle formation resemble the corresponding stages in daughter colony formation. A bowl-shaped mass of 64 or 128 cells is eventually formed (Fig. 29). This mass of cells undergoes a partial inversion (Fig. 30) to form a sperm bundle which is convex on its anterior side. Each sperm forms 2 flagella and an eyespot during post-inversion maturation of the sperm bundle. Mature sperm bundles rock slowly from side to side in vesicles presumably derived from the walls of the parent male initial cell. Release of sperm bundles thru individual escape pores usually occurs after birth of the dwarf male colony.

SEXUAL REPRODUCTION

The NB-3 and NB-7 strains of *V. carteri* are heterothallic (male and female colonies are formed in separate clonal cultures). In order to observe the full cycle of sexual reproduction it is necessary to mix male and female clones.

Fertilization and Zygote Production. Sperm bundles swim rapidly about in the medium after release from the male colony. When sperm bundles come in contact with female colonies an interaction begins (Fig. 31). The sperm bundle attaches to the female colony, usually in the posterior portion, and the colonial matrix of the female colony begins to break down at the point of contact with the sperm bundle. A few somatic cells may be released from the female colonial matrix during this process and swim off into the medium. Concomitant with the breakdown of the matrix of the female colony, the sperm bundle begins to dissociate into individual sperm which penetrate the female colonial matrix (Figs. 32, 33). Sperm are very active at this stage and move in an amoeboid fashion between the somatic cells of the female colony or swim with a corkscrew motion thru the watery material in the interior of the colony. The point of entry of the sperm

Fig. 7. Mature gonidium. Note central nucleus, nucleolus and many pyrenoids. × 525. Note: This and subsequent figures of cleavage stages were photographed with material removed from the parent colony for greater clarity. Fig. 8. Surface view of a mature gonidium. × 525. Fig. 9. Two-celled embryo. × 525. Fig. 10. Phialopore view of 8-celled embryo. Note vesicle surrounding embryo. × 525. Fig. 11. Phialopore view of 16-celled embryo. × 525. Fig. 12. Diagram of the 16-celled embryo shown in Fig. 11. Each cell marked *a* or *b* will divide unequally to form a gonidial initial cell and a somatic initial cell. × 525. Fig. 13. Phialopore view of 32-celled embryo. Note enlarged gonidial initial cells (arrows). × 525. Fig. 14. Diagram of 32-celled embryo shown in Fig. 13. Cells marked a_1 and b_1 are gonidial initials. × 525. Fig. 15. Phialopore view of 64-celled embryo. Gonidial initials indicated by the arrows were formed at the division from 32 to 64 cells. × 525. Fig. 16. Young daughter colony just beginning inversion. Newly formed lobes are visible around the phialopore. × 390. Fig. 17. Side view of an early stage of inversion. × 525. Fig. 18. Later stage in inversion. × 425.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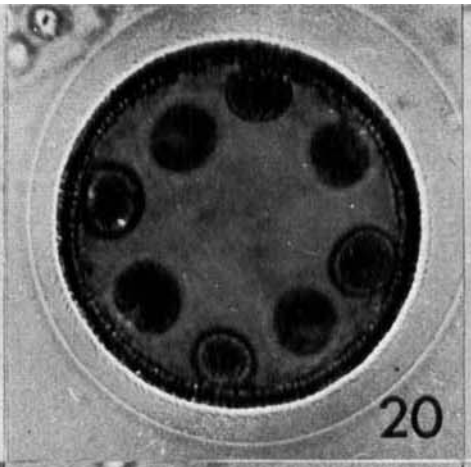
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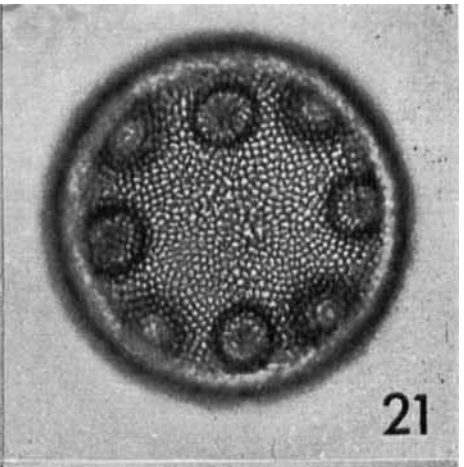
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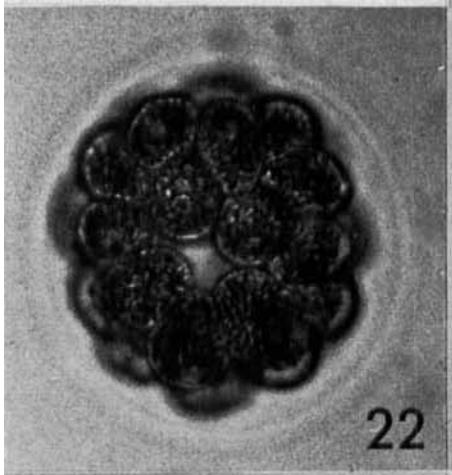
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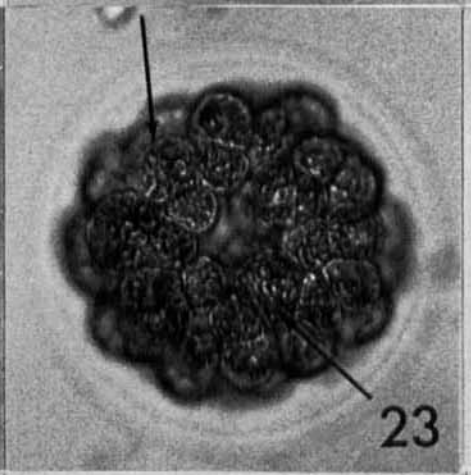
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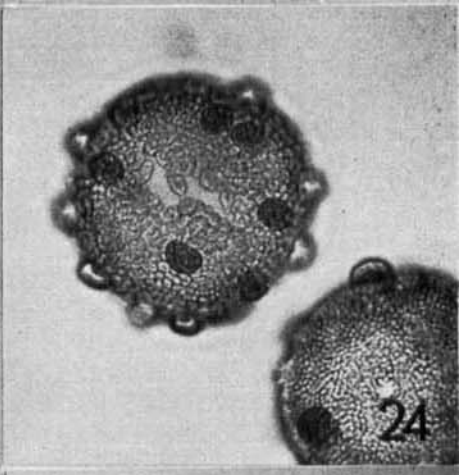
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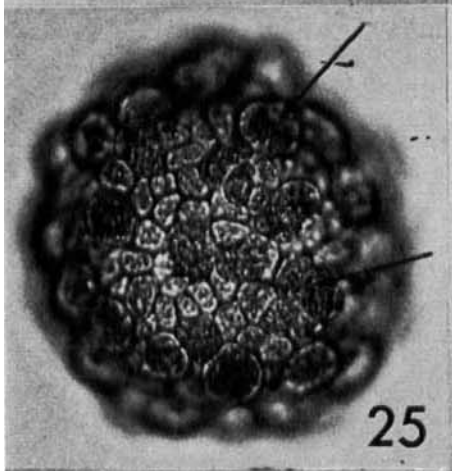
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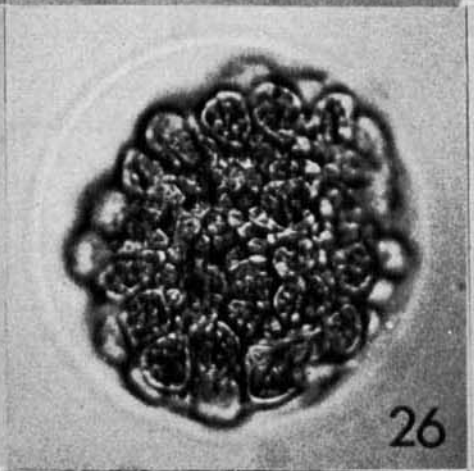
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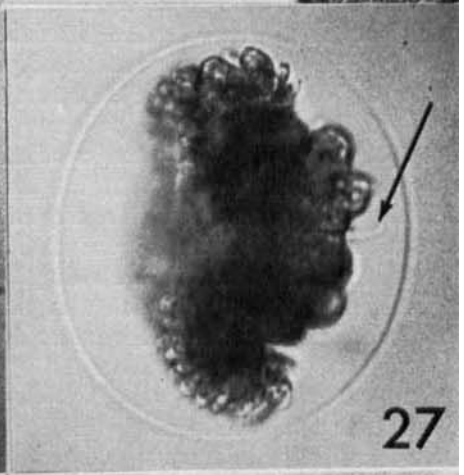
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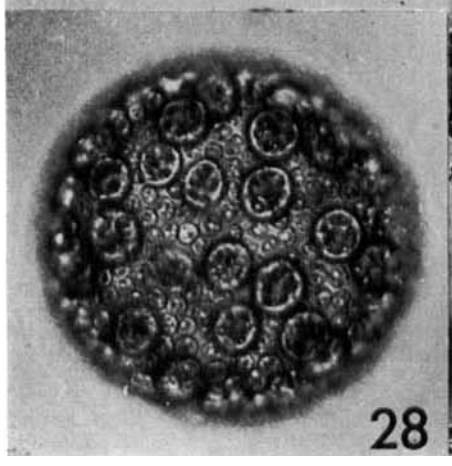
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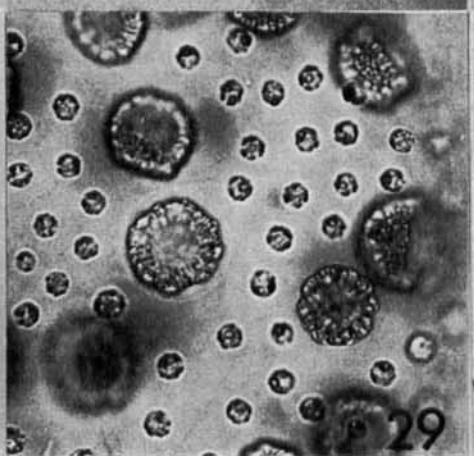
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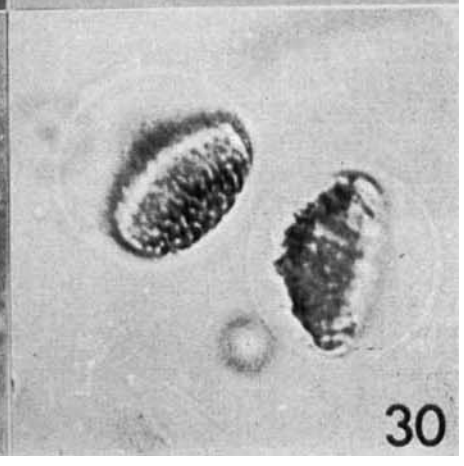
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may be seen as a scar on the surface of female colonies containing young zygotes. Multiple scars are often seen on the surface of a single female colony indicating penetration by more than one sperm bundle.

When sperm bundles come in contact with vegetative colonies they swim about over the colony with their flagella in contact with the surface. This contact is usually short-lived, however, and the sperm bundle soon breaks contact with the colony and swims off into the medium. Thus sperm usually do not penetrate vegetative colonies.

Altho actual fertilization was not seen, genetic evidence presented in another section of this study indicates that true fertilization does occur. The resultant zygotes (Fig. 35) enlarge, develop an orange coloration and secrete a thick, crenulate wall. The parent female colony persists for some time, but eventually it dissociates and releases the zygotes.

Zygote Germination. Zygote germination has been described by Metzner (20) for *V. carteri* var. *hazeni*. Zygote germination as observed in this study was similar to that reported by Metzner.

To induce zygote germination, moribund female colonies containing mature orange zygotes were collected and placed in fresh soil-water medium in the depressions of Pyrex spot plates. These were placed in petri dishes, and a small amount of water was added to the bottom of the dishes to retard evaporation from the spots. Zygotes were incubated under the standard conditions used for growth of stock cultures. Germination usually started in about 2 weeks.

The 1st indication of zygote germination is a slight withdrawing of the protoplast from the zygote wall. The germinating zygote then becomes a lighter orange and decidedly oval (Fig. 36). The outer, crenulate layer of the zygote wall then splits and the middle layer of the wall (mesospore) protrudes thru the resultant opening (Fig. 37). At this stage the protoplast can be seen to possess a hyaline beak and 2 flagella which beat feebly in the watery interior of the mesospore (Fig. 38). As the zoospore begins to emerge thru the fissure in the outer layer of the wall, the innermost layer of the zygote wall (endospore) can be seen along the sides of the emerging zoospore (Fig. 38). After the zoospore is released from the outer zygote wall, the mesospore breaks down (Fig. 39). At no time were the zoospores observed to be actively motile as they are in *Volvox rousseletii* (24).

Cleavage of the zygote protoplast takes place inside the expanded endospore. Initial stages of cleavage are similar to those observed in the cleavage of gonidia to form vegetative daughter colonies (Figs. 40, 41). After cleavage is complete the young gone colony inverts and begins to develop a green coloration. Gone colonies contain approximately 1,000 somatic cells and usually have 4 gonidia in a single tier (Fig. 42).

Inheritance of Sexual Type. Inheritance of sexual type was studied in the following way: Gone colonies were isolated from the germination vessels and clonal populations were established in soil-water tubes. The sexual type of 50 clones derived from gone colonies was determined. Twenty-three were found to be male and 27 female. These results indicate that sexual type is inherited in a 1:1 fashion, as it is in the other genera of the colonial Volvacales so far tested (5, 7, 9, 15, 43).

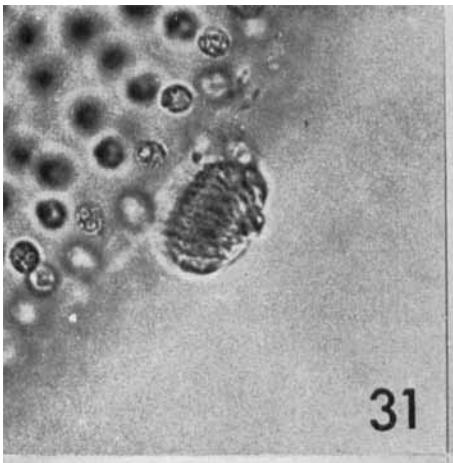
Metzner (20) suggested that parthenospore formation might occur in the strains of *V. carteri* that he studied. In the present study no evidence for parthenospore formation was observed. If female colonies are formed in the absence of male colonies and are allowed to develop with no opportunity for fertilization, mature zygotes are never formed. Eggs from these known virgin female colonies enlarge in size and may begin to show some orange coloration, but true, thick-walled zygotes are never produced. Such unfertilized eggs eventually divide pathologically and often fail to undergo inversion (Fig. 34). Gonidia formed by the resultant, misshapen daughter colonies are, however, capable of developing into normal daughter colonies. Clones derived from gonidia produced in this manner have always proven to be female. Thus it appears that true fertilization does occur in *V. carteri*, and that such fertilization is a prerequisite for zygote formation.

INDUCTION OF FEMALE COLONIES

Dwarf male colonies appear spontaneously in cultures of strain NB-3. Male colonies usually appear 2 or 3 generations after routine transfer of stock cultures to fresh volvox medium. Males are then formed for 1 or 2 generations, after which male production ceases. Growth of the culture continues for some time, but males are not usually formed again until transfer to fresh medium.

Unlike male colonies, female colonies do not appear spontaneously in clonal cultures. Only vegetative colonies are formed thruout the growth of female cultures. Experiments in which the nitrate concentration, total concentration of the medium, temperature and light cycle were varied all failed to induce production of female colonies in potentially female clones. Female colonies were produced only when vegetative colonies from female clones were mixed with and allowed to grow in the presence of colonies from male clones. This finding suggested that colonies

Fig. 19. Inversion nearly completed. Phialopore lobes are coming together in the posterior portion of the young daughter colony. × 390. Fig. 20. Median optical section of a daughter colony which has completed inversion. Note ranking of gonidia into 2 alternating tiers of 4. × 440. Fig. 21. Surface view of the colony shown in Fig. 20. × 440. Fig. 22. Phialopore view of 32-celled female embryo. Note that all the cells are about the same size. × 525. Fig. 23. Phialopore view of 64-celled female embryo. Note enlarged egg initial cells (arrows). × 525. Fig. 24. Late cleavage stage in female daughter colony. × 320. Fig. 25. Posterior view of approximately 256-celled stage in dwarf male colony formation. The slightly larger cells (arrows) are male initials. × 525. Fig. 26. Late preinversion dwarf male colony. × 525. Fig. 27. Inversion of dwarf male colony. Note colorless cytoplasmic extension (arrow) from one of the posterior cells of the colony. × 525. Fig. 28. Post-inversion dwarf male colony. Male initial cells are undivided. × 445. Fig. 29. Division of male initial cells to form sperm bundles. Note the bowl-shaped preinversion stages. × 525. Fig. 30. Formation of sperm bundles. Sperm bundle in the lower right portion of the figure is inverting. × 720.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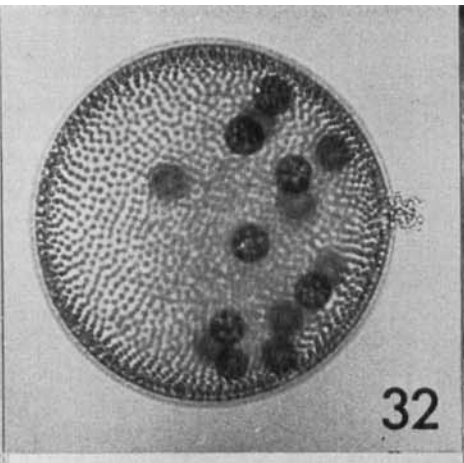
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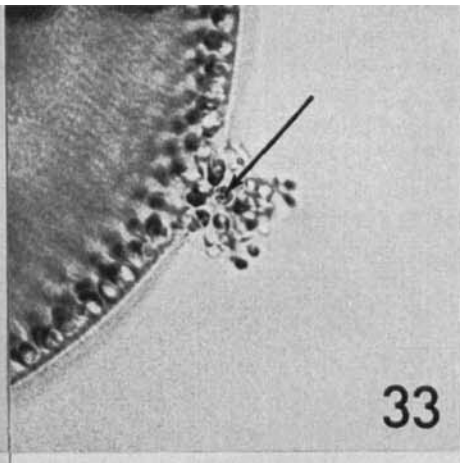
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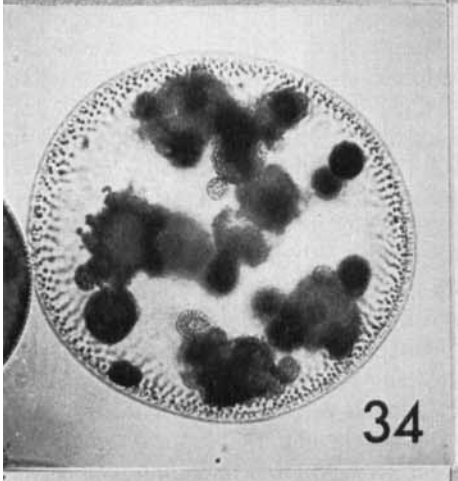
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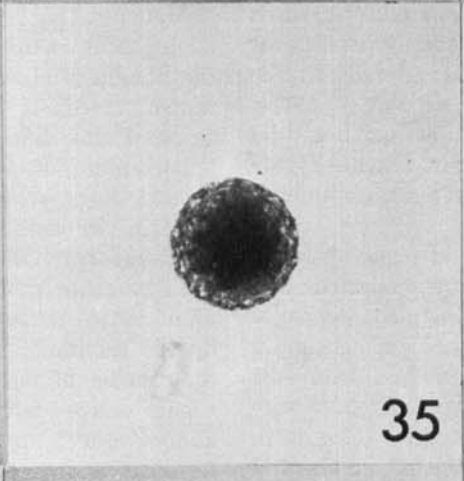
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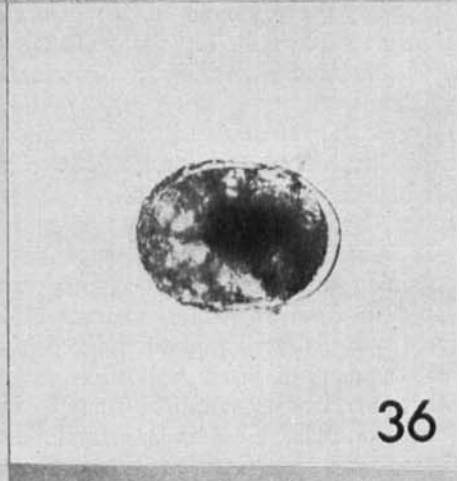
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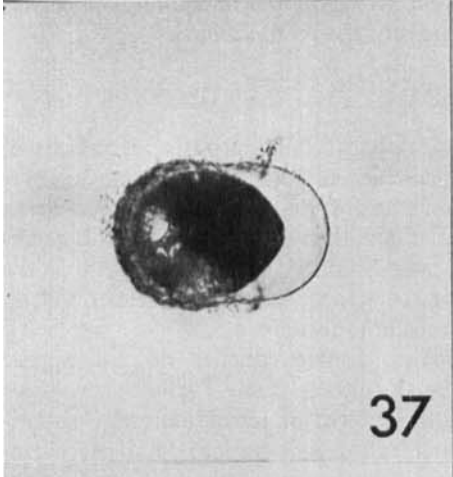
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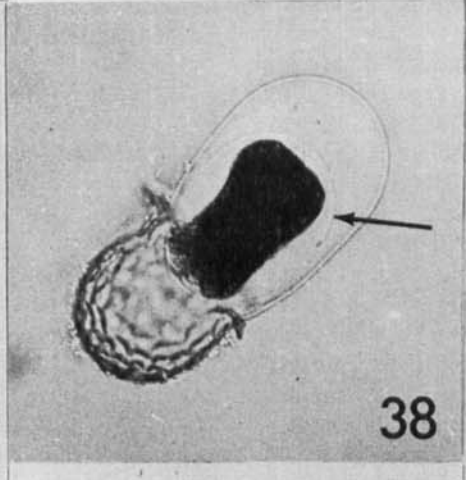
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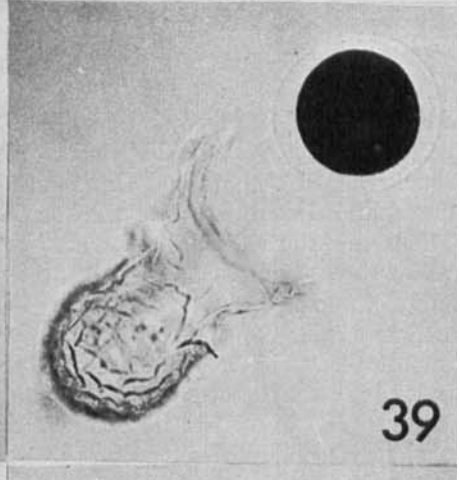
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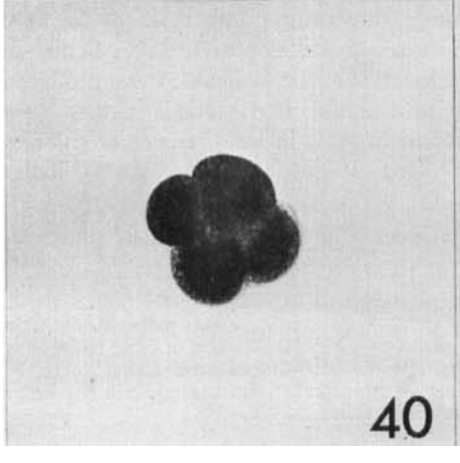
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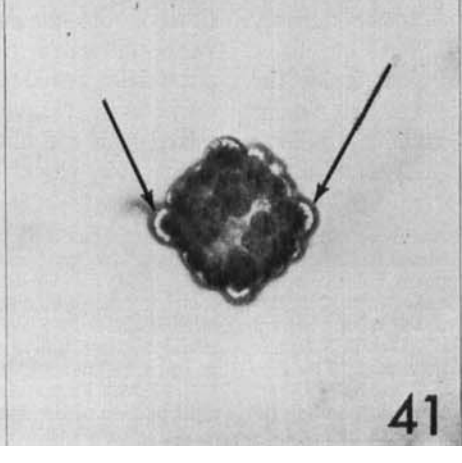
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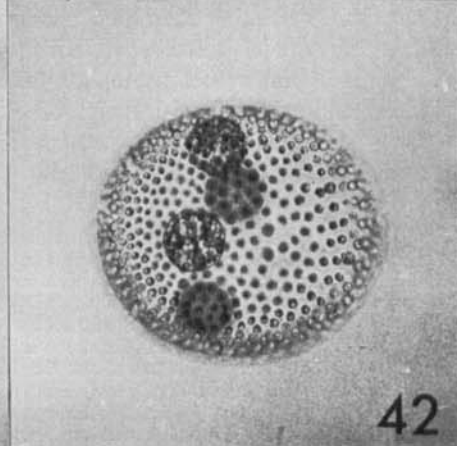
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from male clones might produce some factor which induced the formation of female colonies. To test this hypothesis a male culture was filtered to remove the male colonies, and vegetative colonies from suspected female clones were then inoculated into this medium in which males had been grown (conditioned medium). Fresh volvox medium was used as a control. Females were formed in the conditioned medium, but none was formed in the control. Thus male conditioned medium in some way induced the formation of female colonies in female clones. Colonies from male clones, then, alter the medium in some way during growth and render it capable of inducing the formation of female colonies in female clones. Mere depletion of medial components seemed unlikely since female colonies were not formed when female clones were grown in the absence of male colonies or male conditioned medium. Presumably the medium would be depleted by growth of female clones in much the same manner as it would by growth of male clones. It was still possible, however, that male clones depleted the medium in a different manner than did female clones.

To test whether medial depletion was involved in female induction, 10 ml of male conditioned medium were dialyzed against 400 ml of fresh volvox medium. The medium outside the dialysis bag was changed 4 times during the experiment. The medium inside the dialysis bag was then assayed for female inducing activity. Since all the components of volvox medium are low molecular weight compounds, after dialysis the medium inside the dialysis membrane should contain all the medial components in essentially the same concentration as in fresh medium. Any large, non-dialyzable compounds originally present in the conditioned medium would also be present inside the dialysis membrane. It was found that male conditioned medium retained female-inducing activity after dialysis. Control assays of the volvox medium used for dialysis produced no females. This experiment not only showed that depletion of the medium could not be the cause of female induction by male conditioned medium but indicated that the male conditioned medium contained a non-dialyzable substance which was responsible for the induction of female colonies.

For further experiments on female induction, male clones were grown in 250 ml Delong flasks containing approximately 100 ml of volvox medium. Conditioned medium was prepared by preliminary filtration thru Whatman #1 filter paper followed by 2 successive Millipore filtrations using type HA filters with a pore size of 0.45 μ. The conditioned medium was then distributed into 10 ml aliquots and frozen at —18 C for later use. Activity was retained for several weeks in conditioned medium prepared and stored in this manner.

Assay Method. The bioassay used for detection of the female inducing factor in conditioned medium was modified from that used by Darden (10) in his study of male induction in *V. aureus*. Medium to be assayed for female-inducing activity was placed in each of the 3 depressions of an 85 × 34 mm Pyrex spot plate contained in a petri dish. A few ml of sterile water were added to the bottom of the petri dish to retard evaporation from the depressions in the spot plate. One newly released vegetative daughter colony from the female clone was used as inoculum in each of the 3 depressions. Assay dishes were allowed to develop in an incubator at 26 C under constant light of approximately 350-400 ft.-c. Approximately 8 daughter colonies were formed in each depression of the spot plate after division of the gonidia of the inoculum colony. These 1st generation daughter colonies were allowed to produce daughter colonies of their own so that after 2 cycles of asexual reproduction each depression of the spot plate contained about 64 colonies. These colonies were then counted and scored as vegetative or female. Results were expressed as the percentage of the total colonies in the 3 spots which were female. Some batches of conditioned medium produce 100% females in this assay.

To insure a uniform inoculum, colonies were prepared for use in the bioassay in the following way: A single vegetative colony from the female clone was placed in fresh volvox medium in one of the depressions of a spot plate as described for the bioassay. Care was taken to choose a colony with only 8 gonidia so that daughter colony formation would occur in a fairly synchronous manner. After one cycle of asexual reproduction, 8 colonies would thus be present in the spot. These 8 colonies were allowed to form daughter colonies. These 2nd generation daughter colonies were usually released at about the same time, and the newly released daughter colonies were used as inoculum in the bioassay. Any given experiment was done using a single batch of newly released daughter colonies prepared in this manner.

Dilution of Conditioned Medium. Dilution experiments were conducted to determine the extent to which conditioned medium could be diluted with fresh volvox medium before loss of female-inducing activity occurred. The results of one such experiment are shown in Fig. 43. In this particular batch considerable activity was retained up to a 10^3 dilution.

Production of the Female-Inducing Substance. Preliminary experiments on the kinetics of production of the female-inducing substance indicated that female-inducing

Fig. 31. Sperm bundle on the surface of a female colony. × 525. Fig. 32. Partially dissociated sperm bundle on the surface of a female colony. × 150. Fig. 33. Dissociation of a sperm bundle. A displaced somatic cell from the female colony is indicated by the arrow. × 320. Fig. 34. Cleavage of unfertilized eggs. Note failure of most of the embryos to invert. × 100. Fig. 35. Mature zygote. × 320. Fig. 36. Zygote in early stage of germination. × 320. Fig. 37. Mesospore emerging thru the fissure in the outer zygote wall. At this stage the protoplast is flagellated. × 320. Fig. 38. Protoplast nearly free from the outer zygote wall. Note the endospore surrounding the protoplast (arrow) and the crenulate nature of the zygote wall. × 320. Fig. 39. Mesospore has partially broken down and the zygote protoplast has rounded up inside the endospore. × 320. Fig. 40. Four-celled stage in cleavage of zygote protoplast. × 320. Fig. 41. Thirty-two celled stage in cleavage of zygote protoplast. Note enlarged gonidial initial cells (arrows). × 320. Fig. 42. Mature gone colony. Note ranking of gonidia into a single tier of 4. × 320.

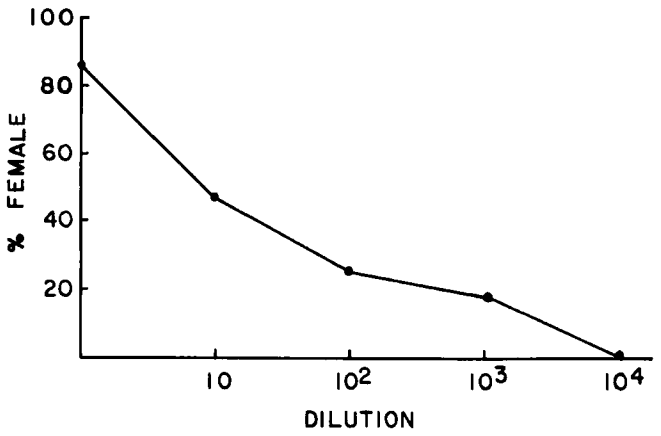

Fig. 43. Conditioned medium dilution curve.

activity appeared in the medium only after males had been formed. Moreover, peaks of activity followed and paralleled peak periods of male colony production. Thus these experiments suggested that male colonies or sperm bundles are involved in the production of the female-inducing substance.

To test the hypothesis that male colonies produce the female-inducing substance, vegetative colonies containing both embryonic male colonies and embryonic vegetative colonies were selected from actively growing male cultures. These were washed thru several changes of fresh volvox medium to remove any female-inducing substance carried over from the original culture. The parent colonies were then broken open and the embryonic daughter colonies removed with a pipette. The 2 types of daughter colonies were sorted into separate batches and each was subjected to a series of additional washes in volvox medium. Twenty embryonic male colonies were placed in each of the depressions of a spot plate and 20 embryonic vegetative colonies were placed in the depressions of a separate spot plate. One newly released vegetative colony from the female clone was then added to each of the depressions. The spot plates were incubated under the bioassay conditions and checked periodically for the production of female colonies. Control assays of the volvox medium used in the last wash in both the male and vegetative series produced no females indicating that no female-inducing substance had been carried thru the washing procedure from the original male culture. The results of this experiment are shown in Table 1. Female colonies were formed only in the spots in which male colonies had been placed. Thus male colonies do produce the female-inducing substance. The experiment does not indicate, however, whether the female-inducing substance is produced by the somatic cells in male colonies, male initial cells or by sperm bundles. Vegetative colonies do not appear to produce the female-inducing substance, but a low rate of synthesis might not be detected under the conditions of this experiment.

These data implicating male colonies in production of the female-inducing substance are consistent with the only published work on the sequence of appearance of sexual colonies of *V. carteri* in nature. Metzner (20) studied

TABLE 1. *Production of the female-inducing substance.*

Colony type	% female
Vegetative	00.0
Male	47.6

populations of *V. carteri* var. *hazeni* growing under semi-natural conditions in tanks used to raise aquatic plants. He observed that at the beginning of a given growth cycle only asexual colonies were present. Male colonies were the 1st sexual colonies to appear, and female colonies appeared only after male colonies had been present for some time.

Developmental Stage of Maximum Susceptibility. Timing experiments patterned after those devised by Darden (10) were conducted to determine the developmental stage of maximum susceptibility to female induction. To obtain colonies at the same developmental stage for use in such experiments the following procedure was used: Vegetative colonies containing mature daughter colonies were selected from actively growing cultures of the female clone and placed in a watch glass containing approximately 15 ml of volvox medium. At hourly intervals all the newly released daughter colonies were collected with a fine glass pipette and placed in a separate vessel. Any one of the hourly collections represented a fairly synchronous population suitable for use as starting material in timing experiments.

To facilitate subsequent reference the life cycle of *Volvox* was divided into the following stages:

Stage 1.....release from the parent colony to beginning of gonidial cleavage.

Stage 2.....beginning of gonidial cleavage to end of inversion.

Stage 3.....end of inversion to release of daughter colonies.

Thus each generation in the life cycle of *V. carteri* is made up of the above 3 stages (Fig. 44).

It was known from the bioassay that when gonidia in the newly released daughter colonies used as inoculum divided to produce 1st generation daughter colonies all of these were vegetative. Thus gonidia in newly released daughter colonies (which would be early stage 1) are past the stage where they can be induced to form female colonies by conditioned medium. In order for females to be formed in any given generation, exposure to conditioned medium must occur during the previous generation.

With this in mind experiments were devised in which exposure to conditioned medium took place in one generation and the results were scored by counting females formed in the following generation.

A pulse experiment was conducted in which newly released daughter colonies were placed in conditioned medium and allowed to develop under the bioassay conditions. Sample colonies were removed from the conditioned medium at timed intervals, washed thru several changes of fresh volvox medium and allowed to continue development in volvox medium. The results of this experiment are shown in Fig. 45. No females were formed by pulses of less than 29 hours. After that time, additional pulse length produced additional induction of females up until hour

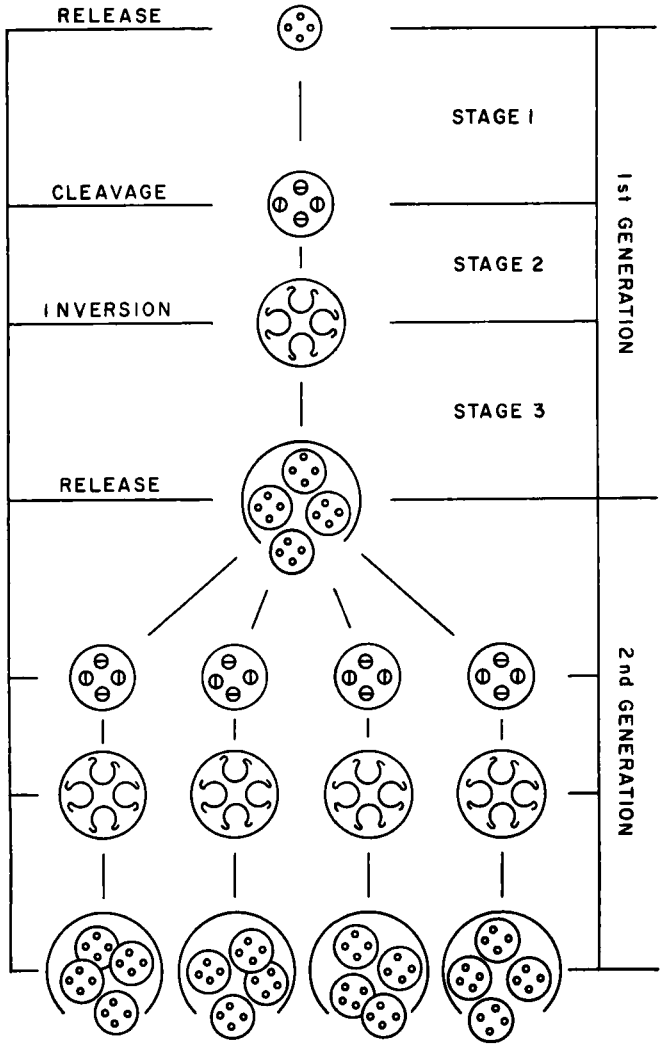

Fig. 44. Diagram of the sequence of developmental stages occurring in the timing experiments.

57, after which additional exposure to conditioned medium caused no further increase in 2nd generation females. Thus, exposure to conditioned medium until about midway thru stage 3 of the 1st generation was necessary for full induction of females in the 2nd generation.

To determine whether exposure was necessary for the full 57 hours indicated by the pulse experiment for full induction, a delay experiment was performed. Newly released daughter colonies were placed in volvox medium and allowed to develop under the bioassay conditions. Sample colonies were periodically transferred to conditioned medium and allowed to continue development. It was possible to delay placing the inoculum colonies into conditioned medium until the start of stage 2 with no loss of female induction (Fig. 46). Thus the delay and the pulse experiments suggest that in order for maximum induction in the 2nd generation, colonies have to be in contact with conditioned medium during the 1st generation from the beginning of stage 2 until approximately midway thru stage 3. In the above experiments this was a period of about 30 hours.

To determine the period during stage 2 or 3 which is most susceptible to conditioned medium, a delay-pulse experiment was conducted. Newly released daughter colonies were allowed to develop in volvox medium as in the delay experiment. Sample colonies were removed at various periods in the developmental cycle, allowed to develop for 12 hours in conditioned medium, and returned to volvox medium to continue development. The 12-hour pulse that proved most effective in inducing females was the 48-60 hour period (Table 2). This period represents the middle part of stage 3 of the 1st generation. At this stage the gonidia affected were in post-inversion daughter colonies which were expanding prior to release from the parent colony. However, even at this apparently most favorable stage, a 12-hour pulse induced only a relatively small number of these gonidia to form females. This is in marked contrast to the situation in *V. aureus*, in which full male induction is possible with pulses as short as 30 minutes (10).

TABLE 2. *Delay-pulse experiment.*

Period exposed (hrs.)	Developmental stage	% female
0-12	Early stage 1	00.0
12-24	Late stage 1	00.0
24-36	Stage 2	00.0
36-48	Early stage 3	00.3
48-60	Middle stage 3	06.2
60-72	Late stage 3	01.6
72-84	Early stage 1 (2nd generation)	00.0
Control (continuously exposed)		55.0

Heat Sensitivity. Preliminary experiments had shown that autoclaving destroyed or greatly reduced the female-inducing activity of conditioned medium. To test the degree of heat lability of the female inducing substance, aliquots of a batch of conditioned medium were heated for 5 minutes at temperatures ranging from 30–100 C in a water bath, cooled rapidly in an ice bath, and then assayed for female-inducing activity in the usual manner. These experiments indicated that 5 minutes at 80 C sub-

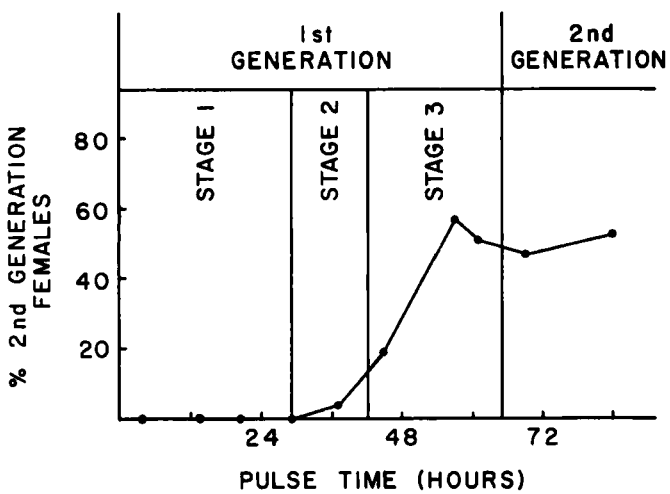

Fig. 45. Effect of increasing exposure to conditioned medium on the production of female colonies.

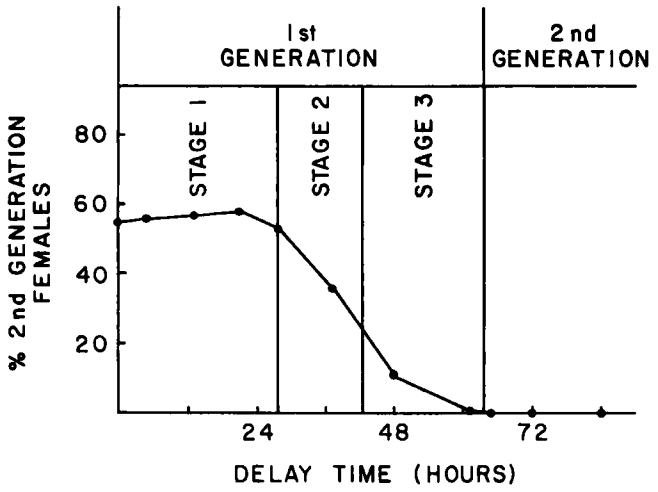

Fig. 46. Effect of decreasing exposure to conditioned medium on the production of female colonies.

stantially reduced the female-inducing activity, and 5 minutes at 90 C completely inactivated the female-inducing substance in the batch of conditioned medium used.

A further expirment was devised in which aliquots of the same batch of conditioned medium were diluted 100:1 and heated at 60 and 80 C. Samples were removed at timed intervals, cooled rapidly, and assayed for female-inducing activity. The results of this experiment are shown in Fig. 47. The female-inducing substance appears to be slowly inactivated at 60 C and rather rapidly inactivated at 80 C.

Effect of Enzymes. The heat lability and non-dialyzable nature of the female-inducing substance suggested that it might be proteinaceous, so the proteolytic enzymes trypsin (Worthington), chymotrypsin (Worthington) and Pronase (Calbiochem) were tested on conditioned medium. All enzymes were used at a concentration of 250 μg/ml and were incubated with conditioned medium for approximately 12 hours at room temperature.

Since the enzymes tested are toxic to *Volvox*, it was necessary to remove them before bioassay of the conditioned medium could be undertaken. This was done by column chromatography with Sephadex gels.

Neither trypsin nor chymotrypsin inactivated the female-inducing substance, but Pronase destroyed female-inducing activity. Pancreatic ribonuclease (Worthington) was also tested and found to have no effect on female-inducing activity.

DISCUSSION

The initiation of sexual reproduction in plants has been found to be under the control of a wide variety of factors, including light, temperature, nutrient supply and specific sexual hormones. Hormonal substances inducing the formation of gametes or sexual organs have been most thoroly studied in the lower plants, particularly the fungi and algae.

The occurrence of sex hormones in the fungi was 1st suggested by de Bary (11) for several saprolegniaceous species. The 1st experimental evidence for such substances was provided by Burgeff (6) when he showed that zygophores of *Mucor mucedo* were initiated in response to a substance produced by the opposite mating type. A similar situation exists in 2 other genera of the Mucorales: *Phycomyces blakesleeanus* (37) and *Pilobolus crystallinus* (18,19). Bishop (2) found that in *Sapromyces reinschii* secretions from female plants induced the formation of antheridial hyphae on male plants. A similar induction of antheridial hyphae secreted by female plants was noted by Raper in *Achlya*. A series of investigations (29, 30, 31, 32, 33, 34, 35) revealed the existence of a complex hormonal system controlling the various stages of the sexual process. A multihormonal system controlling sexual reproduction has also been described for *Ascobolus stercorarius* by Bistis (3, 4). In this genus, induction of ascogonial initials is under the control of a substance secreted by oidia of the opposite mating type.

The 1st report of hormonal induction of sexuality in the algae was that of Diwald (12). He reported that gametes were formed in heterothallic strains of *Glenodinium lubiniensiforme* in response to treatment with filtrates from cultures of the opposite mating type. Coleman (9) also reported the induction of gamete formation by filtrates from cultures of the opposite mating type in *Pandorina morum*. A more complex hormonal system controlling the various phases of sexual reproduction in a heterothallic, nannandrous species of *Oedogonium* was described by Rawitscher-Kunkel and Machlis (36). None of these algal sex substances has been analyzed sufficiently to determine its chemical nature.

The 1st evidence of a sex hormone in the genus *Volvox* was presented by Darden (10) in a study of the factors controlling sexuality in the M5 strain of *V. aureus*. In this homothallic species Darden found that filtrates from sexual cultures would induce the formation of male colonies under conditions in which only vegetative colonies would ordinarily be produced. Male induction was found to be caused by a male-inducing substance which was non-dialyzable, heat-labile, and had a probable molecular weight in excess of 200,000. Activity was destroyed by treatment with trypsin or Pronase, but not by chymotrypsin. Since periodate oxidation also destroys activity

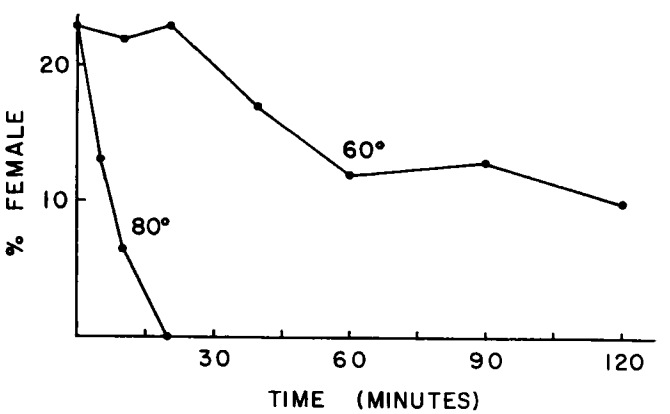

Fig. 47. Effect of heat on the female-inducing activity of conditioned medium.

(Darden, personal communication) it is thought that the male-inducing substance may be a glycoprotein as are the gamones which cause sexual agglutination in *Chlamydomonas* (13, 14).

The present study has shown that colonies from male clones of *V. carteri* produce a substance which induces the formation of female colonies. This female-inducing substance appears to be similar in gross physical and chemical properties to the male-inducing substance from *V. aureus*.

The female-inducing substance from *V. carteri* apparently exerts its effect on gonidia in the early stages of their expansion and determines the type of colony they will produce when they cleave. The situation in *V. carteri* differs from that in *V. aureus* in which induction takes place at the late preinversion stage of daughter colony formation (10).

Proteinaceous substances have been found to be the controlling agents in some forms of embryonic induction in animals. Yamada (45) found that a protein factor isolated from the bone marrow of guinea pigs can induce mesoderm formation by prospective ectoderm cells of the newt *Triturus pyrrhogaster*. This factor is thought to change the developmental pathway of the prospective ectoderm cells by inducing a change in the pattern of gene action.

The female-inducing substance from *V. carteri* also causes a change in developmental pathway. Two pathways of differentiation are possible for gonidia from the female clone: one leading to the formation of a vegetative colony, and the other to the formation of a female colony. Presumably a switch mechanism determining which of the 2 pathways will be followed can be influenced by the female-inducing substance.

Classical experiments on cell differentiation have centered around the formation of an individual by cleavage of a fertilized egg. It is evident from these studies that by the time cleavage begins, the fertilized egg is already a highly differentiated structure. As Spratt (40) said, "...all the necessary instructions for the development of a new organism from an *egg* are present in the egg at the beginning of its development...". Such instructions are expressed in part as patterns of morphogenetic substances in the egg cytoplasm. These patterns are set up during the long period of egg maturation prior to fertilization and the beginning of cleavage. If one alters the pattern of cytoplasmic differentiation formed during the maturation of the egg, the embryo resulting from cleavage of the egg reflects the induced changes. The female-inducing substance from *V. carteri* may act by changing the pattern of cytoplasmic differentiation, but it is not at present known whether the cytoplasm of mature gonidia contains patterns of morphogenetic substances influential in subsequent cleavage as do eggs of higher organisms. The early differentiation of embryos into somatic and reproductive cells would, however, be consistent with such a hypothesis. Experiments are being initiated to test the validity of this hypothesis.

One of the greatest advantages of using *Volvox* as a model system in studies of cell differentiation is its relative simplicity. Since only 2 cell types are usually present in a colony, *Volvox* presents the phenomenon of cell differentiation in one of its most elementary forms. Moreover, one can conduct experiments under bacteria-free conditions with large numbers of genetically identical test organisms. *N. carteri* has the further advantage of allowing one to experimentally control the pathway of gonidial differentiation by means of the female-inducing substance. Further attempts will be made to characterize this substance and to elucidate its mode of action.

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A Continuous Culture Device for Protozoan Cells

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SYNOPSIS. A continuous culture device suitable for controlled growth of *Euglena gracilis* is described. It could probably be adapted to other similar cell types with generation times no greater than 150-200 hrs.

ALTHO the first continuous culture device to be developed was used for growth of the alga *Chlorella* (7), the technic of continuous culture has been exploited most successively with bacterial species (for review, see 5). Similar approaches would have obvious advantages for certain problems relating to protozoan physiology. However, since protozoan cells usually have fairly long generation times, and even active swimmers tend to settle unless agitated, the continuous culture devices commonly described for bacteria (chemostat, turbidostat, bactogen) are unsatisfactory for comparable studies with most protozoan species. Over the past few years we have developed a continuous culture device for *Euglena gracilis;* a few studies resulting from the apparatus have been mentioned elsewhere (1). The basic principle of operation is very simple, but in practice we have learned thru trial and error certain operating procedures which make the device behave more satisfactorily. It would appear to be adaptable to any type cell with a generation time not in excess of 150-200 hours, and dilution rates can be made as continuous as the operator desires. We have obtained highly stable populations of *Euglena* for periods of more than a month with the apparatus, which we refer to as a chemostat, and its detailed description is given here, since others may find this or a modified version of some use in their work.

PRINCIPLE OF OPERATION

Continuous culture in a chemostat relies on continuous dilution with fresh medium of a culture having a fixed volume, with one nutrient in the medium being in limited concentration. The cell population finds and maintains an exponential growth rate at some constant population density, the growth rate and cell density remaining unchanged after equilibrium is established with the rate of dilution. With defined media the limiting nutrilite will be known and considerable control of the experiment is afforded the operator of the chemostat; however, continuous culture is also possible with complex media, even tho the limiting nutrilite may be unknown.

The simplest chemostats add fresh media to the culture at rates fixed by various means, a siphon device being used to hold the culture volume constant. When the siphon volume is small relative to that of the culture, and the cells do not settle, the cells contained in the overflow are representative of those in the culture itself. With *Euglena*, however, the cells settle in a siphon line, and the number of cells needed for routine assays is usually in great excess of those collected over short periods in the overflow. With protozoa which multiply more rapidly and swim more actively, and with very large culture volumes, this would be less of a problem.

THE BASIC APPARATUS

Fig. 1 is a schematic of the basic culture unit, which consists of a pump, the culture flask, and the media reservoir.

Continuous dilution is established by means of a 4-channel continuous infusion-withdrawal pump.[1] Other pumping systems would probably work as well. The pump is fitted with ordinary syringes which can be removed with accompanying rubber tubing for autoclaving with the culture flask and media. Two syringes are used to pump in fresh media; one fills from the reservoir while the other empties into the culture vessel. The other 2 syringes serve a similar function in transferring the cell suspension from the culture flask to the overflow collection flask. The pump is fitted with a reversible drive so that operation to fill or empty a syringe is normally continuous. Note will be made later of the continuity which obtains in reversal of the motor.

Culture vessels could be of many shapes, as long as the cell suspension remained well stirred. Because we occasionally work with

[1] Harvard Apparatus Co., Dover, Mass.