MORPHOLOGICAL DEVELOPMENT OF RESTING CYSTS IN CULTURES OF THE MARINE DINOFLAGELLATE *LINGULODINIUM POLYEDRUM* (= *L. MACHAEROPHORUM*)

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Abstract

The previously undescribed morphological development of resting cysts of the living marine dinoflagellate *Lingulodinium* ("*Gonyaulax*") *polyedrum* has been observed in laboratory cultures. Referred to as *L. machaerophorum* in cyst-based taxonomy, this species has a fossil record extending back at least to the Early Eocene. In our cultures, planozygotes preparing to encyst showed a distinctive interstice in the peripheral cytoplasm and often displayed a characteristic swimming behavior. The transition from motile planozygote to morphologically mature hypnozygote (resting cyst) took approximately 10-20 minutes. Encystment began with several events occurring simultaneously: (1) the cell stopped swimming and came to rest at the bottom of the observation chamber, (2) flagella were expelled from their respective thecal grooves, (3) localized swelling of a membrane external to the theca formed bubble-like protrusions on the surface of the cell, (4) the theca began to dissociate along one or more plate sutures, and (5) a single layer of globules appeared in the interstice between the theca and cytoplasm. External protrusions then enlarged and merged to liberate a continuous membrane which surrounded the entire cell. Subsequent expansion of this membrane gave the encysting cell the appearance of an inflating balloon. In most cases, the outer membrane remained partially attached to the theca so that expansion caused thecal sections to pull away from the underlying globules and cytoplasm. As the outer membrane enlarged, globules on the surface of the cytoplasmic mass grew radially outward (i.e., centrifugally) beneath the dissociating theca to form processes. Morphological development of the resting cyst ended when the expanding membrane ruptured. The maximum lateral dimension attained by this membrane was about twice the diameter of the internal body of the cyst. In these cases, cysts developed the distinctive processes characteristic of *Lingulodinium*. Premature rupture of the balloon-like membrane, however, resulted in processes showing considerable variation in size and morphology. Based on the variability of process morphology observed in laboratory cultures of *L. polyedrum,* three morphotypes currently designated as separate species of the genus *Lingulodinium* are here synonymized with *L. machaerophorum.*

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INTRODUCTION

The ability of some living dinoflagellate species to form cysts was first recognized during observations of natural phytoplankton samples over a hundred years ago. Since periods of rest or dormancy are common in the life cycles of microorganisms, these early reports attracted little attention outside the small group of botanists and zoologists interested in the dinoflagellates around the turn of the century. At this time, difficulties associated with keeping dinoflagellates alive in the laboratory had not been overcome and thus precluded any long-term studies of single populations. Field samples provided isolated glimpses of various life cycle stages, but their significance remained obscure. A good summary of these early observations can be found in Tappan (1980).

Two lines of research, both gaining momentum around the late 1950s and early 60s, began to focus the interest of a large number of investigators on the phenomenon of dinoflagellate encystment. First, studies by paleontologists (e.g., Evitt, 1961; Evitt and Davidson, 1964; Wall and Dale, 1968) made it clear that nearly all fossil dinoflagellates were actually the preserved remains of cysts and not the thecate, motile cells as had been previously thought. Meanwhile, developments in culturing methods finally allowed biologists (e.g., von Stosch, 1964, 1972, 1973; Pfiester, 1975, 1976, 1977) to observe the entire life cycle of dinoflagellates maintained in laboratory cultures. These investigations documented the fundamental role of encysted stages in the reproductive strategy of some species and suggested that it was the hypnozygotic resting cysts rather than the asexual temporary cysts that were most likely to contribute to the fossil record.

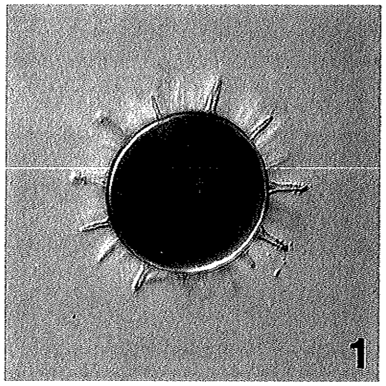
An important consequence of these paleontological and biological studies was the development of both a conceptual framework and an experimental system for subsequent investigation of encystment among living dinoflagellates. However, while notable contributions can be cited in some areas, many aspects of resting cyst formation in laboratory cultures remain to date unexplored. Complete life cycles are known for only about 30 of the approximately 1500 named extant dinoflagellate species (Pfiester, 1989). From a paleontological perspective, perhaps the greatest omission has been the lack of research directed towards the life cycles and modes of encystment of the relatively few extant dinoflagellates which produce resting cysts identical to some of those found in the fossil record. These species (e.g., *Gonyaulax spinifera*, *Pyrodinium bahamense*, *Lingulodinium polyedrum*) clearly represent the strongest link between the biology and paleontology of dinoflagellates.

The primary objective of our present study was to directly observe and document the morphological development of resting cysts in cultures of a paleontologically significant living dinoflagellate. We selected *Lingulodinium polyedrum* (Stein 1883) Dodge 1989, a widely distributed autotrophic and bioluminescent species well known for its ability to form large blooms in coastal waters. Resting cysts of *L. polyedrum*, referred to as *L. machaerophorum* (Deflandre and Cookson 1955) Wall 1967 in cyst-based terminology, have a rich fossil record extending back at least to the Early Eocene (Williams et al., 1993). *L. polyedrum* grows well in laboratory culture and the vegetative cells have been a popular experimental system for investigation of algal cell cycles, bloom phenomena, circadian rhythms, bioluminescence, photosynthesis, physiology, morphology, and ultrastructure (see Taylor, 1987 for references).

PLATE 1

Morphological development of *L. polyedrum* resting cysts. All figs. x500. Figs. 3-12 show a single specimen at successive stages during the transition from planozygote to hypnozygote: figs. 3-6 represent Stage II (Initiation), while figs. 7-12 illustrate Stage III (Expansion). All cells photographed in the living state, surrounded by the culture medium.

1	Living resting cyst (hypnozygote) of *L. polyedrum* produced in laboratory culture. Optical section. Note that processes appear hollow and distally closed; some (e.g., at 3 and 4 o'clock) bear distal spinules.
2	Planozygote in dorsoventral view, Stage I (Pre-expansion). Optical section showing zone of differentiation in peripheral cytoplasm.
3	Planozygote immediately after loss of motility. Outer membrane (external to the theca) beginning to bulge outward. Note longitudinal flagella at upper right of cell.
4	A few moments later, slightly lower focus. Dissociation of theca visible at lower left. Also note transverse flagellum (which has been expelled from the cingulum) at top of cell, and first appearance of globules on surface of cytoplasm (~1 o'clock).
5	Growth of bulges. Expansion of outer membrane begins to pull thecal sections away from underlying cytoplasm (lower right).
6	Continued cell expansion, bulges merging to define a continuous membrane distinct from the theca and cytoplasm. Note "residual" connections between theca and membrane (e.g., 9 and 11 o'clock) which appear to occur at thecal plate boundaries. At this stage, thecal sections are no longer in contact with the cytoplasm. Cytoplasmic mass approaches circularity (in optical section) and shows globules more or less evenly distributed on the surface.
7	Stage III. Outer membrane nearly circular in optical section; globules on the surface of the cytoplasm begin development into processes. Note initiation of process shaft formation between globules and cytoplasmic surface (~7-8 o'clock).
8	Continued expansion of outer membrane occurring simultaneously with deposition of process shafts. Note that (1) thecal sections do not contact developing processes, and (2) globules remain spherical as process shafts grow outwards.
9	Cell at same point as fig. 8, lower focus.
10	Maximum extent of cell expansion. Development of process shafts complete. Most processes still bear a terminal globule.
11	Slightly lower focus of fully expanded cell. Note that boundary of outer membrane is out-of-focus, while the internal cyst shows a clear optical section (c.f. fig. 10). This relationship reflects the lack of concentricity induced by the occurrence of resting cyst formation on the bottom of the viewing chamber.
12	Modification of terminal globules. Spherical globules are beginning to collapse to form process tips. Incomplete or uneven collapse results in terminal spinules which are characteristic of spines of *Lingulodinium* resting cysts.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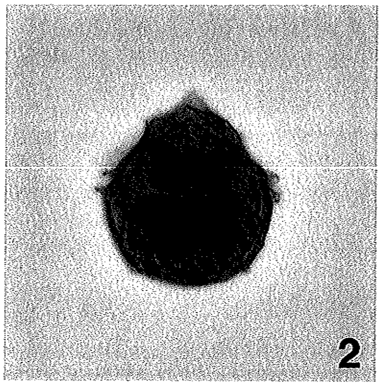
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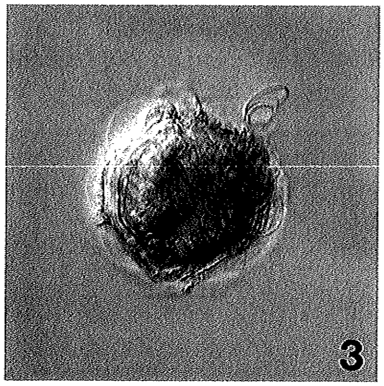
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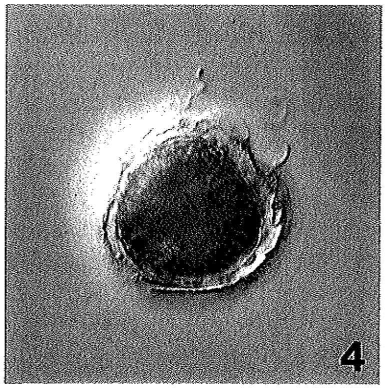
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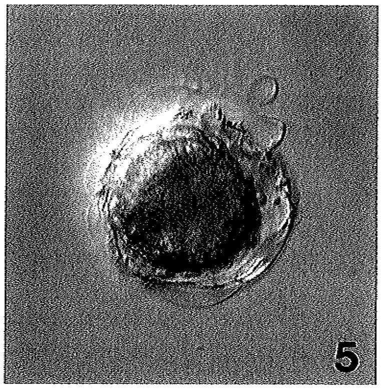
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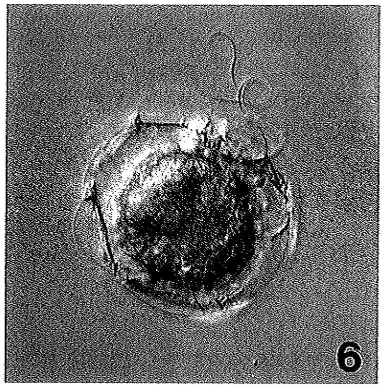
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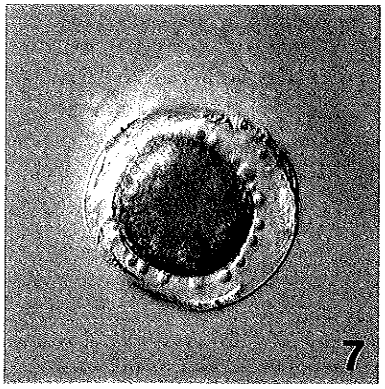
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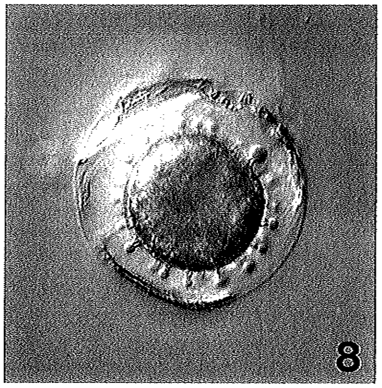
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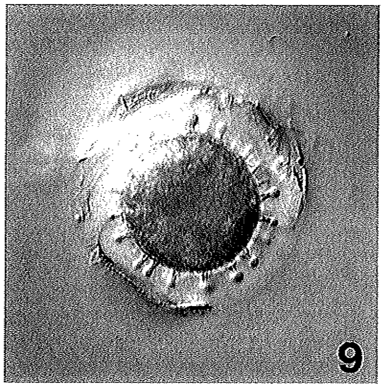
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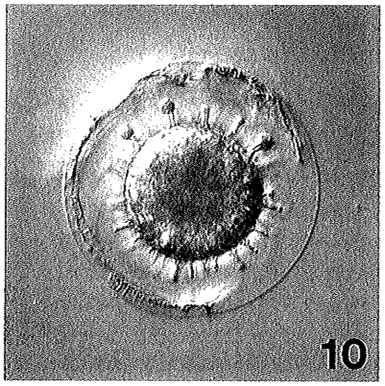
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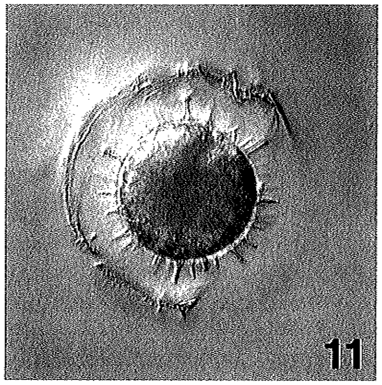
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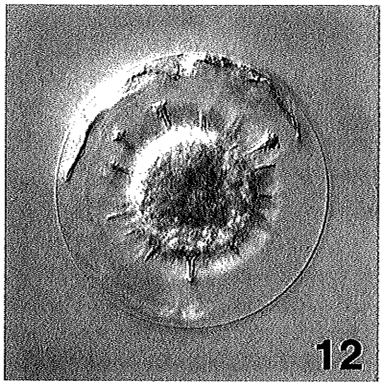
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Resting cysts of this species (Plate 1, fig. 1) consist of a spherical, cytoplasm-containing central body covered by a continuous outer wall which bears numerous hollow spines. These cells were first described by Nordli (1951) who deduced their taxonomic affinity after noting an association with *L. polyedrum* thecal fragments in plankton samples taken from Oslo fjord in Norway. Shortly thereafter, similar hystrichosphaerid forms were identified in plankton tow material and modern sediments by Erdtman (1954), McKee et al. (1959), Evitt and Davidson (1964), and Evitt (1967). Direct confirmation of the cyst/theca relationship in this species was provided by Wall and Dale (1968) who, in a series of laboratory experiments, germinated individual resting cysts isolated from natural sediments to yield the thecate, motile form of *L. polyedrum*. Since that time, many investigators have encountered resting cysts of this species in studies of cyst distribution in modern estuarine and marine environments.

Fossils, now considered representatives of *L. polyedrum*, were first described (as *Hystrichosphaeridium machaerophorum*) by Deflandre and Cookson (1955) from Miocene strata in Australia. Subsequently, Wall (1967) made this species the type for his new genus *Lingulodinium*. Dodge (1989) considered the cyst-based genus *Lingulodinium* to have priority in his taxonomic reassignment of modern *Gonyaulax polyedra*, thus combining previously separate biological and paleontological nomenclature for a single species.

To place the description of resting cyst development contained in this paper in a biological context, a brief review of a general model for the dinoflagellate life cycle is in order. According to the model (here adapted from Dale, 1983, and Evitt, 1985), a typical thecate dinoflagellate is unicellular, biflagellate and swims freely in water. The motile stage is haploid, and reproduction consists of simple vegetative division. When subjected to environmental stress, cells may form asexual temporary cysts which revive when favorable conditions return. Occasionally, and for reasons not well understood, vegetatively dividing cells produce gametes which fuse to form a diploid motile zygote (the planozygote). Except for being larger in size, planozygotes often closely resemble

PLATE 2

Morphological development of *L. polyedrum* resting cysts. All figs. x500. Figs. 1-3 represent a continuation of the developmental sequence illustrated in Plate 1, figs. 3-12. All cells photographed in the living state, surrounded by the culture medium.

1 Resting cyst immediately after rupture of outer membrane. Approximately equatorial focus. Note variability of process morphology. Remnants of planozygotic theca to the left of the cell. At this stage, resting cyst lacks an endospore.

2 Same speciman as fig. 1, high focus on remnants of the epitheca. Note reduced plate ornament.

3 Same specimen as fig. 1, low focus on planozygotic flagella. Dual longitudinal flagella at ~2 o'clock; single transverse flagellum at ~11 o'clock. Note various swellings along each of the flagella, those on the transverse flagellum appear more numerous and variable in size (see also fig. 10). Planozygotic theca to the left, showing wide growth bands.

4 Specimen during Stage II (initiation) of resting cyst formation; view approximately dorsoventral, with apex to the upper right. Bulging outer membrane appears anchored along plate sutures, particularly those between cingular and adjacent (anterior and posterior) plates (e.g., ~3-4 o'clock). This specimen also clearly shows the globule layer on the cytoplasmic surface, as well as longitudinal flagella at ~6 o'clock.

5 High focus on a cell during Stage III (expansion). Mid-dorsal portion of epitheca is more or less intact and remains loosely attached to cingular plates. Hypotheca is underneath the developing cyst (out-of-focus). Again, note significantly reduced intratabular ornament.

6 Optical section (equatorial focus) of cell nearing the end of expansion. Thecal sections appear connected to outer membrane at various points including those at 9, 10, and 11 o'clock. Development of processes proceeding independantly of theca.

7 High focus on cell immediately after rupture of outer membrane. Note single transverse flagellum with regularly spaced swellings.

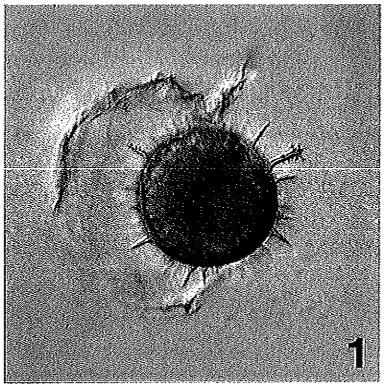
8 Equatorial focus on resting cyst showing an assortment of process morphologies. Note bulbous terminations at 1 and ~3 o'clock. Planozygotic theca to the left of cell.

9 Same specimen as fig. 8; lower focus. Note (1) wide growth bands on the theca, and (2) isolated transverse flagellum at 12 o'clock.

10 Resting cyst immediately after rupture of outer membrane. Low focus on flagella: single transverse flagellum at ~2-3 o'clock; tangled longitudinal flagella at ~5 o'clock.

11 Multiple process morphologies present on a single resting cyst. Compare bases of processes at 1 and 2 o'clock.

12 Same specimen as fig. 11; slightly lower focus. Again, note variability of process morphology. Spine at ~12 o'clock shows widely flared base, and irregular "fist-like" termination. Spines at 2 and 3 o'clock have more compact bases, but the former is capitate and lacks spinules, while the latter is acuminate and bears spinules.



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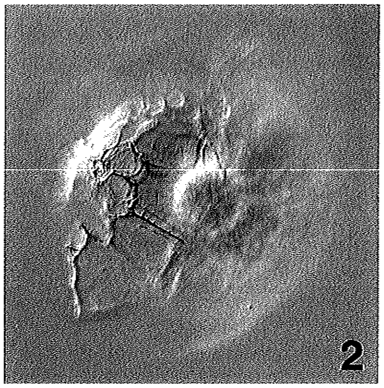

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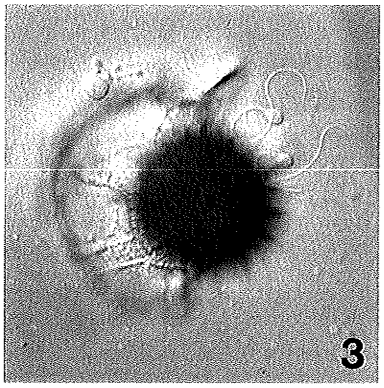

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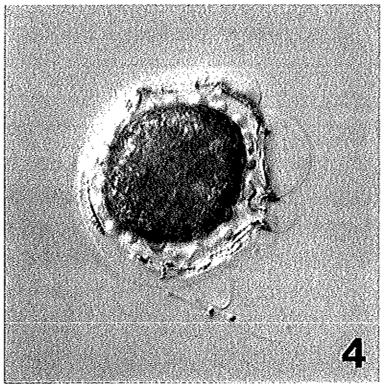

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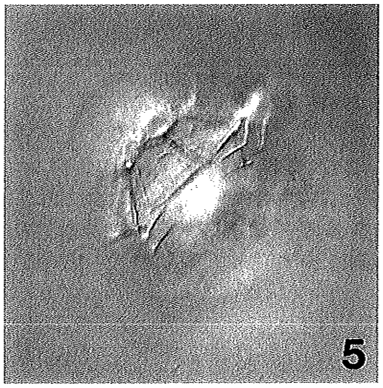

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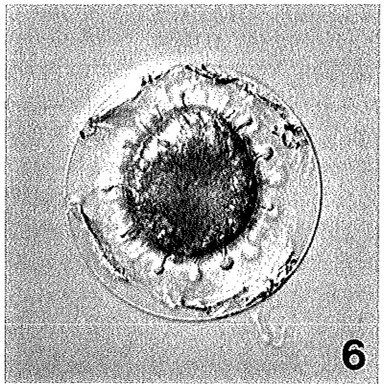

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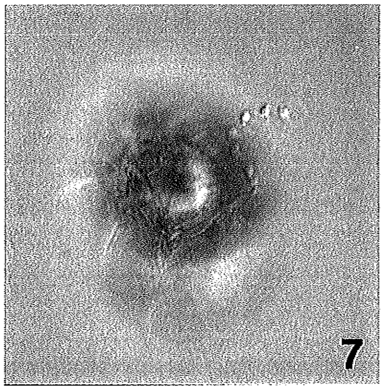

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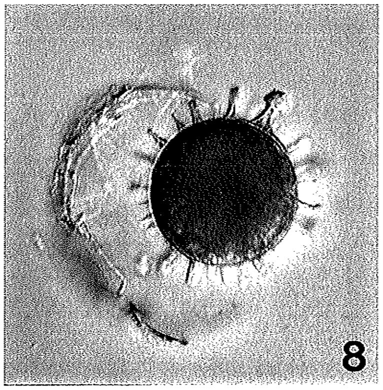

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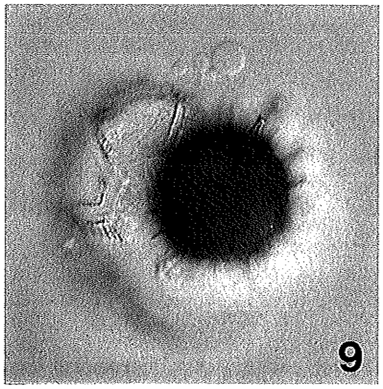

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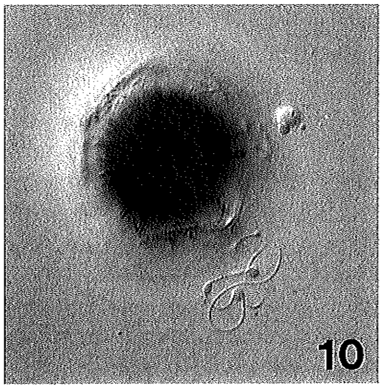

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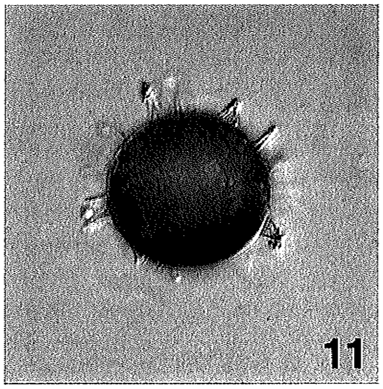

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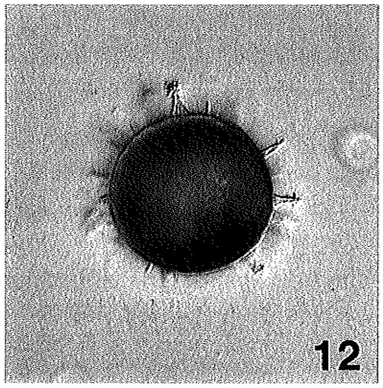

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vegetative cells. In some species, however, the planozygote ultimately loses motility, sheds its theca, and develops one or more new wall layers to become a hypnozygote, or resting cyst. After a period of obligate dormancy, hypnozygotes germinate and the excysting cell undergoes a meiotic division, although the exact timing and nature of this division are not always clear. The resulting daughter cells are motile and resume vegetative growth to complete the life cycle. For more detailed treatment of dinoflagellate reproduction, the reader is referred to summaries in Tappan (1980), Walker (1984), Pfiester (1984, 1989), and Pfiester and Anderson (1987).

In spite of the numerous investigations involving living *L. polyedrum* and related species, their life cycles are incompletely known. Previous morphological studies of living *L. polyedrum* have reported on aspects of asexual reproduction (Kofoid, 1911; Dürr and Netzel, 1974; Dürr, 1979), germination of resting cysts (Wall and Dale, 1968), development of newly excysted cells (Lewis and Burton, 1988), and formation of temporary cysts (Marasovic, 1989). Resting cyst development, however, while certainly one of the most dramatic transitions in the life cycle of this species, is described in this paper for the first time.

MATERIALS AND METHODS

Experimental Organism

All observations were made with isolates GpES-13, GpES-17, and GpES-19 of *Lingulodinium polyedrum* (al. *Gonyaulax polyedra*). The original cultures were established by J. P. Kokinos in March, 1990 using cysts isolated from sediments collected at Essvik Station 1 (90 m), Gullmar Fjord, Sweden. Each strain was started from a single cyst; cultures were not axenic. Experimental strains of *L. polyedrum* were maintained in our culture collection only as long as their ability to produce resting cysts remained significant, a period averaging ~2 years.

Culture Medium

All stock and experimental cultures employed ES medium (Provasoli, 1968; Harrison et al., 1980) modified according to M. Clement Durand (written commun., 1986), with additional minor changes incorporated by our laboratory. Our medium (termed ES-DK) differs from Provasoli's original recipe as follows: (1) Substituting 12.3 mg of anhydrous $MnSO_4$ for 16.4 mg tetrahydrate $MnSO_4$ in the preparation of 400 mL of P_{II} metals primary stock, (2) Adding one third less distilled water in the preparation of ES-DK working stock, (3) Eliminating Tris, (4) Using Guillard's (1975) f/2 vitamin preparation and enrichment protocol (0.5 mL of f/2 vitamin working stock addied to 1 L seawater) instead of Provasoli's vitamin protocol, and (5) Adding 9.3 mL ES-DK working stock (rather than the 20 mL specified by Provasoli) to 1L autoclaved/0.22 µm filtered seawater. Table 1 describes final concentrations of major nutrients, trace metals, and vitamins in 1 L of ES-DK. In general, medium preparation procedures were identical to those described in Anderson et al. (1984) except that glassware was not treated with Surfasil.

Culturing Procedures

Experimental cultures were inoculated with cells in mid- to late-exponential growth to yield an initial concentration of approximately 300 cells per mL. The growth stage of batch cultures was loosely monitored by measuring *in vivo* fluorescence at 2-day intervals. All cultures were kept at 20°C. Irradiance was provided by Cool-White fluorescent bulbs at approximately 250 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR. A 14:10 h L:D cycle was used for all experimental and stock cultures.

Observation

Small volumes of culture were removed aseptically from culture tubes in a laminar flow hood using 3 mL autoclaved glass pipettes and placed into microscope slide chambers. For preliminary observation at 100 to 200x, Palmer-Maloney slides were employed. However, to permit use of higher power objectives with shorter working distances, allow precise focusing of the substage condenser, and minimize spherical aberration due to depth of the water sample, special observation chambers were constructed using spacers cut either from plastic coverslips (American Scientific Products diSPo Slips, M6100), parafilm "M" (American Can Company), cellophane tape (3M, Scotch Brand Tape 3750-G), or plastic film (Saran Wrap, DowBrands Inc.). Spacers were fixed to standard 1" x 3" glass microscope slides by either self-adhesion (cellophane tape), gentle heating (parafilm), or a thin layer of Silicon stopcock grease (coverslips, plastic film). Evaporation of medium during observation of cells was reduced by covering chambers with glass coverslips (Corning No. 1 1/2). All light microscopy was carried out on a Zeiss Axioskop equipped with DIC (Nomarski) optics.

Photography

All cells were photographed in the living state, surrounded by the culture medium. Black and white 35 mm photography was accomplished using a Zeiss MC 100 camera system and Kodak Technical Pan film. SVHS format video employed an Optronics CS-450 camera/camera control system, Sony Trinitron Super Fine Pitch monitor, Panasonic TL (Time Lapse) SVHS AG-6720A video camera recorder, and 3M Blackwatch ST-120 SVHS videotape.

RESULTS

Timing and Observation of Life Cycle Stages

When inoculated with cells in mid-exponential growth, cultures of *L. polyedrum* showed a brief lag phase of typically less than 2 days. Maximum growth rate during exponential phase was approximately 0.7 div./day. *In vivo* fluorescence peaked approximately 3 weeks after inoculation and maximum cell densities (up to 16,000 cells/mL) were recorded 4-5 days after the fluorescence peak. The actual growth limiting factor in batch culture tubes was not determined.

Several previously described life cycle events of *L. polyedrum* were encountered in our study. Vegetative cell division occurred with the highest frequency during exponential growth but could be seen at just about any time except late in senescence. Continuous observations of the entire division process were made twice; in both cases, dividing cells were initially thecate and motile. Early stages of cytoplasmic partitioning were visible while the parent theca was still intact. The general pattern of cell division appeared consistent with a typical gonyaulacoid desmoschisis (see Pfiester and Anderson, 1987); sutures opened along an oblique line and separated the theca into left anterior and right posterior halves. Each daughter cell retained one half of the parent theca and seemed to regenerate their missing halves as cytoplasmic division progressed. When daughter cells finally detached, newly generated and inherited thecal moieties could not be readily distinguished at low power. No shedding of parental thecal remnants was observed either while daughter cells were still joined or immediately after separation. In both episodes, division lasted approximately 1 hour and cells remained motile throughout the entire process.

Although what appeared to be temporary cysts were frequently encountered during our investigation, we did not witness the actual formation of these structures. In some culture tubes, ecdysed thecae were abundant and typically showed some degree of dissociation in the vicinity of the mid-dorsal precingular plate (Kofoid 3'; Taylor-Evitt 4), presumably to allow escape of a temporary cyst.

In general, syngamy (fusion of gametes) was first noted a day or two beyond the point of peak fluorescence. Gametes (average size : 30-40 µm length; 25-30 µm width) appeared smaller than typical vegetative cells (38-45 µm length; 35-45 µm width), were lighter in color, and often showed a simpler outline (pentagonal in dorsoventral view). In a few cases, larger cells seemed to be fusing, but none of these pairs were observed to complete the process. A typical fusion episode took about 45 minutes and involved one or more intervals during which the fusing cells were non-motile and rested on the chamber bottom. During the present investigation, we did not observe any process which could be clearly interpreted as gametogenesis. In most culture tubes, the small gamete-like cells formed a considerable percentage of the swimming population starting about the time of the fluorescence peak.

Development of the Resting Cyst

Resting cyst formation was best observed 5 to 12 days after the fluorescence peak, although in at least two instances it was noted as early as mid-exponential phase. Our description of this process is based on 28 observations of encystment; 13 of these encompassed the entire morphological transition from active planozygote to hypnozygote. The developmental sequence from loss of motility to morphologically mature resting cyst took approximately 10 to 20 minutes. To facilitate description, we divide the encystment process into the following 4 stages: (I) Pre-expansion (cell still motile), (II) Initiation, (III) Cell expansion, and (IV) Rupture of the outer membrane.

I. Pre-expansion. This stage encompasses all encystment-related events occurring while the cell is still motile (i.e., planozygotic). With the light microscope, the first detectable evidence signaling commencement of resting cyst formation was the presence of a prominent zone of differentiation in the planozygote peripheral cytoplasm (Plate 1, fig. 2). In our cultures, planozygotes could be distinguished from cells in other motile life-cycle stages primarily by their large size (up to 60 µm length, 50 µm width) and darker color, but in some cases (using high magnification), wide growth bands and/or dual longitudinal flagella could be detected. At low power (100x), the differentiated zone appeared as a colorless interstice positioned between the theca and a central mass of pigmented vesiculated cytoplasm. Where it was well developed, the zone (hereafter referred to as the peripheral interstice, or PI) was continuously visible at all angles during the swimming behavior of the planozygote suggesting that the PI completely surrounded the cytoplasm (with the possible exception of the area near the flagellar insertion). At higher power (400x) with DIC contrast enhancement, some faint structure was visible in the PI; this consisted mostly of irregular lines (seen in optical cross section) which were roughly parallel to the curvature of the theca. In one specimen, particles in Brownian motion partially filled the PI. Typically, the boundary between the peripheral interstice and central cytoplasmic mass was not smooth, being defined by the outermost extent of rounded cytoplasmic vesicles (Plate 1, fig. 2). The irregular nature of this surface gave the PI of any given specimen a variable thickness. When well developed, a PI averaged ~5 μm.

It should be noted that not all planozygotes displaying the peripheral interstice went on to complete encystment. Specimens observed in "old" cultures (i.e., well beyond the peak of the growth curve) often showed a similar feature, but none were observed to make further progress towards resting cyst formation.

In some cases, planozygotes displayed a unique swimming behavior just prior to their transformation into hypnozygotes. Cells would orient themselves with their polar (longitudinal) axis perpendicular to the bottom of the observation chamber and rotate slowly with very little lateral movement, either swimming upward (towards the viewer) or staying roughly at one level. These episodes would often be interrupted by short lateral swims, after which the cell would resume its slow rotation. This behavior, however, was only observed in Palmer-Maloney slides. The extremely shallow water column of the observation chambers used for higher magnification may have prevented planozygotes from adopting this behavior. Whether the "polar spin" is normal encystment behavior or something induced by our observational methods could not be determined.

II. Initiation. This stage includes those events representing the initial morphological changes accompanying the transition from planozygote to hypnozygote: loss of motility, initial expansion of cell membranes, dissociation of the theca, and the appearance of structures which later develop into cyst processes. In our cultures, these events were rapid and often difficult to observe as they occurred more or less simultaneously during the first 30-60 seconds of resting cyst formation.

Loss of motility. The loss of motility was usually abrupt; normal "corkscrew" swimming stopped and the cell drifted to the bottom of the observation chamber. In some cases, the swimming behavior of the planozygote became less energetic immediately before swimming totally ceased, but again, it is difficult to say whether this is normal behavior or due to observation conditions (e.g., high light intensity, cramped swimming space, etc.). On a few occasions, some flagellar motion continued for several seconds after the cell stopped swimming. In one case, final beating of the longitudinal flagella propelled the planozygote forward for a short distance along the bottom of the observation chamber while cell expansion began.

Fate of the flagella. Once the planozygote came to rest, the flagella were expelled from their respective thecal grooves. The absolute cessation of flagellar movement was often preceded by a highly exaggerated writhing. At this point, both longitudinal and transverse flagella commonly showed spherical swellings along their lengths which gave the appearance of beads on a string (e.g., Plate 2, figs. 3 and 7). These swellings were present during the last stages of flagellar movement and persisted after motion had stopped. Swellings consistently appeared more numerous and showed more variability in size on the transverse flagellum compared to the longitudinal flagella (Plate 2, fig. 10). The writhing movement often left flagella in a tangle; the transverse flagellum usually ending up more tightly tangled than the longitudinal flagella.

All flagella remained connected to the cell during the early stages of morphological development, but normally were found detached around fully developed resting cysts. In some cases, flagella were observed to fragment during resting cyst development. Isolated flagella or flagellar fragments, still retaining the beads or swellings, were commonly encountered while scanning the bottom of a slide containing residue from an encysting population. Around newly encysted cells, flagella were visible at low focus; a typical arrangement consisted of a tangled transverse flagellum located at some distance from the intertwined longitudinal flagella (Plate 2, figs. 3 and 10). In this investigation, we saw no evidence suggesting the presence of a second transverse flagellum. (For a discussion of hypotheses and observations relating to the number of transverse flagella on dinoflagellate planozygotes, the reader is referred to Gao et al., 1989.)

Bulging of membranes external to the theca. Perhaps the most dramatic of the early developmental events is the initiation of cell expansion. One or more membranes external to the theca bulged outward forming blister-like protrusions on the surface of the encysting cell (Plate 1, fig. 3). The number, shape, and location of initial bulges was variable from episode to episode; on any one specimen, these features were modified relatively quickly as encystment progressed (Plate 1, figs. 3-7). On some specimens, bulges seemed to be intratabular with respect to thecal plates. The outer membrane(s) appeared anchored along sutures while bulges developed over plate surfaces (Plate 1, fig. 6; Plate 2, fig. 4). As the encysting cell continued to expand, bulges appeared to grow outward, coalescing and extending laterally to cover more of the thecal surface (Plate 1, figs. 4-5). Eventually, expanding bulges merged to form larger protrusions surrounding the entire cell (Plate 1, figs. 6-7). At no time during cell expansion could the outer membrane be resolved into more than one component with the light microscope. As a result, we will refer to this structure as a single membrane in subsequent description.

Thecal dissociation. As bulging of the outer membrane progressed, the theca began to dissociate. Close inspection of the theca at this stage (and later) revealed that dissociation was often not random, but followed a broad pattern. Major breaks in the theca tended to occur in two places, namely along the upper and lower margins of the cingulum (Plate 2, fig. 5). In many cases, these breaks defined three thecal sections: an epithecal hemisphere composed of apical and precingular plates, a hypothecal hemisphere composed of postcingulars and antapical plates, and a cingular "strip" or band. Sections would occasionally dissociate further along "accessory" sutures, but often remained connected locally.

Thecae of encysting specimens were significantly different from those encountered on cells in other life cycle stages. The former appeared to be thinner and more flexible, with greatly reduced ornament (Plate 2, figs. 2, 5, and 9). Margins of growth bands were often visible, but intratabular areas showed only faint topography.

Although, in general, membrane bulges external to the dissociating theca enlarged and grew together, the original outer membrane never completely separated from the theca, remaining attached locally (Plate 1 fig. 6; Plate 2, fig. 4). Residual attachments often appeared confined to plate sutures. With continued expansion of the cell, the irregularly bulged external membrane became increasingly spherical, and moved radially outward (Plate 1, figs. 6-8). Because of the residual attachment sites to the dissociating theca, this outward movement pulled thecal sections away from the underlying cytoplasm (Plate 2, fig. 6).

Process globules. Simultaneous (or nearly so) with the appearance and early growth of external bulges and the dissociation of the theca, was the modification of the outer surface of the central cytoplasmic mass. During initiation, the irregular surface bounding the vesiculated cytoplasm of the planozygote became better defined (Plate 1, figs. 3-4). On this new surface appeared numerous colorless refractive globules in a single layer (Plate 1, figs. 4-7; Plate 2, fig. 4); the initial number and distribution of these globules varied between specimens. Normally, globules did not contact either the dissociating theca or the expanding outer membrane. On the average, globules measured about 5 µm in diameter.

In the third developmental stage (Expansion, see below), globules migrated radially outward during formation of the characteristic processes of *Lingulodinium* resting cysts. Before this migration, however, globules on some specimens clearly underwent modification in size, shape, and/or location. In some cases, globules completely detached from the cytoplasmic surface and became spherical bodies floating throughout the expansion zone in Brownian motion (Plate 3, fig. 1). Reattachment of free-floating globules to the developing cyst was not observed. Another form of modification involved larger initial globules changing into smaller globules. On any given specimen, globules often varied in size at the beginning of process growth (Plate 1, fig. 7).

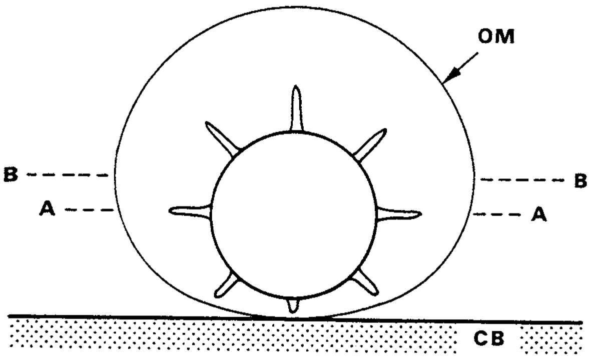
III. Spherical expansion and process formation. Stage III begins when the outer membrane, seen in optical cross section, approaches circularity and globules on the surface of the cytoplasm begin development into processes.

Cell geometry. During this stage, continued expansion of the outer membrane gave the encysting cell the appearance of an inflating balloon (Plate 1, figs. 7-12). Although this expansion tended to be laterally symmetric (i.e., outer membrane approximately equidistant from the surface of the internal cytoplasmic mass), asymmetric geometries were observed early in the developmental sequence of some specimens. These asymmetries resulted when thecal sections adhered to the underlying cytoplasmic surface or globules. Since the outer membrane often remained partially attached to thecal sections, expansion became skewed and the cytoplasmic mass and outer membrane were not concentric. With continued inflation of the cell, however, the growing outer membrane eventually peeled recalcitrant thecal sections away from the cytoplasm to establish a more symmetric geometry.

In spite of this apparent shift from asymmetric to symmetric development, however, the inner cyst body and the outer membrane were never truly concentric. Critical focus on encysting cells showed the maximum circumference of the outer membrane to be consistently higher above the slide bottom (i.e., closer to the viewer) than that of the internal cytoplasmic mass, a vertical asymmetry probably caused by cyst formation against the glass bottom of the observation chamber (Text-Figure 1; see also Plate 1, figs. 11 and 12).

Contents of the expansion zone. During cell expansion, the widening interstice between the cytoplasmic mass and the outer membrane typically showed the same optical character as the external medium (e.g., Plate 1, figs. 10 and 12). In two cases, however, what appeared to be cytoplasmic particles vibrating in Brownian motion occupied a small portion of this space.

Process formation. Process formation involved two basic steps. First, process shafts developed centrifugally (i.e.,



Text-Figure 1. Proposed model of *L. polyedrum* resting cyst development in the observation chambers used for this study. Lateral view. Not to scale; vertical distortion of outer membrane (OM) exaggerated for emphasis. Diameter of resting cyst central body ~45-50 µm. Maximum circumference of outer membrane (level B) is consistently higher than that of the internal resting cyst (level A) due to distortion caused by expansion against chamber bottom (CB).

growth initiated at the cytoplasmic surface and shafts grew outward) beneath the migrating globules, and second, process tips formed by modification of the globules once shaft assembly was completed. Radial growth of processes did not occur immediately after appearance of the globules on the surface of the cytoplasm but commenced after a short interval of time, normally on the order of a few minutes. The first recognizable portion of a developing process appeared between a globule and the cytoplasmic surface (Plate 1, fig. 7). While proximal portions of the processes formed, globules persisted distally, initially maintaining their size, shape, and optical character (Plate 1, figs. 7-10). In some cases, the diameter of terminal globules decreased slightly as development proceeded.

Proximally, process shafts tended to be larger in diameter than the later-formed distal portions (Plate 1, figs. 10 and 11). As mentioned previously, not all globules were identical in size at the start of process development. Larger globules tended to yield wider though not necessarily longer processes. Once initiated, process formation appeared smooth and continuous, and occurred approximately at the same rate for all processes on a given specimen. During early stages of growth, the rigid and structurally competent appearance of the partially-built process shafts contrasted sharply with the delicate bubble-like appearance of the distal globules (Plate 1, figs. 8 and 9). Surface features such as longitudinal striations—often visible on processes of morphologically-mature *L. polyedrum* resting cysts—could be detected at the earliest phase of process material deposition; these features formed in a centrifugal fashion as an integral part of the process shaft.

Thecal interference. As mentioned above, process assembly began several minutes after globules first appeared on the surface of the cytoplasmic mass. Normally, this "delay" allowed for sufficient expansion of the outer membrane (at least laterally) to pull the dissociating theca away from the underlying globules before the initiation of process deposition. As a result, process development usually occurred without contact with the theca (Plate 1, figs. 7-12). In some cases, however, portions of the dissociating theca

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PLATE 3

Morphological development of *L. polyedrum* resting cysts. All figs. x500. Sequences shown in figs. 1-3 and 4-6 illustrate two aberrations of resting cyst formation as observed in labratory cultures. All cells photographed in the living state, surrounded by the culture medium.

Figs. 1-3 = globule detachment.

1 Specimen during initiation (Stage II) phase of development. Note detached globules free-floating in expansion zone.

2 Cell during expansion (Stage III) phase. In equatorial focus, region of cytoplasmic surface between ~11 and 3 o'clock lacks developing processes.

3 Immediately after rupture (premature) of outer membrane. Note: (1) outer membrane is still visible, (2) membrane shows residual attachment to the theca (e.g., 11 and 1 o'clock), (3) premature rupture of membrane resulted in truncation of spines (e.g., 7-8 o'clock), and (4) final distribution of spines on cyst surface is highly uneven.

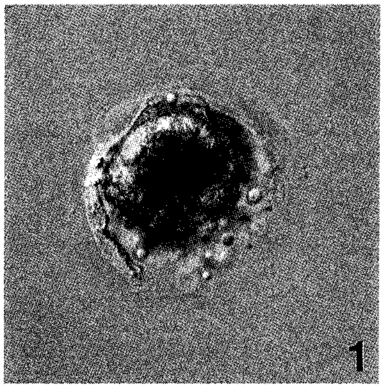
Figs. 4-6 = premature rupture of outer membrane.

4 Specimen during expansion (Stage III) phase, just prior to rupture of the outer membrane.

5 Immediately after membrane rupture, membrane still visible. Terminal globules on processes at ~8 and 9 o'clock maintain spherical morphology while rapidly deflating.

6 Moments after rupture, terminal globules fully deflated leaving sharply truncated processes.

7 Collection of culture-produced resting cysts showing an assortment of process morphologies. Also note (1) well-developed endospore shown by specimen on the far left, and (2) darker colored mass (accummulation body?) adjacent to the wall of the otherwise empty resting cyst at top of photo.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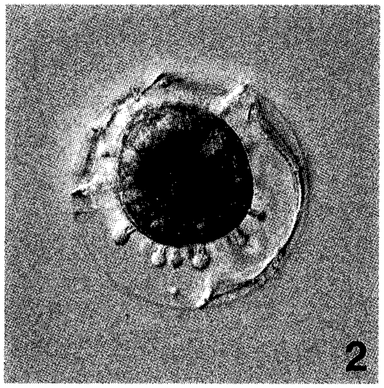
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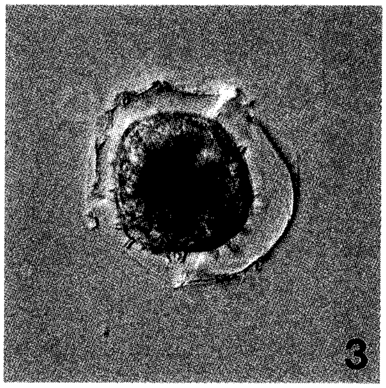
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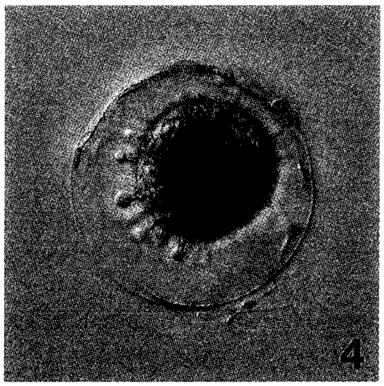
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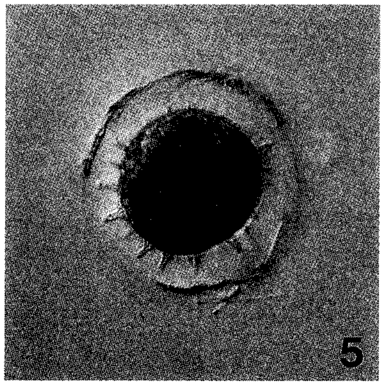
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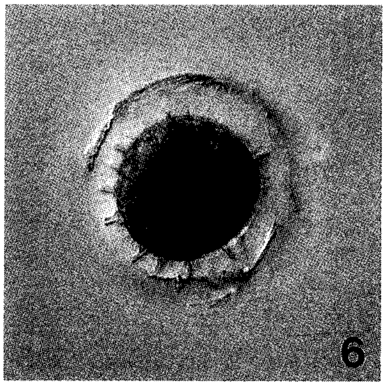
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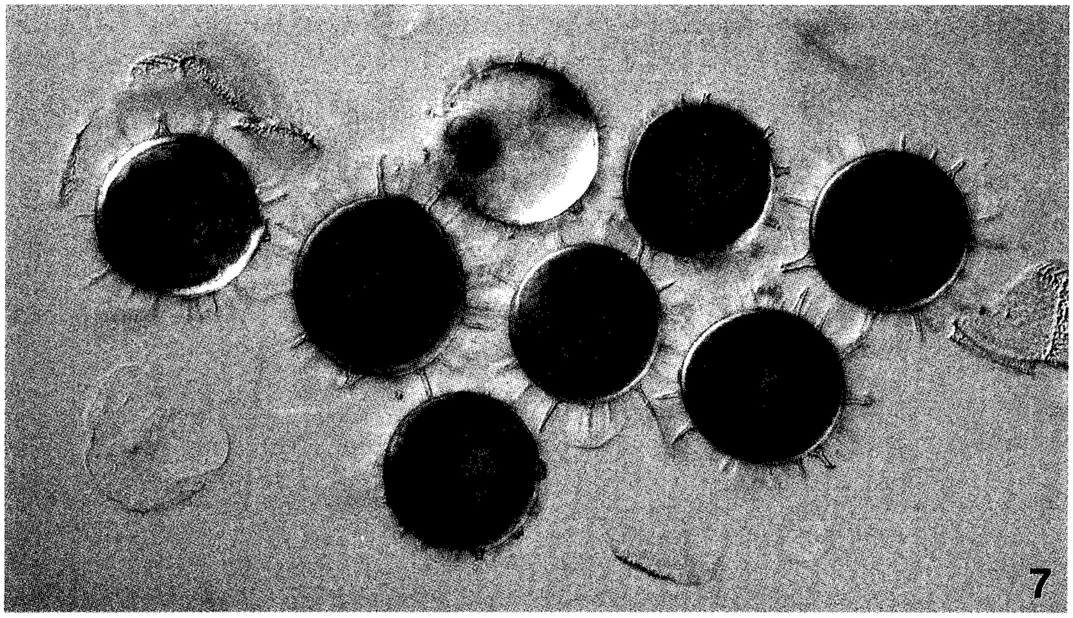
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did not move far enough to avoid contact with the outgrowing processes. (This was certainly true for the area of each encysting specimen in contact with the bottom of the observation chamber; see Text-Figure 1.) However, as long as the theca had room to move (i.e., not pinned between the inner body and the observation chamber bottom or sides), processes appeared to develop normally. It was not clear if process outgrowth pushed back the interfering sections of theca or if sections were pulled away by continued expansion of the outer membrane.

Modification of terminal globules. The final step in the development of process morphology began after deposition of process shaft material was complete and outer membrane expansion reached a maximum, both of which occurred at more or less the same time. As seen in optical section, the maximum diameter obtained by the outer membrane was about twice the diameter of the central body (Plate 1, fig. 10). At this point, modification of distal globules gave each process its final form. In most cases, modification of process tips occurred without contacting either remnants of the theca or the expanding outer membrane. Observed globule modifications were of 3 basic types:

1. Globules constricted to form tapering extensions to the already-formed process shafts (Plate 1, figs. 9-10). These extensions often bore spinules which formed during collapse of the initially smooth, unornamented spherical globules. Often, the first signs of spinule formation could be observed while the globule was still spherical, appearing as irregularities on the surface. These irregularities then remained behind as topographic highs while the rest of the globule surface collapsed around them (Plate 1, figs. 10-12). Spinule presence and morphology was highly variable among different specimens but also between different processes occurring on a single specimen (Plate 2, fig. 1).
2. Globules deflated only slightly, retaining a spherical or bulbous shape (Plate 2, fig. 8) and developed a surface texture similar to that of the process shaft. Bulbous terminations were observed with or without spinule ornamentation.
3. Globules collapsed into irregularly shaped masses. These terminal structures did not appear as simple hollow extensions of the shaft, but served to give the process a more capitate appearance (Plate 2, figs. 11 and 12). During formation of these irregular structures, numerous faint particles of varying size and in Brownian motion could be seen localized near collapsing terminal globules. The origin of these particles was not observed.

Free-floating globules (see *Process globules*, above), if present, were modified in a fashion similar to, and simultaneously with, process tip globules. Detached globules collapsed into irregularly shaped particles which continued to vibrate in Brownian motion. After globule modification, the morphologically mature resting cyst was often observed to twitch and/or rotate slightly within the still-intact outer membrane. The mechanism for this movement could not be determined.

IV. Rupture of the outer membrane. The final stage of development involved rupture of the outer membrane and release of the newly-formed resting cyst. Rupture occurred suddenly, as in the popping of a balloon. In most cases, the outer membrane disintegrated immediately. Occasionally, however, portions of this membrane persisted for several seconds and could be seen collapsing onto the cyst processes (Plate 3, figs. 3 and 6). Whether occurring immediately after rupture or slightly delayed, disintegration of the membrane was instantaneous and complete, breaking into small and very faint particles which dispersed rapidly in the surrounding medium.

Rupture of the outer membrane allowed the back-migration of thecal sections, which could then come into contact with the processes. As a result, morphologically mature resting cysts encountered at the bottom of our culture vessels were often closely associated with, or even partially enclosed by, portions of the theca belonging to the original planozygote (Plate 2, figs. 1, 8, and 9).

Newly liberated resting cysts appeared morphologically mature and looked more or less identical to cysts recovered from natural sediments. The spherical inner body of a mature resting cyst averaged 45-50 μm in diameter; with well-formed processes, the total resting cyst diameter could increase to ~70 μm. Continued observation of some specimens for up to 3 months revealed no additional changes in gross morphology. Resting cysts that had formed in our observation chambers typically showed one side bearing shorter, and in some cases bent, processes (Plate 1, fig. 1) presumably reflecting their impeded growth against the chamber bottom. Immediately after formation, the typical resting cyst cytoplasm appeared evenly colored brownish-grey with dispersed orange-red pigment. Within 24 hours, the cytoplasm appeared lighter, almost colorless, and orange-red pigment was localized in a small number of patches. On some specimens, these patches were very well defined, to the point were they resembled mineral grains (i.e., showing planar faces and sharp edges). Also developing within this time was the endospore, a conspicuous clear band between the outermost cyst wall and the cytoplasm (e.g., Plate 3, fig. 7). In most specimens, this band appeared irregular in thickness and displayed a characteristic interference figure (reminiscent of a uniaxial cross) when viewed with crossed nicols at low magnification. Such an interference figure has been noted for other dinoflagellate cysts (Erdtman, 1954; Reid and Boalch, 1987). Particles in Brownian motion were commonly observed throughout the cytoplasm. Periodic examination of resting cysts over several months revealed that the orange-red bodies changed both in position and number over time.

Premature Rupture of the Outer Membrane

In our observation chambers, encysting cells attaining full expansion, with subsequent formation of morphologically mature processes, were the exception rather than the rule. In most cases, the outer membrane ruptured prematurely, before modification of process tip globules was complete.

The most visible result of premature rupture was the instantaneous interruption of normal process development. If rupture occurred before significant deposition of process material, a high percentage of globules tended to detach from the surface of the cytoplasm and disperse in the surrounding medium (Plate 3, fig. 1); the resultant "cyst" was sub-spherical and typically showed fewer processes with an irregular distribution (Plate 3, fig. 3). In the present investigation, no attempts were made to determine the viability of such aberrant cysts. If rupture occurred during process shaft deposition, terminal globules completely collapsed leaving behind sharply truncated processes (Plate 3, figs. 3, 6, and 7). This "premature" collapse of terminal globules occurred in two different ways. (1) The initially smooth and spherical globules developed a granular surface texture which appeared to quiver as though surface granules were in Brownian motion. This was followed by rapid and complete deflation of the globule (globule material appeared to collapse into the process shaft) to yield a truncated process. (2) After rupture, terminal globules deflated smoothly while maintaining their spherical morphology (Plate 3, figs. 4-6). With the light microscope, we were unable to determine if truncated processes were distally open or closed.

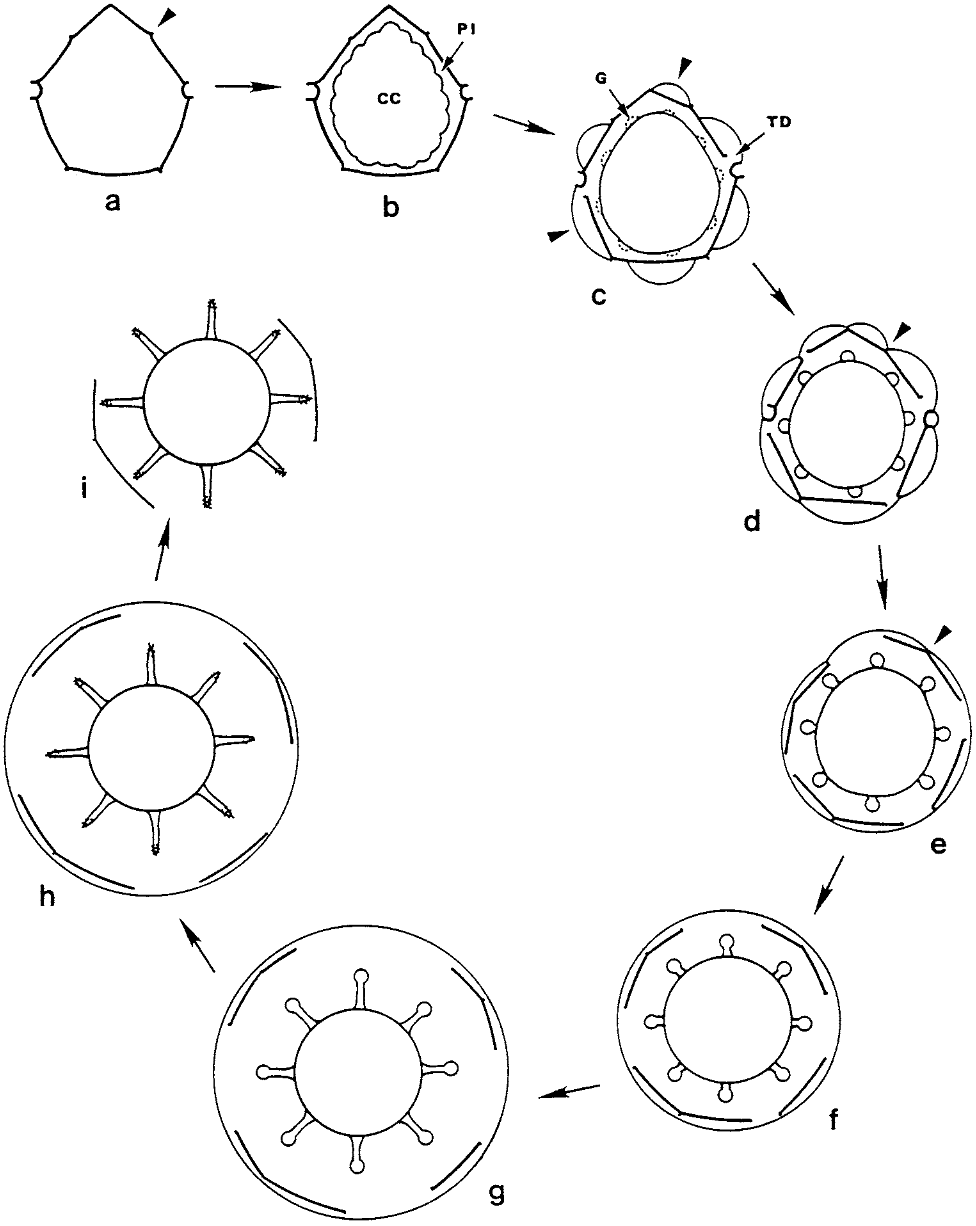
DISCUSSION

In this paper, the morphological development of *L. polyedrum* resting cysts is described for the first time. The basic mechanism consists of rapid cell expansion resulting from the widening of an interstice between the planozygotic cytoplasm and a membrane external to the theca (Text-Figure 2). Key morphological events in the development of the resting cyst cell wall take place within this expanding interstice. These include early dissociation and outward migration of the theca, synthesis of the dinosporin cell wall, and centrifugal development of cyst processes. The level of morphological maturity attained by the encysting cell depends primarily on how much development occurs before rupture of the expanding outer membrane. If rupture is premature, a wide variation of cell wall morphology may occur, particularly with respect to the size, number, and distribution of processes. From a paleontological point of view, these results are significant because considerable morphological variation is produced by a single biologically defined species.

Life Cycle

Since the complete life cycle of *L. polyedrum* has never been described, it seems appropriate to briefly comment on some of the life cycle stages, in addition to resting cyst formation, observed in our investigation. Vegetative cells in the process of asexual division were frequently encountered, particularly during exponential growth. The morphological details of desmoschisis appeared essentially consistent with previous descriptions by Dürr and Netzel (1974) and Dürr (1979) for this species. However, in the two complete episodes of asexual division we followed, daughter cells were not observed to shed their inherited thecal moieties as was reported by the latter author. Such shedding may have taken place after the physical separation of daughter cells, but we did not track cells beyond this point. It may be significant that most of the thecal material which accumulated at the bottom of our culture tubes consisted of relatively entire thecae. If cells routinely shed well-defined thecal "halves" during vegetative division, one would expect to encounter large numbers of these moieties in culture vessel residues. Although both Kofoid (1911) and Dürr and Netzel (1974) mention vegetative division among naked cells, no clear indications of this phenomenon were encountered during our study.

When batch culture growth became limiting (as indicated by maxima in fluorescence and cell density), morphologically distinct gametes appeared which were later observed fusing in pairs to form motile zygotes. Since experimental cultures in this investigation were started from cysts, all mating types (if they exist) are assumed to be present in any given batch culture. As a result, it was not possible to determine if *L. polyedrum* was homothallic (all gametes identical and capable of indiscriminate fusing) or heterothallic (mating types exist, and "plus" and "minus" strains required for fusion; see Pfiester and Anderson, 1987). After a period of cell fusion, large, darker-colored cells with two longitudinal flagella and wide thecal growth bands began to form a significant percentage of the swimming population. We consider these cells to be planozygotes. As reported in this paper, planozygotes transformed directly into resting cysts. Since we did not follow individual



Text-Figure 2. Cartoon illustrating major features of *L. polyedrum* resting cyst morphogenesis. Approximately to scale; diameter of resting cyst central body ~45-50 µm. Sketches represent optical sections through center of cell; view approximately dorsoventral. Flagella omitted. Illustrations *a-b* = Stage I; *c-d* = Stage II; *e-h* = Stage III; *i* = Stage IV; see text for details. *a.* Planozygote; theca intact. Plate boundaries (i.e., sutures) shown as raised points on theca (arrow). *b.* Planozygote just prior to resting cyst formation. Peripheral interstice (PI) results from consolidation of cytoplasm (CC) in center of cell. *c.* Initiation of resting cyst formation. Cell expansion begins with localized swelling of a membrane external to the theca (arrows). Theca begins to dissociate along one or more plate boundaries (TD = thecal dissociation). A single layer of globules (G) appears on surface of central cytoplasmic mass. *d.* External swellings enlarge and merge to liberate a continuous outer membrane (OM) surrounding entire cell. OM often remains anchored to theca along plate boundaries (arrow). *e.* Continued expansion. Remaining points of attachment (e.g., see arrow) between OM and thecal sections ensures that theca is pulled away from underlying globules and theca. Globules begin to migrate radially outward; process bases appear between globules and surface of cytoplasmic mass. *f.* Midpoint of cell expansion. Process shaft formation occurs without direct thecal influence. *g.* Maximum extent of cell expansion; formation of process shafts complete. *h.* Modification of terminal globules yields process morphology characteristic of *L. polyedrum* resting cysts. *i.* Rupture of OM liberates resting cyst. Back-migration of thecal sections often leaves portions of "parental" theca in contact with resting cyst.

cells throughout all stages of sexual reproduction (cf. Pfiester, 1975, 1976, 1977), a rigorous definition of the complete life cycle of *L. polyedrum* is not possible at this time. Nevertheless, our observations strongly suggest a course of events consistent with the basic dinoflagellate sexual cycle model summarized by Dale (1983).

Cells appearing to be temporary or pellicle cysts were quite common in some of our cultures, although formation of these forms was not observed directly. In our study, there was no obvious connection between the occurrence of temporary cysts and any recognizable event in the sexual life cycle outlined above. This observation contrasts with the preliminary report of Marasovic (1991) which suggested that temporary cyst formation may be a precursor to resting cyst formation among natural populations. In our laboratory cultures, resting cysts of *L. polyedrum* formed directly from actively swimming planozygotes without any pellicle or temporary cyst intermediates.

It is interesting to consider the implications surrounding the observation of resting cyst formation during early to mid log-phase growth of batch cultures. If our interpretation of the life cycle is accurate, encystment must be preceded by gametogenesis, syngamy, and planozygote maturation. These events, however, are commonly hypothesized to occur as a result of limitation following exponential growth (Anderson et al., 1984; Anderson and Lindquist, 1985; Pfiester and Anderson, 1987). One possible revision of this hypothesis might cast the first step towards resting cyst formation (namely gametogenesis) as a broad response to several kinds of stresses—not necessarily involving limitation—which cells may experience in the batch culture environment. For example, early gametogenesis induced in a small percentage of cells by inoculation shock might be one explanation for the hypnozygote formation occasionally observed during log-phase growth.

Morphology of Hypnozygote Development

To date, most reports of dinoflagellate resting cyst formation mention the deposition of one or more new walls which surround the cytoplasm as the cell loses motility. In armored species, these layers typically form beneath the theca which tends to remain more or less intact until cyst formation is complete. The only published encystment mechanism that closely parallels what we have observed for *L. polyedrum* appears to be that of *Gymnodinium pseudopalustre*, an athecate, freshwater dinoflagellate described by von Stosch (1973). Planozygotes of this species were observed to secrete an outer membrane (termed the preliminary wall by von Stosch) which, upon swelling, formed a "hyaline interstice" around the cytoplasm. Granules then formed on the surface of the cytoplasmic mass and these elongated outward into short, hair-like spines as the outer wall continued to expand to a maximum diameter (based on our interpretation of von Stosch's illustrations) of about 1.5 times the diameter of the inner body. Rupture of the preliminary wall liberated the hypnozygote. The entire sequence described by von Stosch lasted 9 minutes.

Similar mechanistic elements are also mentioned by Taylor and Gaines (1989) in a note describing resting cyst formation in cultures of *Gonyaulax spinifera* and *Polykrikos kofoidii*. Unfortunately, this report is very brief and no illustrative material is provided, making interpretation and comparison to the present study somewhat difficult. It is noteworthy, however, that resting cyst formation by these species appears to involve conspicuous cell expansion, during which time the cell is bounded by a growing outer membrane. In both cases, expansion seems to occur by mucus secretion beneath this membrane, and major elements of cyst wall relief form within the resulting mucoid layer.

Although quite striking as a dynamic morphological phenomenon, resting cyst formation in *L. polyedrum* was relatively difficult to observe in its entirety. The loss of planozygote motility and beginning of cell expansion is extremely rapid (on the order of seconds) and the chance of encountering this initiation event in a random low-magnification survey of lab cultures or natural populations is correspondingly low. In our investigation, planozygotes likely to encyst (i.e., showing a well developed peripheral interstice) were identified and tracked under the microscope at 200x so that initiation events could be observed and documented. Tracking episodes lasting several hours were not uncommon and as a result considerable time was invested at the microscope. In addition, key encystment structures such as the balloon-like outer membrane and process globules were usually very faint and required contrast enhancement and relatively high magnification (at least 400x) for observation and photography. Given these difficulties, it is not surprising that detailed descriptions of dinoflagellate encystment have been rare in the literature.

Planozygote cytoplasm. Our account of the morphological development of resting cyst formation in *L. polyedrum* begins with the observation of a zone of cytoplasmic differentiation beneath the planozygote theca. Although changes in the cell leading to the development of this peripheral interstice must begin at some point during planozygote maturation, the exact nature and timing of these events were not detectable with the light microscope. The actual mechanism of cytoplasmic differentiation was not observed in the present study but it is noteworthy that when multiple specimens showing the peripheral interstice could be seen simultaneously, some variability in PI definition and character was clearly visible. It is unknown whether these differences represented different stages of cytoplasmic differentiation or, alternatively, if differentiation occurred to different degrees in different specimens.

Regarding the significance of the peripheral interstice, it seems likely that this feature represents consolidation and/or modification of cytoplasmic components in preparation for cyst formation. Extensive rearrangement of cytoplasm was noted during an ultrastructural investigation of encystment of freshwater *Woloszynskia tylota* by Bibby and Dodge (1972). Similar observations were reported in studies of *Scrippsiella* sp. (Gao et al., 1989) and *Alexandrium tamarense* (Fritz et al., 1989; Doucette et al., 1989). In an investigation of the fine structure of the *L. polyedrum* cell surface, Dürr and Netzel (1974) mention but do not illustrate the presence of a "hyaline zone" around the cytoplasm of cells preparing for ecdysis. This suggests that a peripheral interstice is not unique to planozygotes but may signal the reorganization of cytoplasm during other life cycle stages as well.

Theca. Degradation of thecal cellulose has been observed by Netzel and Dürr (1984) during vegetative division of *Peridinium cinctum*. In our study of *L. polyedrum*, cellulose degradation clearly occurs prior to and/or during dissociation of the theca during resting cyst formation. Again, the precise nature and timing of this phenomenon could not be determined. Intuitively, a reduction in the rigidity of the thecal plates seems consistent with an encysting cell trying to shed its planozygotic amphiesma. Another hypothesis might invoke resorption of thecal cellulose just prior to encystment in order to build reserves which later are used in the deposition of the cellulosic endospore. Regarding the mechanism of degradation, our observations suggest some sort of enzymatic dissolution. The moderate to severe reduction of all surface features indicates that dissolution is not localized with respect to any part of the thecal plates. Our description of thecal degradation during encystment contrasts with Dale's (1983, p. 103) observation that "thecal plates attached to fully developed cysts [of *L. polyedrum*] usually show no evidence of decay."

Outer membrane. This feature appears homologous to the "preliminary wall" described for *Gymnodinium pseudopalustre* by von Stosch (1973) and may also be related to the "outer membranes" mentioned by Taylor and Gaines (1989) for *Gonyaulax spinifera* and *Polykrikos kofoidii*. At the level of the light microscope, it is difficult to correlate the balloon-like outer membrane with any specific amphiesmal component described in ultrastructural studies of *L. polyedrum* (e.g., Schmitter, 1971; Gaudsmith and Dawes, 1972; Sweeny, 1976; Dürr 1979; Lewis and Burton, 1988). At the very least, it seems safe to assume that this membrane incorporates the plasmalemma, but it may be possible that other membranes are involved as well. In some encystment sequences, initial bulging was clearly intratabular; i.e., the outer membrane remained temporarily anchored along thecal sutures while bulges formed over plate areas. Although connections between outer membrane and theca are largely broken during the early stages of cell expansion, some degree of physical attachment persists throughout encystment. These remnant attachments allow the expanding outer membrane(s) to pull thecal sections away from the underlying cytoplasm and process globules. One way to explain these observations is that expansion may initiate *within* amphiesmal vesicles. If this is so, bulges exterior to the theca are bound by both the plasmalemma and the outer vesicular membrane (see Loeblich, 1970; Dodge and Crawford, 1970; Morrill and Loeblich, 1983; Höhfeld and Melkonian, 1992; and Bricheux et al., 1992 for amphiesmal ultrastucture). Elucidation of these ultrastructural details will require investigation at the level of the electron microscope.

Cell expansion. A model of outer membrane expansion invoking a simple sphere increasing in diameter from 50 to 125 µm, gives an increase in cell volume of roughly 16x, this occurring in less than 20 minutes. There are three hypotheses which may account for this phenomenon: (1) Soluble compounds from outside the cell are transported across the outer membrane; (2) Volume increases "internally" either by alteration of existing cytoplasmic contents or the production of new material; and (3) A combination of internal and external sources. As noted previously, the optical character of the material filling the space between the cytoplasmic mass and outer membrane appears identical to that of the external medium, suggesting that the expanding "balloon" may be filled with fluid rapidly pumped from the cell's exterior environment. This observation contrasts with the report of Taylor and Gaines (1989) which described mucus secretion as the driving force for outer membrane expansion during encystment of *Gonyaulax spinifera* and *Polykrikos kofoidii*. Interestingly, von Stosch (1973) mentioned a mass of "coagulum" adhering to a newly formed resting cyst of *Gymnodinium pseudopalustre* and speculated that it represented the remnants of material filling the space between the outer ("preliminary") wall and inner body. There was no obvious release of such material during rupture of the outer membrane in our investigation of *L. polyedrum*.

Endophragm formation. In the Evitt et al. (1977) terminology of cyst morphology, the preservable portion of the *L. polyedrum* resting cyst cell wall consists of two components: the spherical, cytoplasm-enclosing layer is referred to as the "endophragm," while the processes represent the "periphragm." The precise nature and timing of endophragm formation are unclear. Close examination of photographed sequences show the cytoplasmic mass to be more or less irregular in shape without a well-defined boundary layer throughout the initiation stage of encystment. At the level of the light microscope, a wall surrounding the cytoplasm first becomes detectable near the time of preliminary globule development. Globules, however, are clearly present (although perhaps not fixed in terms of size and location) before this "proto-endophragm" becomes well defined suggesting some overlap in the formation of periphragm (i.e., processes) and endophragm. According to von Stosch (1973), spine growth and "exospore secretion" occur concurrently during encystment of *G. pseudopalustre*. As mentioned above, a more detailed description of wall formation will require investigation at the level of the electron microscope.

Globules. Process globules presumably derive from the cytoplasmic mass, although this origin was not visibly obvious during the flurry of activity accompanying the initiation of the planozygote to hypnozygote transition. The structure, composition, and contents of the globules as well as their exact relationship to the developing cyst processes are unknown. With the light microscope, globules appear as bubbles which form the terminal structures of hollow processes. What drives the outward migration of the globules? Is this movement solely the result of adding process shaft material beneath the globules or perhaps a response to chemical and/or physical forces such as pressure acting within the cell?

The dynamic nature of globule shape, size, and position during the early stages of cyst formation suggests that some aspects of process distribution and morphology are not fixed until expansion of the cell is well underway. The degree to which distribution and morphology are directly controlled by the cell as opposed to "generic physical mechanisms" (buoyancy, surface tension, adhesion, etc.; see Newman and Comper, 1990) is unknown.

Processes. On fully developed resting cysts, the material which forms the processes is visually similar to that of the endophragm and both layers respond similarly to chemical treatments such as acetolysis. Our observations on the morphology of spine development prompt questions regarding the chemical and ultrastructural details of cyst wall formation. For example, what is the source of the dinosporin which ultimately forms the chief structural component of the processes? Is it newly synthesized during the relatively rapid deposition of processes or are precursors formed and stored in the planozygote so that the dynamics described in this report represent merely an assembly phase? The rapidity of wall formation, together with the dependence of this phenomenon on the maintancnce of an isolated and specialized environment (the expansion zone) suggest that contruction of *L. polyedrum* resting cyst cell walls may occur by the self assembly of precursor units (R. J. Miller, personal commun., 1994).

During process elongation, it is clear that the composition and/or structure of the terminal globules differ from that of the process shaft. This is supported by the reaction of these features to premature rupture of the outer membrane—terminal globules collapse and disappear while process shafts appear unaffected. At what point do process shafts and terminal structures become chemically indistinguishable and what is the nature of this transition? During uninterrupted encystment episodes (i.e., those that escape premature rupture), any unattached free-floating globules undergo collapse simultaneously with the modification of terminal globules into process tips which occurrs at least several minutes before rupture of the outer membrane. This suggests that some kind of signal, acting throughout the entire region between the inner cyst body and the outer membrane, triggers the structural and chemical modification of terminal globules into terminal process structures.

Variability of process length. Resting cysts formed in our laboratory cultures occasionally showed reduced processes on one side of the inner body. This presumably relates to cyst formation as cells rested against the bottom of the various culture vessels employed in this study (Text-Figure 1). In contrast, resting cysts recovered from sediments display a more even distribution of long and short processes suggesting that cyst formation in natural waters occurs while cells are in soft sediments or (more likely) still suspended in the water column. In the absence of external obstruction, expansion of the outer membrane and subsequent process development is likely to be symmetrical.

Rupture of the outer membrane. Thus far, outer membrane rupture has been referred to as either "normal" (i.e., releasing a morphologically mature resting cyst) or premature. Details relating to how the outer membrane actually expands and what causes it to rupture are unknown. In most encystment sequences, rupture of the outer membrane appears to be the result of this feature exceeding its physical limit of expansion, although this certainly does not rule out a mechanism involving some kind of physical and/or chemical disruption. Regarding the physical integrity of the outer membrane, it is noteworthy that encysting cells in the late stages of expansion, in spite of their delicate appearance under the microscope, were occasionally observed to withstand being rammed by thecate swimming cells.

As to the causes of premature rupture, an important question concerns whether cells are "internally" predisposed towards this phenomenon (e.g., early rupture due to aberrant physiological state of the cell) or whether the cause is external and due to a more immediate physical and/or chemical "disruptant" in the culture environment. In our study, there was no obvious correlation between any visual character of an encysting cell and its disposition to premature rupture. All observed episodes of encystment involved cells resting against the bottom of covered, motionless chambers suggesting that turbulence is not a necessary factor for premature rupture. Other possible disruptants might include heat from microscope illumination, salinity changes due to evaporation, pH imbalance, bacterial metabolites, and other contaminants in the culture medium.

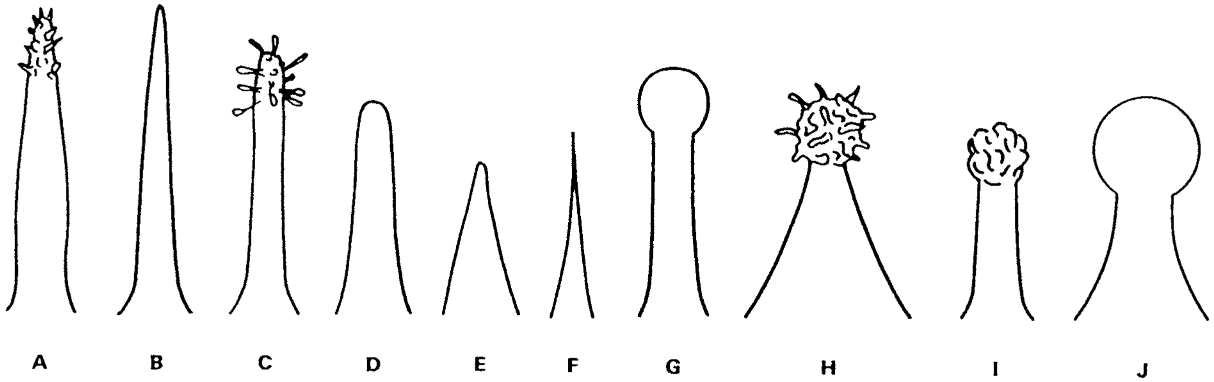
Surveys of cysts collected from the bottom of our culture tubes often showed a relatively high proportion of the population bearing truncated processes (Plate 3, fig. 7). This indicates that premature rupture is significant in both the tube environment and the chambers used for microscopic observation. Resting cysts of *L. polyedrum* recovered from modern sediments also occasionally show incomplete development of processes indicating that premature rupture of the outer membrane occurs during encystment of natural populations. A more precise statement concerning the frequency of this phenomenon in natural settings will require a quantitative analysis of resting cysts from sediments. The identity and viability of naturally occurring cysts with truncated processes has been confirmed by germinating such cysts in the laboratory, identifying the motile stages, inducing resting cyst formation, and observing the range of cyst morphology produced (unpublished observations).

Morphological Variability (Evolutionary vs. Environmental Control)

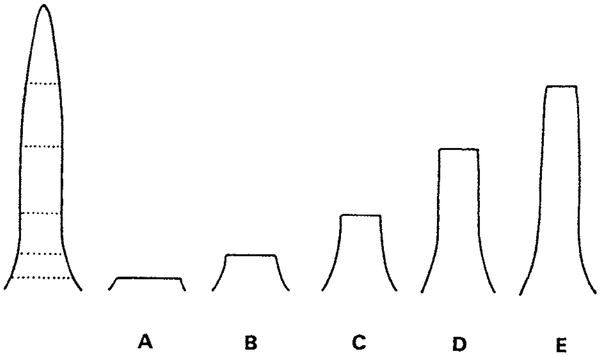
Resting cysts formed in our cultures showed an interesting range of morphology, particularly with respect to the number, distribution, and character of processes (Text-Figures 3 & 4). Typical processes were 5-10 μm long and tapered distally from a fibrous base to a rounded tip bearing short spinules. Process length varied widely, sometimes on a single specimen, and shapes ranged from conical to slightly lagenate. Terminations varied from relatively sharp points to irregular fist-like structures while others showed abrupt truncations. Some specimens lacked spines completely.

Given the degree of morphological variability of cysts formed in our cultures, it is interesting to consider similar forms encountered both in modern natural sediments and the fossil record. Traditionally, classification of dinoflagellate cysts rests primarily upon interpretation of their morphology. As a result, resting cyst "species" represent morphotypes whose true biological affinities are often unknown. The definition of how much morphological variability is validly encompassed by any given taxon is largely subjective in nature and highly dependent on the background, experience, and philosophy of the taxonomist.

Lentin and Williams (1993) list 12 species and 4 subspecies of fossil resting cysts which have been assigned to the genus *Lingulodinium*. Several of these forms appear very similar to the type species (*L. machaerophorum*) but are distinguished on the basis of the processes; indeed, Islam (1983a, p. 90) cites process morphology as "one of the main criteria for specification in *Lingulodinium*." The variation in process number, distribution, and morphology shown by resting cysts produced in our cultures of *L. polyedrum* suggests that one need not invoke additional species/subspecies to account for morphotypes such as those just mentioned. As a result, we propose the synonymy of several forms (see Systematic Paleontology, below). Although the suggestion that process morphology (particularly length) may not always be an optimal criterion for the delineation of cyst species is certainly not new, our study



Text-Figure 3. Sketches showing the variability of process morphology among *L. polyedrum* resting cysts formed in laboratory culture. Figures approximately to scale; length of longest process about 10-12 μm. Examples to the left of the figure (A-F) are fairly common, and, when present, tend to dominate the process type on any given specimen. Processes illustrated in sketches G-J are less common. All process types shown here can also be found in their "truncated form" as explained in Text-Figure 4.



Text-Figure 4. Process truncation. Figures to scale; length of longest process 10-12 µm. Interruption of normal process growth (by premature rupture of the outer membrane) at one of the points represented by dotted lines in the illustration at left would yield a corresponding truncated form as shown in A-E. Truncated processes were very common on culture-derived resting cysts of *L. polyedrum* (e.g., Plate 3, fig. 7).

lends considerable weight to this notion by providing a look at the magnitude of process variability encountered in laboratory populations.

Many investigators have suggested that some morphological variability displayed by resting cysts of *L. polyedrum* may be environmentally controlled. The bulbous process terminations described by Wall et al (1973) on *L. machaerophorum* recovered from Black Sea sediments seemed to correlate with low salinity (estuarine) environments. Turon (1984) also correlated reduced process length in this species with lower salinity. A similar suggestion was made by Dale (1988) regarding the shorter spine length shown by some *Operculodinium centrocarpum* and *Spiniferites bulloideus* from recent sediments of the Baltic region. Other variations in fossil *Lingulodinium* morphology have been noted by many investigators. Processes occasionally appear slightly flattened or bladed and, in terms of ornament, can be smooth, granular, or fibrous. Similarly, the number of paraplates involved in archeopyle formation varies from one to at least five, and may be as high as nine in some cases (J. H. Wrenn, written commun., 1987). The ability to correlate specific morphologies to environmental conditions would greatly enhance the paleoenvironmetal application of fossil dinoflagellates. To firmly establish such correlations, if they exist, requires morphological analysis of resting cyst assemblages produced in laboratory cultures grown under different environmental conditions. This approach was successfully adopted by Sandgren (1983) in a study of the effects of temperature on the morphology of chrysophycean resting cysts.

Previous Models of Resting Cyst Morphogenesis

Observations of the development of dinoflagellate hypnozygotes with multi-layered cell coverings (e.g., Pfiester, 1975 and later) have generally confirmed earlier speculation (Evitt and Wall, 1968) that wall layers form in a centripetal sense; i.e., outer layers form first and inner layers later. The development of processes and other major elements of surface relief (high ridges, septa, etc.), however, has long been a topic of debate, particularly in the paleontological literature (e.g., Dale, 1983; Gocht, 1983, 1987; Evitt, 1985; Sarjeant et al., 1987). Evitt (1961, 1985) discussed three hypotheses: (1) process formation is purely centripetal, beginning distally and proceeding inward as the protoplast contracts (Dale, 1983, refers to this as the "contractional growth hypothesis"); (2) processes are elongated in a purely centrifugal fashion, growing outward from the surface of the protoplast; and (3) process growth combines both centripetal and centrifugal mechanisms; tips form in contact with the still-intact theca and subsequent outward elongation proceeds by addition of material to process bases. Two additional possibilities are suggested by the report of Taylor and Gaines (1989): (4) process growth is neither centripetal nor centrifugal but occurs simultaneously along the entire length of the process as it polymerizes in a mucoid layer between protoplast and outer membrane, and (5) processes are constructed directly from an outer membrane (i.e., cell expansion halts, the still-intact outer membrane collapses inward and locally fuses with the underlying endophragm, and unfused portions of the membrane are modified into processes).

Although he did not directly observe resting cyst morphogenesis, Nordli (1951) speculated that *L. polyedrum* processes grew outward from the central body. This opinion was also expressed by Dale (1983) who inferred centrifugal process formation based on comparison of planozygote and hypnozygote volume in both plankton tow and cultured material. The results of the present study clearly demonstrate that processes of the *L. polyedrum* resting cyst develop in a centrifugal sense. As mentioned above, a similar mechanism was documented by von Stosch (1973) during observation of encystment by *Gymnodinium pseudopalustre*.

Although it is tempting to speculate, based on observations of these two very different extant species, that centrifugal growth may be the general mechanism of surface relief development in dinoflagellate resting cysts, we believe that such a conclusion is not warranted at this time. Processes on resting cysts of both *L. polyedrum* and *G. pseudopalustre* are randomly distributed (i.e., nontabular) over the central cyst body and a given specimen of either species often displays processes of significantly different length. This situation contrasts sharply with resting cysts of many other species both fossil and modern which clearly show tabular arrangements of surface relief, and morphology consistent with a developmental model invoking a rigid outer wall and centripetal growth. After careful morphological analysis of fossil specimens, Evitt (1985) favored a centripetal mechanism (at least in part) for many species. Some biological evidence for the potential significance of contractional growth during dinoflagellate encystment has been provided by Blackburn et al. (1989) who documented the reduced size of the *Gymnodinium catenatum* hypnozygote (a "proximate" cyst) relative to the planozygote. Finally, it is interesting to consider the comments of Taylor and Gaines (1989) regarding encystment among living species of *Gonyaulax* and *Polykrikos*. In summary, we believe that the existence of multiple developmental mechanisms among a group as diverse as the dinoflagellates is entirely possible and it remains for continued investigation of encystment in living species to shed more light on this issue.

SYSTEMATIC PALEONTOLOGY

Division DINOFLAGELLATA (Bütschli 1885)
Fensome et al. 1993
Class DINOPHYCEAE Pascher 1914
Order GONYAULACALES Taylor 1980
Family GONYAULACACEAE Lindemann 1928

Genus *Lingulodinium* Wall 1967 emend. Wall et al. 1973

Lingulodinium machaerophorum (Deflandre & Cookson 1955) Wall 1967
Plate 1, figs. 1-12; Plate 2, figs. 1-12; Plate 3, figs. 1-7

Baltisphaeridium funginum Morgenroth, 1966, p. 17, pl. 3, figs. 7-8.
Lingulodinium funginum (Morgenroth 1966) Islam, 1983, p. 341, pl. 3, figs. 10.
Lingulodinium sadoense Matsuoka, 1983, p. 124, pl. 10, figs. 1a-c, 2-3, 4a-b, 6-7.
Lingulodinium brevispinosum Matsuoka & Bujak 1988, p. 60-61, pl. 7, figs. 8-9, pl. 8, fig. 1.

Comments. *Lingulodinium machaerophorum*, first described from the Miocene of Australia (Deflandre and Cookson, 1955), is the accepted name for the resting cyst formed by the extant dinoflagellate *L. polyedrum*. Based on the range of resting cyst morphology observed during germination and culturing studies of *L. polyedrum*, the above cyst-based species are here synonymized with *L. machaerophorum*. Islam (1983b, p. 341) stated that *L. funginum* "differs from other species of *Lingulodinium* in possessing mushroom-like or distally truncate processes." Both of these process types (but especially the latter) were common on our culture-produced resting cysts. *L. sadoense* (Matsuoka, 1983) was distinguished from *L. machaerophorum* in having larger but fewer processes. This morphotype, while rare in our cultures, was common in modern sediment samples examined during the early stages of this project; germination of these forms yielded motile cells of *L. polyedrum*.

The suggestion that *L. brevispinosum* may be a taxonomic junior synonym of *L. machaerophorum* has been made previously by M. J. Head after detailed restudy of the holotype (see Head et al., 1993). According to Matsuoka and Bujak (1983), *L. brevispinosum* is similar to specimens of *L. machaerophorum* described from Quaternary Black Sea sediments by Wall et al. (1973), but differs in that processes lack spinose ornamentation. As demonstrated in the present study, spinule development on processes of *L. polyedrum* resting cysts can be highly variable (Text-Figure 3).

Other morphotypes now designated as separate species of the genus *Lingulodinium* may well be synonymous with *L. machaerophorum*, but further work is necessary to rigorously assess this possibility. Among those species deserving restudy are *L. pycnospinosum* Benedek & Sarjeant 1981; *L. siculum* Wall et al. 1973; *L. strangulatum* Islam 1983; and *L. varium* Sütö-Szentai 1986. Although the exact process types described for these species were not directly observed in our *L. polyedrum* cultures, they nevertheless appear to fall within the range of possible process morphology produced by the developmental sequence described above. From this perspective, further cyst germination and laboratory culture investigations of *L. polyedrum* are warranted.

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TABLE 1. Final composition of ES-DK medium.

	mg/L	Concentration (µM)
Major Nutrients		
1. $NaNO_3$	32.55	[N] = 382.99
2. Glycerophosphate of Na	4.65	[P] = 14.35
Trace Metals		
1. EDTA (Na_2) (2 sources)	3.860	[EDTA] = 10.37
2. Fe $(NH_4)_2 (SO_4)_2 \cdot 6H_20$	1.632	[Fe] = 4.16
3. $FeCl_3 \cdot 6H_2O$	0.114	[Fe] = 0.422
4. H_3BO_3	2.651	[B] = 42.88
5. $MnSO_4$	0.286	[Mn] = 1.89
6. $ZnSO_4 \cdot 7H_2O$	0.051	[Zn] = 0.177
7. $CoSO_4 \cdot (x)H_2O$	0.011	[Co] = 0.071
Vitamins (cf. Guillard, 1975)		
1. Thiamin · HCl	0.1	0.296
2. Biotin	0.5 µg	0.002
3. B_{12}	0.5 µg	$3.7x10^{-4}$
Seawater	1L	