A STUDY OF THE ENCYSTMENT PROCESS IN THE ZOOSPORES OF BLASTOCLADIELLA EMERSONII

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ABSTRACT

A STUDY OF THE ENCYSTMENT PROCESS IN THE ZOOSPORES OF BLASTOCLADIELLA EMERSONII

By

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The posteriorly uniflagellate zoospores of Blastocladiella emersonii measure ca. 9 x 12 u, do not possess a cell wall, and do not synthesize detectable quantities of nucleic acid or protein. The transformation of the spore into a spherical cyst-like cell marks the first stage of germination. This process is referred to as encystment. About 10 minutes later, the cyst, which ultimately grows into the thallus of the fungus, produces a germ tube that develops into a branched rhizoidal system.

The structural changes that precede germ tube formation have been followed by light and electron microscopy. At the gross morphological level, five major changes were recognized: retraction of the flagellum with concurrent rotation of the "nuclear apparatus"; formation of vesicles which appear to fuse with the cell surface; deposition of cyst wall material; change in cell shape; and decrease in cell volume. Flagellar retraction and rotation of the nuclear apparatus are viewed as a consequence of the overall structural-mechanical processes of encystment, and thus require no special mechanisms or forces not already provided by the other associated events.

At the level of fine structure, the decay of gamma particles was examined, as well as changes in other cell components. The electron dense

gamma matrix released small vesicles that fused with the gamma-surrounding membrane, while the latter released vesicles (corresponding to those observed via light microscopy) which fused with the plasma membrane. It was proposed that the decaying gamma particles function in cyst wall formation, and that analagous particles may function in a similar manner in the zoospores of other aquatic fungi.

The influences of some environmental factors on zoospores have also been investigated. During incubation at low temperatures (ca. 0-3° C), spores swelled, absorbed their flagella and eventually lysed. The rates of these events were dependent on the composition of the suspending medium. Furthermore, the longer the duration of cold incubation, the greater was the percentage of spores which encysted when the suspension was brought to a higher temperature.

Within the range 10^6 to 10^7 spores/ml., the percent of a spore suspension encysting is inversely proportional to the population density. Evidence suggests that an inhibitor released into the suspending medium may account for this. The nature of the suspending medium and pH influences the inhibition while temperature does not.

Spores can be induced to encyst by the addition of high concentrations of many simple inorganic salts, dilution, cold incubation, and sulfonic acid azo dyes. Frequently, encystment levels off at less than 100%. If the spores which encyst are treated as a separate population, the normal distribution becomes an appropriate model for describing the time course of encystment. Using the two parameters which characterize this distribution, the mean and the standard deviation, comparisons between encysting populations were conveniently made. The encystment kinetics of spores induced to encyst by KCl, cold incubation, and sulfonic acid azo dyes were compared. The mean times of encystment for the three

induction methods were independent of the final encystment level attained. The KCl-treated spores displayed a mean time of encystment about twice as great as the cold and dye treated spores.

It is suggested that the zoospore of B. emersonii is a highly ordered propagule that undergoes a cascade of rapid and integrated transformations (conveniently measured in seconds or minutes) as it encysts. The controls which maintain this system are dispersed throughout the structural and chemical organization of the cell. Perturbations of these may lead to their breakdown, which in turn may cause others to be disengaged, until the stability of the non-encysting spore is lost, and the encysting spore emerges. Extreme perturbations may, however, cause death of the cell. The various inducing agents may disrupt different control sites. How quickly these disjunctions lead to the breakdown of the remaining controls will be reflected in the mean time of encystment.

A STUDY OF THE ENCYSTMENT PROCESS IN THE ZOOSPORES OF *BLASTOCLADIELLA EMERSONII*

By

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To

Mother and Father

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TABLE OF CONTENTS

TABLE OF CONTENTS - Continued

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES - Continued

FIGURE

I. INTRODUCTION

Numerous aspects of the developmental biology of Blastocladiella emersonii have been extensively reviewed, e.g., see Cantino and Lovett (1964) and Cantino (1966). A number of discrete phenotypes are recognized, although no sexual stage has been described and all existing cultures have been derived from a single spore isolate (Cantino, 1951).

Ordinary colorless (OC) plants develop under conditions conducive to growth and sporulation. Each consists of a multinucleate cell with a branched rhizoidal system at one end. The cell grows exponentially through most of its generation time in laboratory cultures, but, eventually, the rhizoidal system is partitioned off by a cross-wall near the base of the thallus, and growth ceases. The protoplasm in the terminal cell (opposite the rhizoids) cleaves into uninucleate zoospores which, at the end of their genesis, are discharged through one or more exit pores. The spores swim for a time before they germinate and begin the cycle once again.

In the presence of bicarbonate (Cantino, 1951; Griffin, 1964), high population density (Cantino, 1951), or high concentrations of potassium (Griffin, 1964; Horgen and Griffin, 1969), sodium or ammonium chlorides (Horgen and Griffin, 1969), the resistant-sporangial (RS) plant forms. It is generally larger than the OC plant and has a much thicker, pitted, melanin rich wall. Maturation takes about four times as long as it does for the OC organism, and it is not accompanied by spore formation.

Instead, the RS cells remain dormant until induced to sporulate by the correct environmental conditions. The RS pathway has been extensively investigated over the last two decades by Cantino and coworkers; this work is summarized in the two reviews cited above.

Aside from the major OC and RS phenotypes, two minor ones are also recognized: late colorless (LC) and orange (O). These usually comprise a small percentage of an OC population, and both are slower growing than the OC type. The O cells remain colorless through most of their development but turn orange at sporogenesis. They produce zoospores smaller than those from OC and LC plants and of extremely low viability. On the other hand, LC plants remain colorless and produce zoospores with a viability comparable to those derived from OC cells. The major difference between LC and OC cells is the slower growth rate of the former.

To account for the phenotypic variation (OC, O, LC) in the absence of apparent genetic variation, Cantino and Hyatt (1953) postulated that the random distribution of a cytoplasmic factor was responsible. Using an orange mutant of Blastocladiella (BEM) which produced only orange offspring, Cantino and Horenstein (1954) were able to demonstrate a transfer of mutant characteristics among the cells in a mixed population of mutant and wild type zoospores. This was correlated with the appearance of cytoplasmic bridges, too small to permit the transfer of nuclei, between wild type and mutant spores. In 1956 the above authors (Cantino and Horenstein, 1956) reported that they had "...observed small [ca. 0.5 u] deeply-staining, cytoplasmic particles whose distribution among swarmers derived from plants of BEM and from the O, OC, and LC, and RS plants of B. emersonii corresponds to that predicted for our hypothetical cytoplasmic factor, 'gamma'...." Counts indicated that zoospores from RS and OC cells

contained an average of 12.5 particles/spore, LC spores contained an average of 15.5 particles, and - spores contained an average of 7.5 particles. The contents of orange mutant spores, 8.0 particles/cell, corresponded closely with the average number found in wild type O spores. Since the small cytoplasmic particles behaved in a manner compatible with the gamma factor hypothesis, they were named gamma particles.

The only class of propagules produced by Blastocladiella is zoospores. Those of OC, LC, and RS plants display no morphological differences when examined with the light microscope, except for the average number of gamma particles each contains. There have been no comparative electron microscope studies. As mentioned previously, however, the zoospores of O cells are smaller, show a slight orange pigmentation, and are of much lower viability than the others. Because the spores from RS, LC, and O plants are difficult to obtain in sufficient quantities for many experimental procedures, the zoospores derived from OC cells are most commonly used.

Zoospores are unlike most spore types. They are rather frail and ephemeral, and possess no obvious resistive capacity against adverse environmental conditions. Those of B. emersonii have an endogenous Q_{O_2} as high as ca. 50 to 100 (McCurdy and Cantino, 1960; Cantino and Lovett, 1960) ranking well with that of many vigorously metabolizing organisms. The two factors which probably warrant this high respiratory rate also place the zoospores in a unique position among other spore types: they spend much of their time swimming actively, and they are constantly battling with the aquatic environment to maintain osmotic balance because they are not provided with a cell wall.

On these and other bases, it could well be argued that they are not dormant cells. But, if we accept a definition of dormancy such as Sussman's (1965; p. 934), namely, "any rest period or reversible interruption of the phenotypic development of the organism," then the zoospores of B. emersonii have to be labeled dormant. They do not "grow," they do not display pronounced morphological changes (other than amoeboid movements), they do not synthesize detectable amounts of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), or protein (Lovett, 1968; Soll, 1969,); in fact, they actually consume protein as well as polysaccharide, as they swim about (Suberkropp and Cantino, unpublished data). Thus, while keeping most if not all of their biosynthetic machinery shut down, the zoospores must simultaneously maintain energy producing pathways in operative condition.

The first gross morphological change in germination is the conversion of the zoospore into an essentially spherical, cyst-like cell. This is accompanied by retraction of the single flagellum into the main body of the spore, loss of motility, extensive changes in fine structure (Lovett, 1968; Soll et al., 1969, Truesdell and Cantino, 1970), increase in oxygen uptake (Cantino et al. , 1969), and onset of macromolecular synthesis (Lovett, 1968; Schmoyer and Lovett, 1969). These transformations occur so rapidly that they can be measured conveniently in seconds or minutes. This process will be referred to as "encystment."

A small germ tube subsequently emerges from the cell, and later gives rise to a branched rhizoidal system. The cyst itself enlarges and eventually becomes a large, multinucleate, coenocytic thallus.

It is the encystment process that is the focus of attention in this thesis. In sections I-VII, the structural changes and other coordinated

events which together comprise encystment are presented. Special attention is devoted to the interpretation of the structural interrelationships involved. In sections VIII-X the influence of exogenous factors on encystment is presented with the hope of elucidating some controls and mechanisms of the process. A large portion of these data has resulted from exploratory experiments performed to develop methods for manipulating and maintaining zoospores.

II. STRUCTURE OF NON-GERMINATING ZOOSPORES

Since structural changes play a very important role in encystment, selected aspects of the internal make-up of zoospores must be summarized briefly at the outset to provide essential background.

A. GENERAL APPEARANCE

A zoospore is about 7 x 9 u in size, and propels itself with a single posterior, whiplash flagellum. The cell does not possess a wall, but it is delimited by a single, continuous, unit membrane. My measurements indicate that it is ca. 90-100 A thick, a value typical of many other plasma membranes (Fawcett, 1966). The lack of a cell wall and plasticity of its plasmalemma permit the zoospore to take on a continuum of quickly changing, undefinable shapes and amoeboid characteristics. During these changes in contour, the cellular organelles, with the exception of gamma particles, do not significantly change in their relative positions to one another within the cell. The gamma particles may move throughout all areas of the cytoplasm, but in amoeboid spores they are usually confined to the posterior portion of the cell, and in swimming spores, to the anterior regions.

B. FINE STRUCTURE

The first electron micrographs of thin sections through zoospores of B. emersonii were made in 1963 (Cantino et al., 1963); this was followed with additional descriptions of their fine structure by Reichle and Fuller (1967), Lessie and Lovett (1968), Soll et al. (1969), Cantino

(1969), Cantino and Mack (1969), Truesdell and Cantino (1970), and Cantino and Truesdell (1970). Figure 1 is a diagrammatic representation of the zoospore, and is supplemented with a few electron micrographs (Figures 3, 4, 5, 6). The important structural features are as follows:

1. The nucleus and nuclear cap.

The nuclear cap (NC, Figures 1, 2, 3) partially encircles the nucleus and is the most massive single structure in the spore. It is an aggregate of basophilic particles (Turian, 1962) identified as ribosomes (Lovett, 1963), and is entirely delimited by a system of double membranes (i.e., two parallel unit membranes), parts of which are continuous with the nuclear membrane and other structures. The double membrane separating nuclear cap from nucleus contains a network of evenly distributed pores found in no other region of the nuclear membrane.

2. The kinetosome, flagellum, and banded rootlet.

Two centrioles (Figure 5) of different lengths (Reichle and Fuller, 1967; Lessie and Lovett, 1968; Soll et al., 1969) are located at the posterior end of the nucleus; the longer one, the kinetosome (K, Figure 1, 4; see Cantino et al., 1963), is continuous with the flagellar axoneme. Perpendicular to the long axis of the kinetosome, and in intimate contact with it, is a banded rootlet (R, Figure 1, 5). Although it had been thought (Reichle and Fuller, 1967) that three such rootlets were present, it now seems likely (Cantino and Truesdell, 1970) there is only one, it being bent at the point along its length where it makes contact with the kinetosome, with its two "arms" extending into open-ended channels in the mitochondrion.

3. The single mitochondrion.

The single mitochondrion (M, Figure 1, 3) is situated asymmetrically around part of the nuclear apparatus (nucleus plus nuclear cap) where it also surrounds the kinetosome. Sometimes, especially in amoeboid spores, the portion of the mitochondrion nearest the spore's anterior is flattened (Arrow, Figure 4), almost devoid of cristae, and exceptionally rich in particles similar in size and staining properties to nuclear cap ribosomes; many of them are aligned along the inner mitochondrial membrane. Usually, such particles are also found in the mitochondrion where it contacts lipid bodies.

4. Lipid bodies, SB matrix, and backing membrane.

The lipid bodies are dispersed along the outer surface of the long "arm" of the mitochondrion, and usually in intimate contact with it. Molded against them is an organelle bound by a unit membrane, the SB matrix (SB, Figure 1, 2, 3). Although originally viewed as a collection of individual SB bodies, serial sections suggest (Cantino and Truesdell, 1970) that they are part of a continuous structure. The SB matrix is confined to a region around the lipid bodies, does not obstruct the openings to the two mitochondrial rootlet channels, and consists of a granular to amorphous substance of moderate electron density; its composition is unknown.

A sheet of double membrane, the backing membrane (BM, Figure 1, 2, 4) covers the SB-lipid-mitochondrial complex and is attached at several places to the outer unit membrane of the nuclear apparatus (Figure 1, 2). The innermost portion of the backing membrane stains intensely with OsO_4, UO_2^{++}, or Pb^{++} in those areas adjacent to the SB matrix; in other regions, it does not. Portions of the backing membrane also enter into and extend along the surfaces of the mitochondrial rootlet channels.

5. Gamma particles.

These cytoplasmic organelles (G, Figure 1, 3) undoubtedly correspond to the "gamma" particles described (Cantino and Horenstein, 1956), and recently reinvestigated (Matsummae et al., 1970) by way of light microscopy. The gamma particle consists of two major components (Figure 27). A unit membrane (the gamma surrounding membrane, or GS-membrane) which encloses an ellipsoid, bowl-shaped matrix (gamma matrix) about 0.5 u in length. The matrix is tightly packed with amorphous and membranous osmiophilic material (Figure 6) (Truesdell and Cantino, 1970). Cantino and Mack (1969) provide a detailed, three-dimensional description of the gamma particle, All available evidence (Myers and Cantino, in press) suggests that this organelle contains DNA.

6. Other cytoplasmic inclusions.

The cytoplasmic ground substance is homogeneous and contains numerous, rather evenly dispersed, polysaccharide particles (Lessie and Lovett 1968), and a few vesicles. Aggregations of particles ("satellite ribosome packages"; Cantino, 1969; Cantino and Mack, 1969; Shaw and Cantino, 1969) identical in size and staining properties to nuclear cap ribosomes, and surrounded by a double membrane, appear with high frequency in amoeboid spores.

FIGURE 1. Diagrammatic view of B. emersonii zoospore. For purposes of clarity, the relative proportions of some structures are exaggerated, while a few ingredients (e.g., the second, short centriole, satellite ribosome packages, etc.), are not included. Overall dimensions of spore are approximately 7 x 9 u. Abbreviations: V, vacuole; M, single mitochondrion; BM, backing membrane; SB, side body; L, lipid body; K, kinetosome; P, prop (following terminology of Olson and Fuller, 1968); F, flagellum; R, rootlet; G, gamma particle; NC, nuclear cap; N, nucleus.

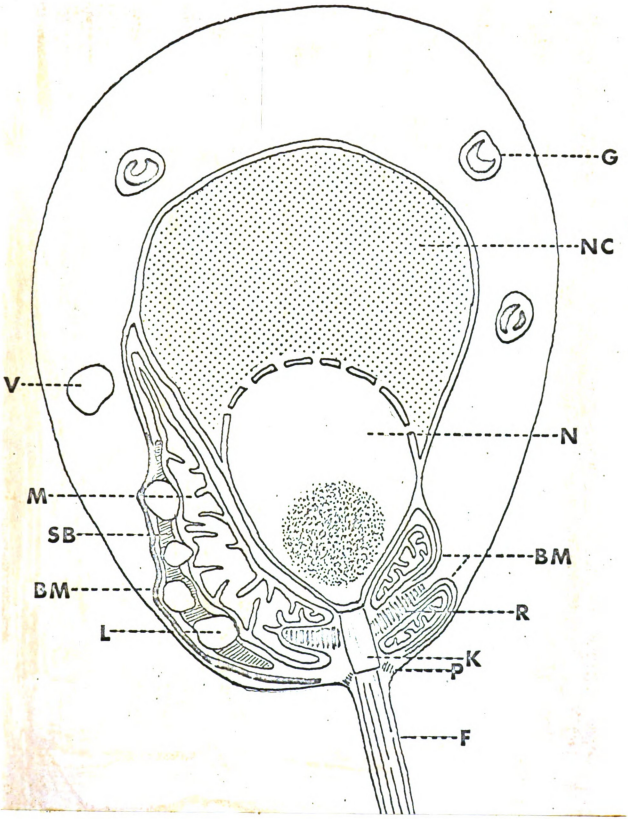
G
NC
V
N
M
SB
BM
BM
R
L
K
P
F

FIGURE 2. Relationship of components associated with nuclear apparatus. A. SB matrix (SB) with lipid particles "embedded" therein. B. Position of L, SB, and mitochondrion (M) along posterior end of nuclear apparatus: NCOM = nuclear cap outer membrane, R = rootlet canal in M, K = kinetosome. C. The manner in which the backing membrane (BM) is attached to the NCOM and covers the structures shown in B. (Figure copied from Cantino and Truesdell, 1970.)

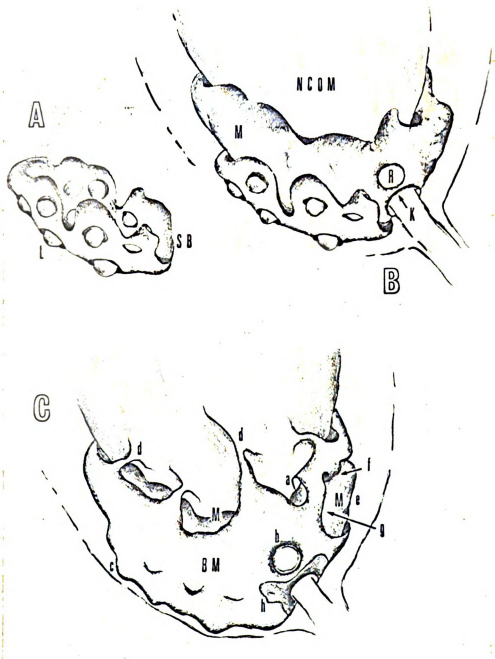
A
NCOM
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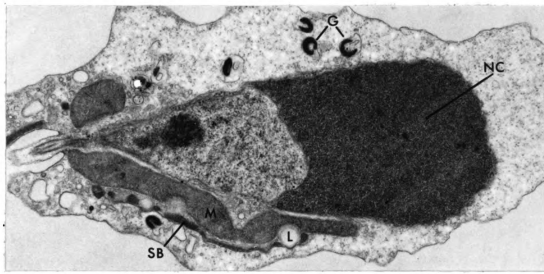
FIGURE 3. Amoeboid zoospore. Longitudinal section displaying many of the structures diagrammed in Figure 1; nuclear cap (NC), mitochondrion (M), gamma particles (G), lipid (L), sb-matrix (SB). Fixation 1, ca. 10,000X.

FIGURE 4. Posterior portion of zoospore. Lipid (L), sb-matrix (SB), backing membrane (BM), kinetosome (K), axoneme (A). Fixation 1; 35,000X.


G
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M
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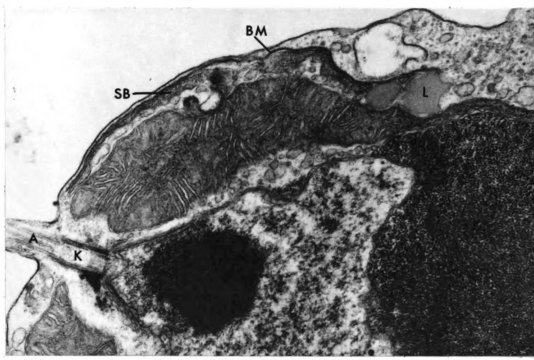
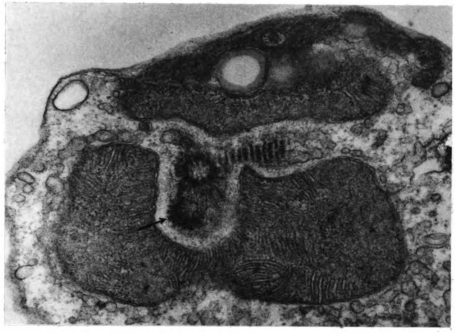
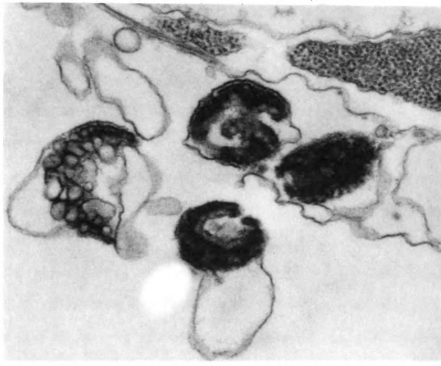

BM
SB
L
A
K

FIGURE 5. Relationship of rootlet, kinetosome, and centriole. This micrograph shows a portion of the banded rootlet passing from the kinetosome into a mitochondrial channel. Arrow points to second centriole. Fixation 1; ca. 50,000X.

FIGURE 6. Gamma particles from lysed zoospore. After spore lysis the membranous component of the gamma matrix is easily visable. Normally, this membrane is masked by the intensely staining, compact gamma matrix (see Figure 2). Fixation 1; ca. 60,000X.





III. BEHAVIOR OF THE SPORE DURING ENCYSTMENT

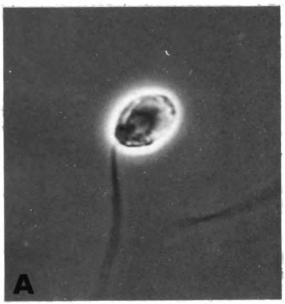
As mentioned previously, two basic spore "types," swimming and amoeboid, are recognizable under conditions compatible with zoospore maintenance. These types differ with respect to flagellar activity and the general conformation of the spore body. Swimming spores propel themselves with planar waves of lateral displacement along their flagella (Miles and Howill, 1969), while no propulsive force results from the erratic and slow lashing motion of the flagellum of amoeboid spores. The body of the swimming spore almost always conforms to an egg-like shape (Figure 7, A); the amoeboid body usually exhibits a more elongate form (Figure 7, B), or less frequently, a non-descript "lumpy" contour (Figure 7, C).

An individual spore may alternate between the two types, but a population generally consists mainly of one or the other depending on environmental conditions. It is my impression that all spores which germinate pass through an amoeboid condition. This has also been observed by Soll and Sonneborn (1969) for cells encysting in their "germination solution." The following events comprise encystment at the gross morphological level as it commences from the amoeboid state.

A. FLAGELLAR RETRACTION AND ROTATION OF THE NUCLEAR APPARATUS

These two intimately linked phenomena, previously described in detail (Cantino et al., 1963, 1968), are summarized below and supplemented with observations not reported earlier. Figures 8 and 9 provide

FIGURE 7. Phase contrast micrographs of the B. emersonii zoospore. A.) Swimming spore. B.) Elongate amoeboid spore. C.) Irregular amoeboid spore. ca. 2,300X.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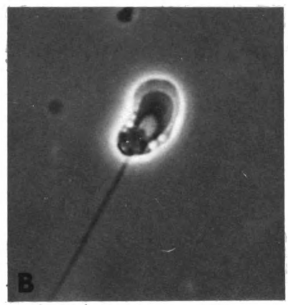
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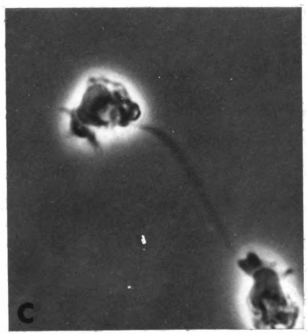

C

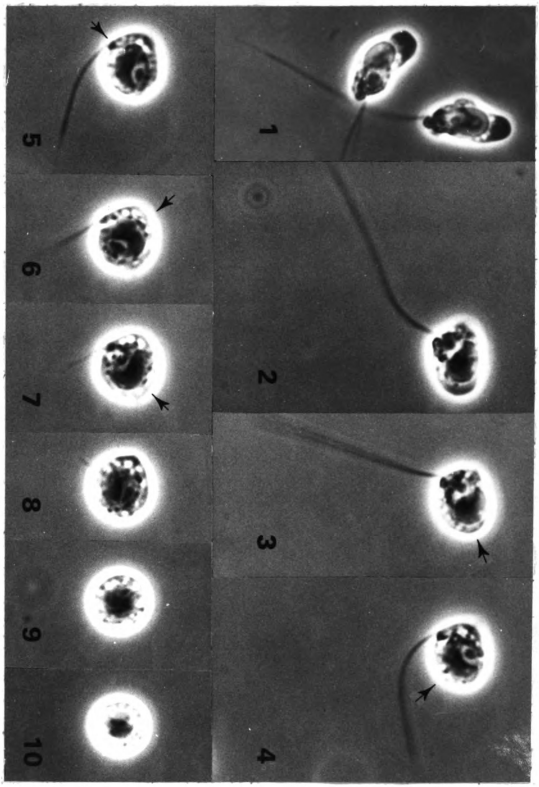
a time lapse series of photomicrographs (see methods) of zoospore encystment to accompany the description.

When the time for encystment draws near, the spore gradually becomes spherical. Figure 8, frame 2, shows the spore just as it is coming out of the amoeboid state. Usually all flagellar activity has ceased, although the flagellum may occasionally swing slowly back and forth or curl to one side. Eventually the flagellum straightens out, vibrates rapidly (Figure 8, frame 3), and stops, all usually within a few seconds. The nuclear apparatus shifts slightly toward the spore's anterior, then begins to rotate in the direction of the short arm of the mitochondrion. As rotation proceeds, the extended flagellum sweeps into an arc, following the path of rotation (Figure 8, frame 4). The nuclear apparatus continues turning until the entire axoneme has been retracted through a fixed locus, the original point of entry on the spore's surface. Thus, the body of the spore does not rotate simultaneously; it simply becomes progressively more spherical until, eventually, it resembles an encysted cell. It may take only a few seconds or longer than a minute (depending on conditions) for the flagellum to disappear, and an additional ninety seconds or so for the cell to attain its final cyst-like morphology. By the end of this short interval, an initial cyst wall is already detectable in electron micrographs, as has already been observed by Lovett (1968) and Soll et al., (1969). Some ten minutes later, a small germ tube emerges from which the rhizoidal system eventually develops.

B. VACUOLE FORMATION

In actively amoeboid spores, vacuoles may be present along the nuclear cap or at the posterior end of the cell. When the cell begins to lose its amoeboid features prior to flagellar retraction, vacuole-like structures become more numerous (Figures 8, 9). As encystment progresses,

FIGURE 8. Time series of a spore retracting its flagellum. As the flagellum is retracted, the nuclear cap rotates within the spore (follow arrow which points to apex of nuclear cap). Frame 1 shows the typical appearance of elongate amoeboid zoospores. As encystment commences, the spore begins to take on a more spherical shape (frame 2); the flagellum straightens and vibrates (frame 3); followed by flagellar retraction and concurrent rotation of the nuclear apparatus (frames 4-8). A cyst-like cell results (frame 9, 10). Throughout the process numerous vacuoles are observed in the cytoplasm, some of which disappear at the spore surface. Frames 2-8 were taken at ca. 7 second intervals; frame 8-10 ca. 30 second intervals. Spore was induced to encyst by cold incubation (see Section V). ca. 2,000X.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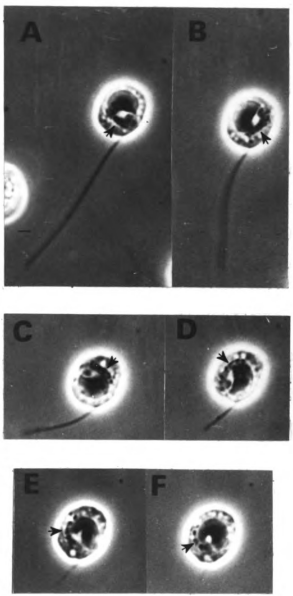
10

FIGURE 9. Zoospore retracting flagellum. Shows rotation of the nuclear apparatus during flagellar retraction induced by biebrich scarlet (see Section V). Arrow points to what is probably the mitochondrion being swept along with the rotating apparatus. A vacuole in frame E and F (bottom of spore) is fusing with the surface. ca. 1,600X.


A
B
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D
E
F

the vacuoles move to the spore surface and appear to fuse with it. The fact that gamma particles are frequently observed *in vivo* in close association with vacuoles led to early speculations that such vacuoles arose from gamma particles. This idea will be developed further in Sections V and VI, B.

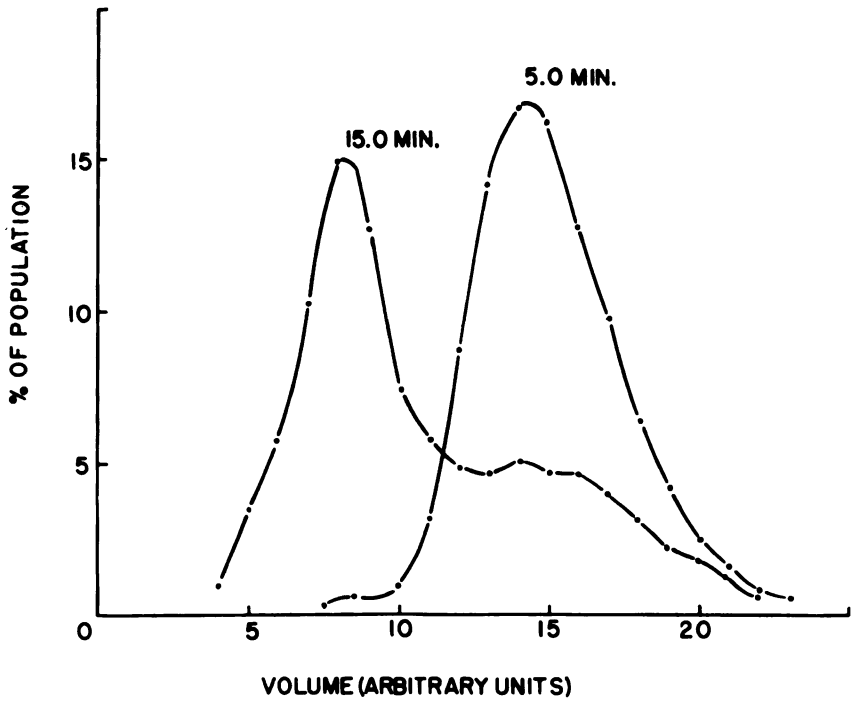
During encystment, cells take on adhesive properties (Cantino *et al.*, 1968; Soll *et al.*, 1969), *i.e.*, spores begin to adhere to one another or to their containers. This occurs at about the time vacuoles migrate to the spore surface. If spore suspensions are agitated during this period, cumulative collisions among encysting spores give rise to increasingly large clumps that may contain up to 100 or more cells. After encystment, such clumps are not broken up by very strong agitation, even in the presence of high concentrations of urea, sodium chloride, or mercaptoethanol.

C. VOLUME CHANGES DURING ENCYSTMENT

When observing encystment through the light microscope, I received the impression that spores decrease in volume. Yet, this might have been illusory because changes in spore shape were occurring simultaneously. In order to test the reality of this observation, the volumes of encysting zoospores were monitored by a Coulter Counter equipped with a size distribution plotter (see methods).

A spore suspension was induced to encyst by dilution into a NaCl-KCl solution, and the distribution of spore volumes was determined after 5 and 15 minutes. At 5 minutes no spores had encysted; at 15 minutes, about 86% had encysted. The size difference is obvious (Figure 10). At 5 minutes, the mode for the population is 14.5 volume units; at 15 minutes, it is 8.2 volume units. Thus, under these experimental conditions, there is a ca. 43% volume decrease during encystment.

FIGURE 10. Change in cell volume during encystment, as measured with a Coulter Particle Size Distribution Plotter. Distribution of spore volumes at 5 and 15 minutes after inducing encystment by diluting a freshly harvested spore population with a 2 mM NaCl-KCl solution to 5.7×10^4 spores/ml.


5.0 MIN.
15.0 MIN.
% OF POPULATION
15
10
5
0
5
10
15
20
VOLUME (ARBITRARY UNITS)

A small bimodal component in the 15 minute curve is positioned beneath the 5-minute peak. One of these minor modes, located in 14.2 volume units, represents spores which did not encyst. The other minor model is there because a few encysted spores adhered to one another; note that is is positioned at about twice the relative volume of encysted spores. The slight displacement of these two minor modes toward one another, and away from their theoretical values, results from the summing effect of the overlapping distributions.

D. RESUMÉ OF MAJOR STRUCTURAL CHANGES IN ENCYSTMENT

I regard the structural changes described in Section III, A to III, C as major transformations in zoospore encystment, i.e., retraction of the flagellum, formation of vacuoles, deposition of cyst wall material, change in cell shape, and decrease in cell volume. These processes must be rooted in cellular chemistry and physics; but, to achieve some understanding at these levels, the relevant structural interrelationships among them must first be comprehended. In the following section, these events are examined in more detail.

IV. MECHANICS OF FLAGELLAR RETRACTION AND ROTATION OF THE NUCLEAR APPARATUS

After observing encystment *in vivo* in hundreds of spores of *B. emersonii*, and examining their fine structure in detail (Section V), it is my impression that retraction of the flagellum and concurrent rotation of the nuclear apparatus can be rationalized as a purely mechanical process. I argue as follows:

The portion of the flagellum that extends outward from the rest of the spore body is composed of two major parts: the axoneme and the membranous axonemal sheath. During retraction and rotation, the axoneme coils up along the inside periphery of the encysting spore. This can be seen with phase optics under optimal conditions, but the most conclusive evidence has been seen in electron micrographs of newly encysted spores (Lovett, 1967b; Reichle and Fuller, 1967; Soll *et al.*, 1969). After the axoneme has been withdrawn, the membranous sheath is no longer associated with it (See Figure 18, Section V, D). Since the sheath is continuous--and probably identical--with the plasma membrane, it seems highly likely that the membranous sheath *is retained as part of the plasma membrane after flagellar retraction*. This conclusion is indirectly strengthened by the fact that the axonemal sheaths in some other water molds also seem to have a similar fate, judging from the observations of Meir and Webster (1954) who noted that hairs on the flagella of primary zoospores of some Saprolegniaceae seem to appear on the cysts derived from them, and the conclusion of Fuller and Reichle

(1965) that laterally projecting "flimmer filaments" (mastigonemes) attached to the axonemal sheath of Rhizidiomyces apophysatus are subsequently observed on part of the cyst surface following flagellar retraction.

I should emphasize that the flagellar axoneme is never outside the cell, but is always contained within by the continuous plasma membrane. Thus, it is not necessary to envision a mechanism with which the flagellum is drawn inside the spore, as it is already there. In light of the argument being developed, it would be more logical to speak of axonemal translocation than of flagellar retraction.

In B. emersonii, the axoneme is translocated only and always when the nuclear apparatus is rotating. The two structures probably remain connected by some means throughout the process (supporting evidence for this comes from electron microscopy; Section V, C). In such a linked device, the force responsible for the simultaneous translocation and rotation could theoretically be applied at either "end" of the system, e.g., a force that causes the nuclear apparatus to rotate would, in effect, wind in the axoneme or, conversely, a force applied to the axoneme would, in turn, push the nuclear apparatus in its circular path. It is the latter alternative that appears most reasonable.

During encystment, the spore is assuming a spherical shape, i.e., its surface area is being minimized with respect to its volume, and its volume is decreasing. Forces must be operating which oppose any extension of the cell surface, i.e., the membrane extending around the protruding axoneme. These forces may cause the membrane around the flagellum to assume a new position confluent with the increasingly spherical contour of the cell. Axonemal translocation and rotation of the nuclear apparatus will be the result.

However, there is evidence for the presence of binding sites between the nuclear apparatus and the plasma membrane which must first be broken before rotation can occur. The plasma membrane is closely associated with the electron scattering portion of the backing membrane which, in turn, is continuous with the outer unit membrane of the nuclear apparatus (Cantino and Truesdell, 1970). Furthermore, an amorphous substance (P, Figure 1) connecting the kinetosome with the plasma membrane at the base of the flagellum is visible in B. emersonii (as well as in B. britannica, other water fungi, and even an alga ; Cantino and Truesdell, 1970; Olson and Fuller, 1968; and Olson and Kochert, 1970, respectively). If all these binding sites are broken, the nuclear apparatus--axoneme assemblage would presumably "float" without restraint and be free to rotate.

If the causal force of rotation is being transmitted via the axoneme, rotation should not be detected in those spores which do not possess a flagellum. Therefore, zoospores were deflagellated by rapidly bubbling air (see methods), and examined for rotation of the nuclear apparatus during encystment.

Rotation was never observed in any of the deflaggelated zoospores (Table 1). Figure 11 shows a time series of such a spore encysting (the arrow points to the anterior end of the cap). Throughout encystment, the cap remains essentially stationary, although a very slight shift does occur between frames 2 and 3. A similar shift (or, more accurately, a very slight rocking motion) was observed in other deflaggelated spores examined, and may signify the breakdown of the binding site holding the nuclear apparatus to the plasma membrane. The changing shape of the zoospore may also be responsible for some of the movement.

FIGURE 11. Encystment of deflagellated zoospore. Encystment proceeds normally except for the lack of rotation of the nuclear apparatus (and flagellar retraction). Arrow points to the apex of the nuclear cap. ca. 4,000X.

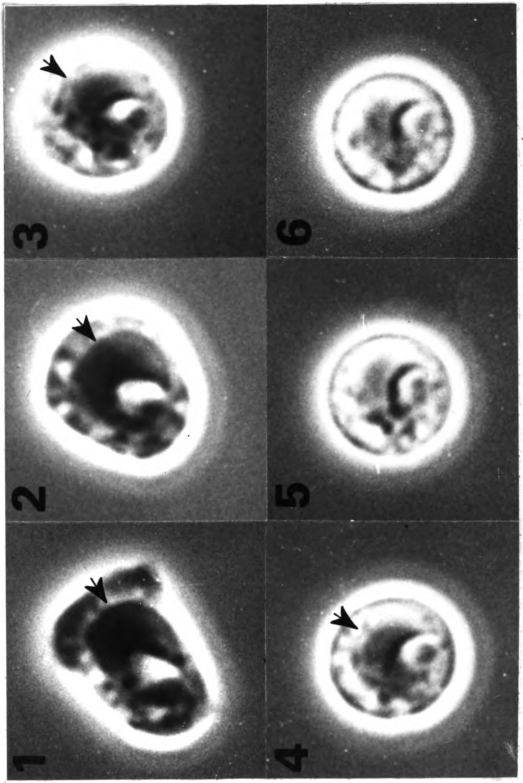

1
2
3
4
5
6

Table 1. THE EFFECTS OF MECHANICAL DEFLAGELLATION ON ZOOSPORES.

Characteristic	Control Population	Aerated Population
Possessed flagella	100%	39%
Rotation of nuclear apparatus	all of 10	none of 10
Viable count/plate	137	131
Clones from viable spores	93	92

Deflagellated cells also showed no decrease in viability over flagellated cells from the same initial population (Table 1), suggesting that severe injury resulting from the procedure was unlikely and, furthermore, that retention of the axoneme is unnecessary for the completion of normal growth and development after encystment.

The results of these experiments clearly provide supporting evidence for the mechanical explanation of retraction and rotation that has been advanced in this section. I conclude, therefore, that flagellar retraction is simply an integral part of the overall structural-mechanical process of encystment, and requires no special mechanisms, or forces, not already provided by the other associated events.

V. FINE STRUCTURAL CHANGES DURING ENCYSTMENT

This section delineates the fine structural changes that occur from the time spores are induced to germinate to the time the primary rhizoid, or germ tube, is formed. Although an integrated view of all recognizable changes is presented, events involving gamma particles are given major emphasis. Two observations about these particles elicited questions which provided the basic motivation for this study. Phase microscopic evidence (Section III, B) suggested that gamma particles were associated with vacuole formation during encystment; and Lessie and Lovett (1968) reported that the particles were absent from growing cells, i.e., they must disappear during or shortly after zoospore germination.

For the study, spores were induced to encyst by a variety of methods, outlined in methods Section G and discussed in Section VIII. Samples of spore suspension were fixed by any one of five fixation methods (see methods) at 2-4 minute intervals from the time of induction to the time of germ tube formation.

All spores examined displayed the same structural changes during germination regardless of the method by which encystment was induced. Slight differences in the appearance of zoospores fixed by different methods resulted from the varying ability of a fixative to preserve specific structures; but these, except for the formation of myelin-like figures, were not sufficiently serious to influence the interpretation of structural changes.

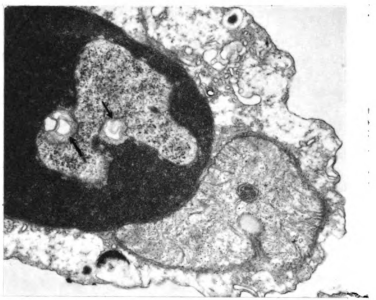
A. FORMATION OF MYELIN-LIKE FIGURES

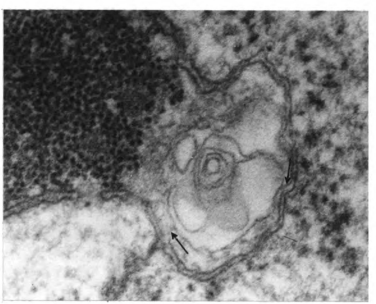
Glutaraldehyde-post osmium tetroxide is a standard fixative used by electron microscopists. When it was used on spores induced to encyst (by cold treatment or biebrich scarlet), myelin-like figures were frequently observed. These appeared along the cap side of the double membrane separating the nucleus from the nuclear cap (Figure 12, 13), within the first few minutes after induction. Some sections were obtained in which the membranous material of the myelin-like figure was continuous with the inner membrane of the nuclear cap double membrane (Figure 13). As the time of flagellar retraction approached, the figures began to appear outside of the cap as well; most of these were now near the plasmalemma, frequently contained in vacuoles, and apparently about to break through the spore surface.

This sequence of events led to the supposition that the myelin-like configurations represented the migrating, vacuole-like structures (Section III, B) observed by phase microscopy in germinating spores. However, this notion was soon confused by the fact that no such configurations were ever observed leaving the nuclear cap for the cytoplasm. By extending the wash period normally used between glutaraldehyde and osmium tetroxide fixations to 24 hours, the incidence of myelin-like figures was greatly reduced. When new combinations of fixatives were used (see methods, Fixatives I, II, III), the figures were always absent. Consequently, I have now consigned them to the rank of artifact. Nevertheless, this does not deny their possible significance as indicators of real changes in spores prior to and during encystment; their formation could well result from phospholipid released during cytomembrane alterations. (For a discussion of the origin and significance of myelin-like bodies in other organisms, see Anderson and Roels, 1967, and references therein.)

FIGURE 12. Two myelin-like figures. The two figures (arrows) along the periphery of the nucleus are actually on the cap side of the nuclear membrane. Fixation IV, IP-1, 16,000X.

FIGURE 13. High magnification view of myelin-like figure. Figure may be continuous with inner membrane of nuclear cap (arrows). Fixation IV, IP-1, 150,000X.





B. STRUCTURAL CHANGES PRIOR TO FLAGELLAR RETRACTION

During germination, a major re-ordering of cellular architecture and a breakdown of compartments occurs, yielding the less rigid structural arrangements that seem necessary to accommodate the more dynamic states of germination and growth.

The first changes detected after induction are in the backing membrane. To reiterate, this membrane is joined to the outer membrane of the nucleus-nuclear cap and covers the mitochondrion-sb-lipid complex (Section II, A, 4). It consists of two portions: one undifferentiated (typical double membrane) and one differentiated (the interior of the double membrane is filled with an osmiophilic substance). The undifferentiated portion of the membrane begins to fragment after induction of germination (Figure 14, arrows) and continues until only a layer of vesicles remains. The differentiated portion of the backing membrane does not fragment as quickly, but is slowly consumed as vesicles bud from its periphery (Figure 14; 15C; broken arrows).

Recall that the backing membrane is one of the focal points envisioned as a possible "binding site" (Section IV) between the nuclear apparatus and plasma membrane. Thus, it would have to be broken before rotation of the nuclear apparatus occurs--and this is observed. Neither the differentiated nor the undifferentiated portion has ever been found after normal flaggelar retraction.

As the backing membrane is breaking down, changes can also be detected in the gamma particles. The membrane surrounding the gamma particles (GS membrane), in contrast to its somewhat spherical appearance in non-germinating spores, is now amorphic (Figures 14, 15, A, B, C, D) and frequently gives the impression of budding off vesicles. At times it extends so irregularly through the cytoplasm that it can only be traced

FIGURE 14. Spore 3.5 minutes after induction of encystment. Backing membrane is vesiculated (arrows) and GS-membrane is amorphic. Fixation II, IP-3, 30,000X.

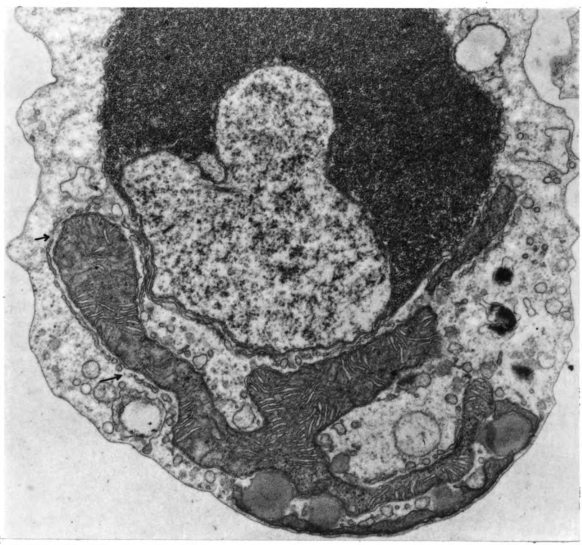
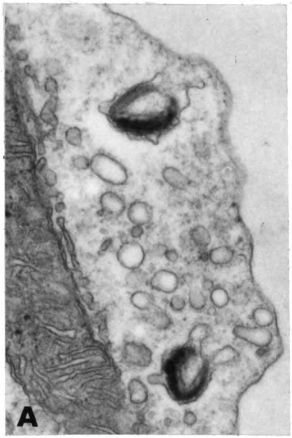
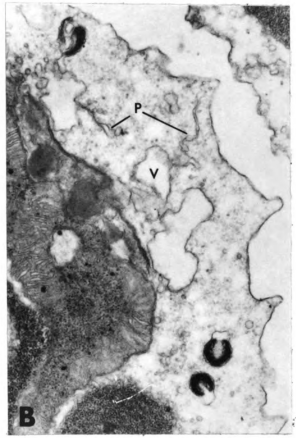
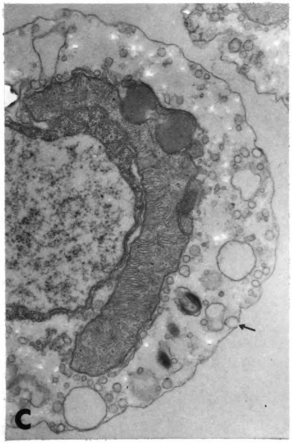


FIGURE 15. Behavior of GS-membrane prior to flagellar retraction. A.) GS-membrane giving the appearance of budding vesicles. Note the numerous vesicles in the surrounding cytoplasm. Fixation I, IP-3, 40,000X. B.) Gamma particle with amorphic GS-membrane. Narrow passages P are connected to large vesicles V. Fixation III, IP-3, 24,000X. C. and D.) Vesicles fused with plasma membrane (arrows). Fixation II, 17,000X and Fixation I, 45,000X, respectively. IP-3.


A


P
V
B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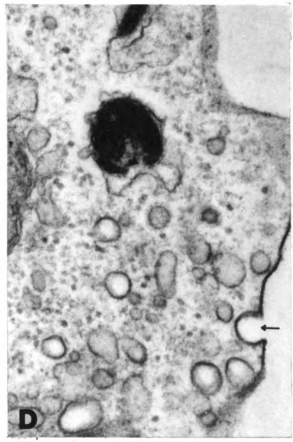

D

to its full extent via serial sections. Long narrow vesicular passages (Figure 15, B) are likely to be continuous with the GS-membrane. Concurrent with the amorphic behavior of the GS-membrane, numerous vesicles begin to appear in the vicinity of the gamma particles (Figure 15, A, D). Some of these, in serial sections, are continuous with the gamma surrounding membrane; others are not. The non-continuous vesicles frequently fuse with the plasma membrane (Figure 15, C, D); their proliferation and fusion paralleling in time the similar observations made via phase optics.

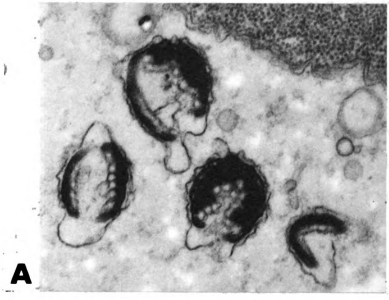
Along the inner surface of the gamma particles, numerous small vesicles are visible, partially embedded in the matrix (Figure 16, A, B). These are also observed, although less prominently, along the gamma matrix of non-germinating zoospores. Their major component resolves into the characteristic unit membrane structure of electron micrographs. A few vesicles of similar size, but free from the matrix, may also appear within the confines of the GS-membrane. Most likely these originate from the matrix.

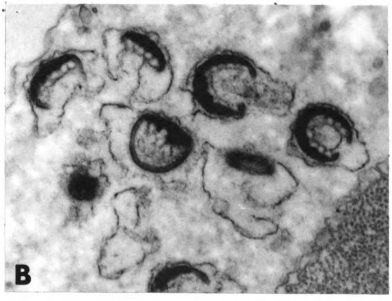
Sometimes the GS-membrane extends inward and contacts the small matrix-vesicles (Figure 16, A, B), possibly fusing with the "free" ones. In this manner it could replenish itself of material lost to budded vesicles. At times, more frequently in the later stages of germination, the free matrix-vesicles become trapped within the larger vesicles budded by the GS-membrane. The result is the formation of multivesicular bodies (MVB), single large vesicles containing many smaller vesicles. MBV are most common just after flagellar retraction (Figure 19).

C. STRUCTURAL CHANGES DURING FLAGELLAR RETRACTION

Of the many hundreds of electron micrographs examined during the course of this study, only two contain a profile of a zoospore that may

FIGURE 16. Small vesicles along gamma particle matrix. Small ca. 80 um vesicles are partially embedded along the inner surface of the gamma matrix. In B the arrows point to the GS-membrane where it extends inward along the matrix vesicles. Fixation II, IP-3. A.) 50,000X B.) 38,000X.


A


B

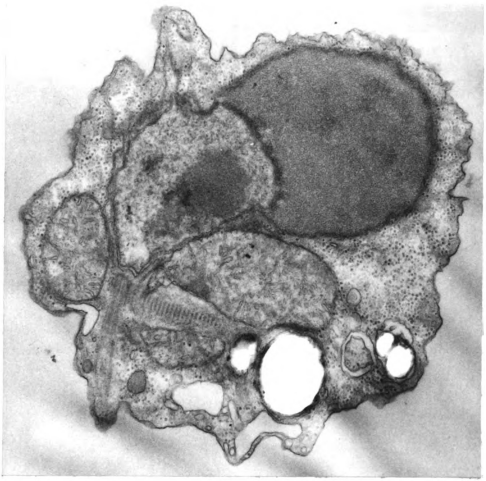
be in the process of retracting its flagellum (Figure 17). Nevertheless, some conclusions can be drawn from these pictures and from those of spores which have recently completed retraction.

Perhaps the most important observation is that the linkage between the axoneme, kinetosome, and nucleus remains intact, an assumption necessary for the proposed mechanism of retraction and rotation. Also of importance is the positioning of the translocating structures along an arc, for this is the arrangement that would result, I believe, if the axoneme were pushing the nuclear apparatus along its circular path.

The plasma membrane of the spore (as well as that of spores observed after retraction) is irregularly folded. This could reflect the actual in vivo state, or be an artifact resulting from hypertonic fixation. In weighing these alternatives, I should mention that non-germinating spores fixed at the same osmolarity possess relatively smooth surface membranes. If the folding is real, it signifies that the surface area decrease during retraction (see Section IV) is accomplished, at least in part, by a folding process instead of an actual reduction by uniform contraction. It is therefore an apparent decrease in surface area only.

Throughout retraction, the banded rootlet remains embedded in the mitochondrial channels and connected to the kinetosome (Figure 17) so that to some degree, the mitochondrian must rotate with the nuclear apparatus. Sections of spores examined shortly after retraction still show the lipid bodies and sb matrix in close association with the mitochondrion (Figure 18), although a slight shift in the relative position of the three constituents is noted. The extent to which the sb matrix and lipid bodies move with the mitochondrion during rotation is uncertain, but my impression, based on phase and electron microscopic observations, is that the movement is much more confined than that of the mitochondrion.

FIGURE 17. Zoospore retracting flagellum. Note banded rootlet is still embedded in mitochondrial channel. Fixation 1, IP-2, 20,000X.



The mitochondrion probably slides by them and, in the process, causes some mixing of the other two components as it tends to carry them along as well.

D. STRUCTURAL CHANGES AFTER FLAGELLAR RETRACTION

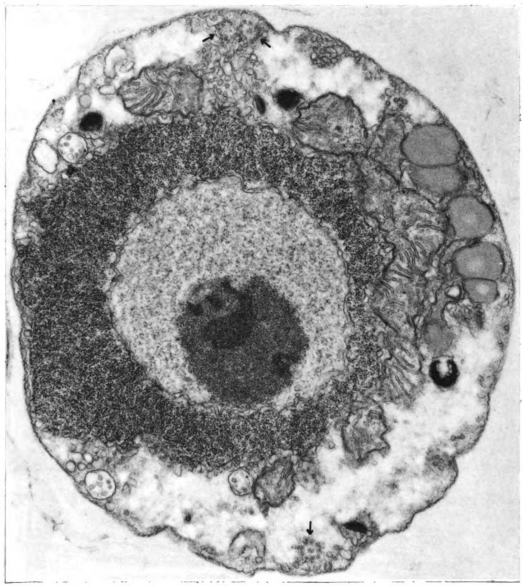
The most conspicuous structural changes associated with encystment occur after flagellar retraction when various morphological units in the cell continue to break down and become more evenly distributed throughout it. Polysaccharide particles (Lessie and Lovett, 1968) evenly distributed within the cytoplasm of non-encysted zoospores (see cytoplasm of spore in Figure 17) aggregate into discrete regions of the cell. In most sections that I have cut (i.e., less than 70 um) these regions appear as light areas of lower electron scattering power than the embedding medium,but in slightly thicker sections the granular effect is preserved (Figure 22,A).

Traces of an amorphous substance along the spore surface are detectable immediately after retraction. This is the first sign of the cyst wall which continues to increase in thickness during germination.

An example of a spore within the first few minutes after flagellar retraction is given in Figure 18. Three cross sections of flagellar axoneme (arrows) reveal that the nuclear apparatus rotated more than 360°. Lipid bodies still lie adjacent to the mitochondrion and were probably carried with the rotating nuclear apparatus; but the sb matrix has shifted slightly in some areas, and now lies between the lipid bodies and the mitochondrion. Although more than one mitochondrial profile is seen, serial sections would likely prove that they are all parts of the same structure.

During this early encystment stage, the mitochondrion surrounds a greater area of the nuclear cap surface than it did at any time prior to retraction. This is more obvious in Figure 21, and other micrographs not shown, than it is in Figure 18. Mitochondrial channels like those housing

FIGURE 18. Spore within a few minutes after flagellar retraction. Three cross sections of axoneme, without surrounding membrane, are visible (arrows). A thin layer of amorphous substance adheres to cell surface. Lipid and the sb-matrix are still adjacent to the mitochondrion and the ribosomes are still confined to the nuclear cap. Numerous small vesicles and MVB are distributed through the cytoplasm. Fixation II, IP-3, 24,000X.



the banded rootlet are no longer observed; and, their absence suggests that the mitochondrion partially subdivides along them to yield a many-armed, yet single, structure that spreads out over the nuclear cap. Such a process could be the source of the obviously branched (Lovett, 1968; Soll et al., 1969) mitochondrion that appears as the time of germ tube formation is approaching. Long, thin mitochondrial profiles are very typical of recently encysted spores (although it represents a later encystment stage, see Figure 21 for an example). These narrow sections always have few cristae and contain numerous small particles that are possibly mitochondrial ribosomes.

The gamma particles in Figure 18 are not typical of newly encysted spores. Usually most of them are oriented such that their major openings face the nuclear cap, and portions of their GS-membranes protrude into indentations in the cap double membrane (Figure 19, 20). Similarly, but much less frequently (perhaps a function of available surface area), the gamma particles also tend to adhere to the nucleus and retracted axoneme. However, gamma particles have never been found adhering to the mitochondrion, sb-matrix, or lipid bodies.

Within a few minutes after the gamma particles adhere to the cap, the cap double membrane fragments extensively and the particles lose their hold (Figure 21). The GS-membrane expands extensively and is filled with small vesicles released from the gamma matrix (Figure 21). The expansion probably results from a difference in the rate at which the small vesicles fuse with the GS-membrane and the rate at which the GS-membrane buds off material. This latter activity appears to diminish after retraction, while the activity of the former appears to accelerate.

The flagellar axoneme begins to break down shortly after the disruption of the cap membranes. Two sections of axoneme are visible in Figure 21.

FIGURE 19. Budding MVB attached to gamma particle. Fixation III, IP-4, 58,000X.

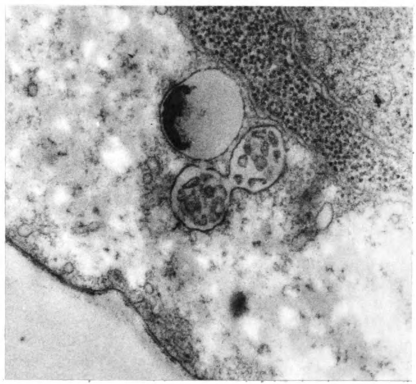
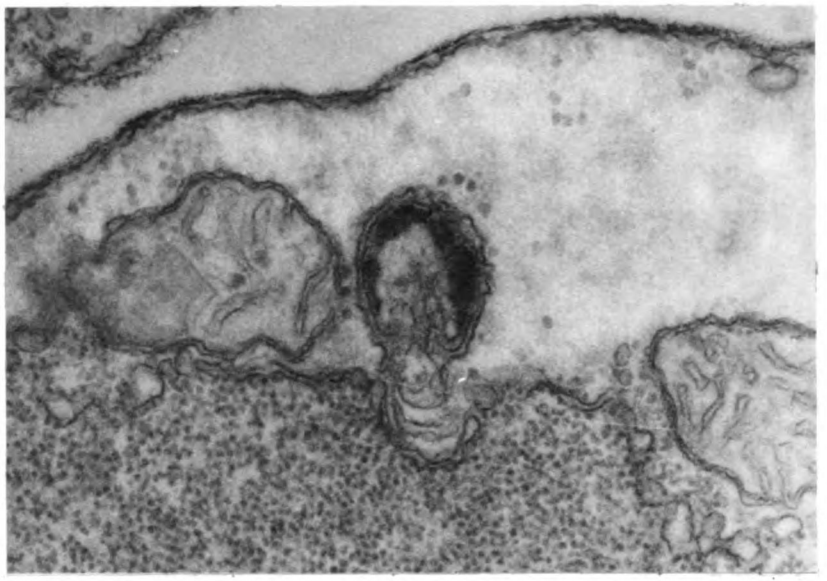


FIGURE 20. Gamma particles adhering to nuclear cap. A.) Fixation I, IP-3, 43,000X. B.) Fixation III, IP-4, 36,000X.



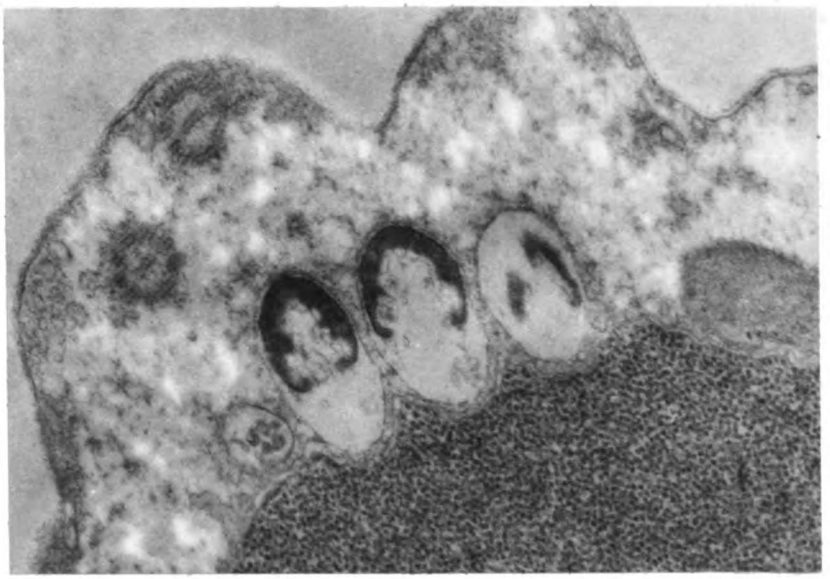


FIGURE 21. Encysted spore midway between flagellar retraction and germ tube formation. Nuclear cap membranes are disrupted and ribosomes scattered. Small vesicles fill expanded GS-membrane. Note the microtubules of decaying axoneme (arrows). Fixation II, IP-3, 29,000X.

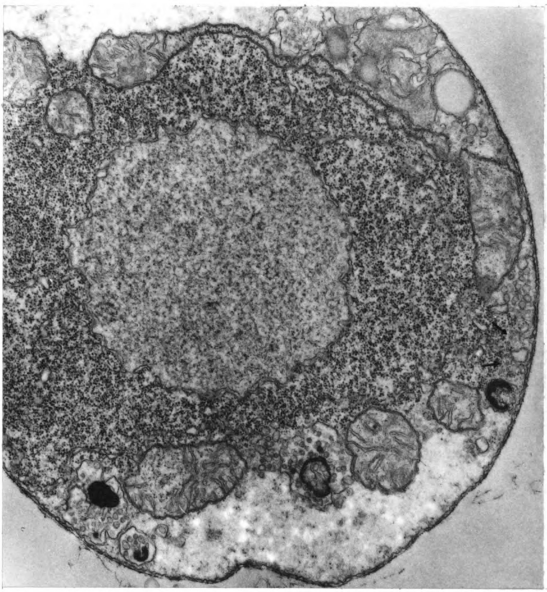
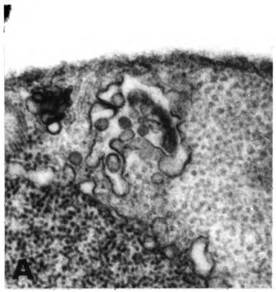
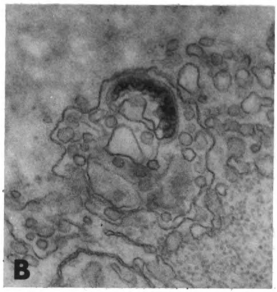
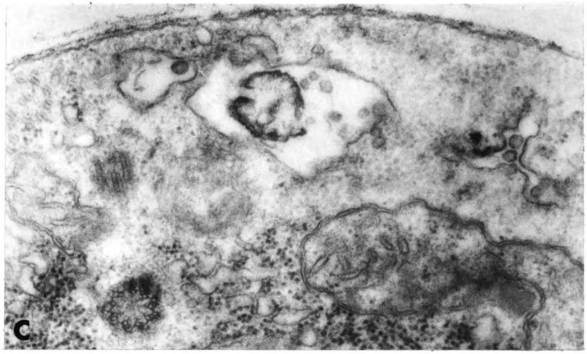


FIGURE 22. Gamma matrix releasing small vesicles. The vesicles accumulate within the GS-membrane and sometimes cisternae derived from the GS-membrane (B, arrow). A.) Fixation I, IP-3, 46,000X. B.) Fixation II, IP-3, 48,000X. C.) Fixation I, IP-3, 48,000X.


A


B


C

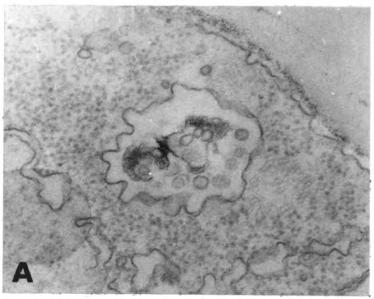
The one located between two mitochondrial profiles near the nucleus is probably closest to the kinetosome, while the other, located near the cell surface, is probably furthest from the kinetosome. The latter has lost the typical 9+2 fibrillar arrangement. Thus the axoneme breaks down from the terminal end (furthest from the kinetosome) first in a manner similar to a rope fraying. Fragments or sub-units of decaying fibrils have never been observed.

About two-thirds of the way between flagellar retraction and germ tube formation, the final stage in the breakdown of the gamma particles begins. The GS-membranes of the different particles fuse together and form large vacuoles containing one or more of the decaying gamma matrices (Figure 23). As many as seven matrices have been counted in serial sections through a single vacuole. In these vacuoles the gamma matrices completely break up into small vesicular elements (Figure 25).

By the time the gamma particles are fusing with one another, ribosomes are scattering throughout the cell. Notably, however, the ribosomes do not invade large areas of cytoplasm that are exceptionally dense in polysaccharide particles. This uneven distribution could be an important factor in determining cellular polarity and influencing the point of germ tube emergence; in fact, I have never observed germ tubes forming in areas rich in polysaccharide.

After the nuclear cap membranes break down and while the ribosomes are dispersing, the mitochondrion transforms into a more tubular structure weaving through the cytoplasm. In this configuration, it is obviously branched. But, the branching may actually have occurred, in the manner previously suggested, shortly after retraction when the mitochondrial channels disappeared. Growth of the branches could make them more noticeable

FIGURE 23. Fused gamma particles. The GS-membrane of two or more gamma particles fuse with one another to produce large vesicles which contain more than one gamma matrix. Note similar appearance of gamma matrix material and amorphous cyst wall material (arrows). A.) Fixation II, IP-3, 55,000X; B.) Fixation I, IP-3, 35,000X.


A

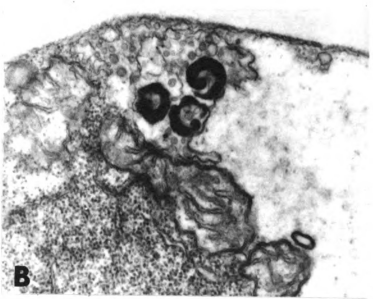

B

FIGURE 24. Cell with germ tube. Portion of the mitochondrion, in typical fashion, extends into germ tube. Banded rootlet is still attached to kinetosome and some microtubule remnants of the axoneme remain (arrow). This section also contains part of an amoeboid spore which did not encyst. Note that the gamma particles and the almost vesicle free cytoplasm. Fixation II, IP-3.

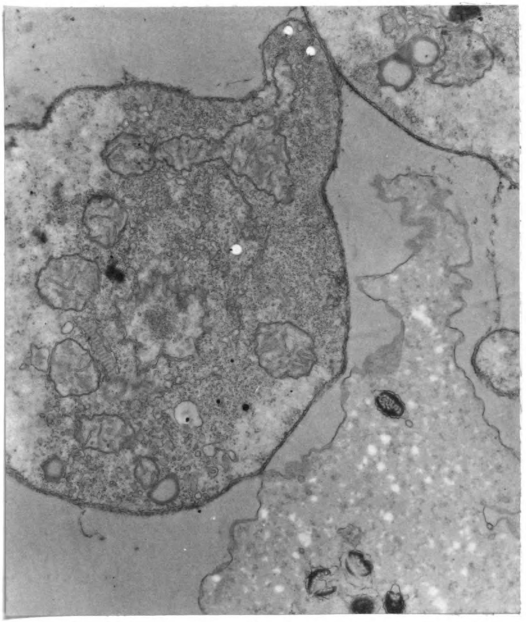
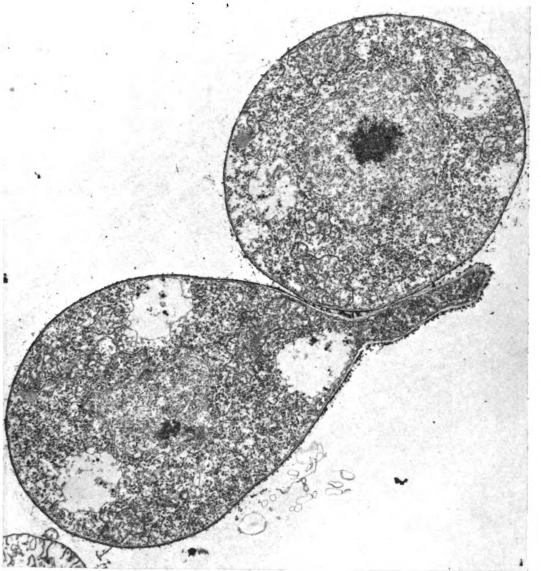


FIGURE 25. Two young germlings. These cells were grown in PYG for two hours. Both have a slightly branched rhizoidal system. Note the large vacuoles with remnants of decayed gamma matrices. Fixation I, 10,000 X.



at this stage. The mitochondrion divides into two or more separate structures (Soll et al., 1969), at least in some instance, before germ tube formation, and profiles are commonly seen extending into the emerging germ tubes (Figure 24, F; Lovett, 1968).

The flagellar axoneme has usually disappeared in cells with a germ tube. But in Figure 24, axonemal fibers (arrow) are still present, as is a normal looking banded rootlet. Profiles of lipid bodies and sb-matrix are dispersed in the cytoplasm. These frequently lie close to the mitochondria and near the cell wall. The large vesicles which have resulted from gamma particle fusion continue to bud material that migrates to the spore surface.

VI. PUNCTUATION ON THREE IMPORTANT STRUCTURAL CHANGES IN GERMINATION

The breakdown of certain, specific, cytoplasmic membranes is a crucial step in the germination of B. emersonii zoospores. Vesiculation of the backing membrane seems to destroy a binding site (see Section IV) that holds the nuclear apparatus in place, thereby allowing it to rotate. Its disruption is also necessary if lipid bodies, sb-matrix, and the mitochondrion are to be released and distributed to the cytoplasm. Similarly, disruption of nuclear cap membranes is a prerequisite for the scattering of its ribosomes. Although there is little information available about the causes of these membrane alterations, their importance leads to some speculations.

A. CHANGES IN THE BACKING MEMBRANE

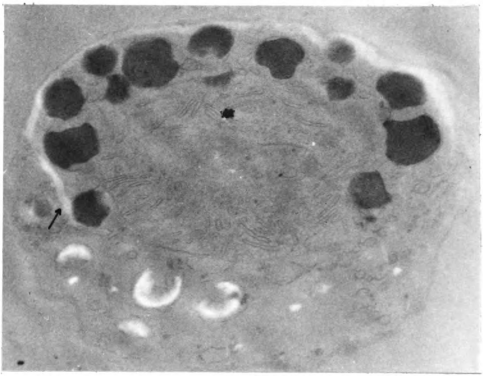
The undifferentiated portion (as contrasted with the densely staining section) of this membrane is one of the most labile structures in the spore. It is the first membrane to be disrupted any time fixation is below par. It is also the first to change in vivo during germination. There is even some indication that the undifferentiated portion of the backing membrane may become fractured in non-germinating spores if but a few hours have passed since sporulation. When this happens, the sb matrix, lipid bodies, and mitochondrion still maintain their relative positions to one another, indicating that there are attractive forces among them. Frequently, the lipid bodies and mitochondrion are so tightly molded to one another that the line of demarcation is not even visible.

The densely staining portion of the backing membrane is much more stable than the undifferentiated section. In this connection, we have made an interesting observation on the electron dense region between the unit membranes of this double membrane. If thin sections of zoospores are floated on 10 mM NaOH for 2 minutes and subsequently stained with Pb citrate, the dense interior shows no staining whatsoever. Only one other structure in the spore of B. emersonii displays this characteristic behavior, namely, the gamma matrix (Figure 26). It could well be, therefore, that the densely staining amorphous material of the gamma matrix and the densely staining interior of the backing membrane are composed of the same substance. Indeed, this may help explain why decay of the gamma matrix and the electron dense portion of the backing membrane is initiated simultaneously.

B. BREAKDOWN OF NUCLEAR CAP MEMBRANE

It can be logically argued that the disruption of the nuclear cap membranes and the scattering of its ribosomes may be necessary for efficient synthesis and distribution of proteins in the germinating spore. Gamma particles adhere to the nuclear cap membranes only minutes before these two events occur. The firmness with which they cling is dramatized by the distortions they seemingly produce in the usually smooth contour of the cap membranes. Thus, it would be tempting to link such events to the breakdown of these membranes. However, two other facts complicate this interpretation. First, the gamma particles also seem to stick to the nucleus, which does not fragment, and to the retracted axoneme. Second, the backing membrane (an extension of the nuclear cap outer membrane) begins to rupture before this alignment stage occurs, and without any prolonged contact with the gamma particles. It may be more realistic,

FIGURE 26. Spore section treated with NaOH. Densely staining material in gamma matrix and backing membrane (arrow) has been dissolved away by floating section for 2 minutes in 10 mM NaOH. Dark areas are lipid. Fixation II, 20,000X.



therefore, to view the adhesion of gamma particles to the membranes of the nuclear cap as a result, not a cause, of subcellular changes. Many modulations are taking place in the cell at this time: alterations in cell shape and volume, onset of cell wall synthesis, etc. These things may be associated with ionic shifts which could cause new arrangements in attractive forces and give gamma particles their adhesive properties, albeit properties which may or may not serve specific functions.

I suspect, in fact, that the mitochondrion is more involved in membrane breakdown than are gamma particles. It will be recalled (Section V, D) that it spreads over the nuclear cap immediately after flagellar retraction, and remains there until membranes have fragmented. Then, it changes in form and extends to other places in the cell. Specific ionic uptake and release by mitochondria is so well known as to need no documentation. If specific ionic changes are required for membrane alterations (Kavanau, 1965), then the mitochondrion is a likely candidate to provide them. The flattening of the mitochondrion may generate increased surface area to maximize a membrane mediated flow of substances.

When the double membrane around the nuclear cap fragments, its inner and outer unit membranes fuse to form vesicles derived from both membranes. Such a fusion of different unit membranes may be necessary for fragmentation. This could explain why there is no obvious morphological change in the outer membrane of the nucleus, even though this membrane is continuous (see Figure 1) with the outer membrane of the nuclear cap. Specifically, the inner nuclear membrane is the only membrane that makes direct and extensive contact with the nucleoplasm, which may exert a controlling influence upon it. If, as a result, the inner nuclear membrane cannot fuse with the outer nuclear membrane, then vesiculation of this

particular part of the double membrane around the nuclear apparatus cannot take place.

C. THE GAMMA PARTICLE

1. Summary of gamma particle decay.

The breakdown of gamma particles may be visualized as a continuous four stage sequential process depicted in Figure 27. The quiescent particles of the non-encysting zoospore exist in an essentially vesicle free cytoplasm and display a smooth, almost spherical contour in their surrounding membranes (Figure 27, stage 0). But, soon after induction of germination (stage 1), the GS-membrane loses any definable shape and begins to bud vesicles (including a few MVB) which, in turn, fuse with the zoospore plasma membrane.

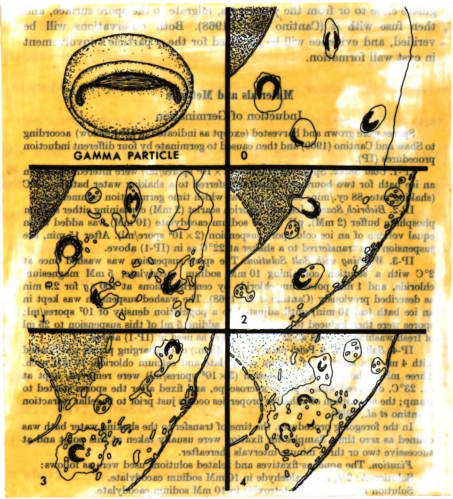
The next stage of decay (stage 2) is noted just after flagellar retraction and is characterized by many of the gamma particles taking a position along the nuclear cap, nucleus, or axoneme. The occurrence of MVB reaches a peak at this time.

In the third stage, the gamma particles drift away from the cap, and the GS-membrane expands greatly. Numerous small vesicles released from the gamma matrix accumulate within it.

The last stage of decay begins when the GS-membranes of the separate gamma particles fuse with one another to yield large vacuoles containing more than one decaying gamma matrix. In these large vesicles the matrices continue to break up until completely exhausted.

Although the decay process may be divided into four separate stages, the unity of the sequence is maintained by the continuous transfer of vesicular material from the gamma matrix to the GS-membrane, and eventually to the cell surface.

FIGURE 27. Interpretive sketch of gamma particle decay. See text for description of stages 0-4.


GAMMA PARTICLE
0
2
3
4

2. Role of the gamma particle in cell wall formation.

Although the earliest, visable (via electron microscopy) evidence for the presence of a cyst wall is not found until the axoneme has been translocated, it is likely that wall formation is initiated prior to retraction. As mentioned earlier (Section III, B), the spore surfaces acquire adhesive properties leading to mutual attractiveness shortly before retraction. There is reason to believe this is a direct result of newly deposited wall material, at least in those cells that still adhere to one another after flagella have disappeared. Electron micrographs show that the wall material around the cysts merges into an undifferentiated continuum in areas where cysts adhere to one another (this may explain why all attempts short of vigorous hydrolysis have failed to break up clumps of encysted cells). Also, zoospores acquire adhesive properties at approximately the time that vacuoles are fusing with the plasma membrane. These vacuoles originate from gamma particles (Section V, B), as do, probably (Section V, D), the MVB observed (Soll et al., 1969) at the spore surface. These events establish a link between gamma particle decay and cell wall formation. Other correlations include the facts (Truesdell and Cantino, 1970) that (a) gamma particles always begin to break down shortly before the cell wall is detected; (b) they always (and only) continue to break down as the cell wall is being deposited; and (c) electron dense material resembling the gamma matrix accumulates at the cell surface while simultaneously the electron dense substance of the gamma matrix is disappearing. Finally, the argument finds support in the results of some enzymatic assays. Camargo et al. (1967) studied chitin synthetase in B. emersonii and established that, in spore homogenates, the activity of this enzyme was highest in fractions containing mainly smooth

membranes. Their electron micrograph of this fraction also showed that it contained gamma particles. Work with cell free preparations of gamma particles in this laboratory has demonstrated (Myers and Cantino, in press) that smooth membranes can originate from decaying gamma particles during their isolation. Thus, the available evidence is at least consistent with the idea that gamma particles may contain the "cell wall" enzyme chitin synthetase.

The growing plants of B. emersonii produce chitin (Cantino et al., 1957) but they do not contain gamma particles (Lessie and Lovett, 1968). The swimming zoospores apparently do not produce chitin, but they do contain gamma particles. Perhaps the extraordinarily compact nature of the gamma matrix provides the mechanism whereby chitin synthetase activity is suppressed in the motile stage of this organisms's life cycle.

Although it does not pertain directly either to encystment or uniflagellate fungi, a recent paper from Bracker's laboratory (Grove et al., 1970) on the ultrastructural basis for hyphal tip growth in Pythium is especially relevant here. In discussing the literature and their own work on the deposition of hyphal wall material, the authors hypothesize a sequence of events that includes: (a) secretion of vesicles by dictyosomes, with eventual loss of the entire dictyosome cisternae;(b) migration of the vesicles to the hyphal tip, with increase in size and/or fusions among some of them to yield larger vesicles; and (c) fusion of the vesicles with the plasma membrane, and concomitant deposition of vesicle contents in the wall region. There are, of course, obvious differences in origin and structure between the dictyosomes of Pythium and the gamma particles of Blastocladiella; yet, their functional activities may have much in common. An earlier statement by Bracker (1967; p. 349) is appropriate here:

"The apparent ahsence of dictysomes in so many fungi raises the question of what cell component, if any, carries out the expected functions of dictyosomes in cells where none are present.... It seems logical for this role to fall upon a membrane-bounded structure capable of packaging materials within a membrane for transport."

The results of one other study should also be cited here. Manton (1964) believes that vesicles beneath the plasmalemma in zoospores of the fresh water alga Stigeoclonium provide the first components of the cell wall. After flagellar retraction, the spore secretes a flocculent, adhesive material probably derived from the contents of superficial vesicles.

3. The genesis of gamma particles.

At present, two theories co-exist concerning the genesis of gamma particles. The first was proposed by Lessie and Lovett (1968) in a paper on ultrastructural changes during sporogenesis. Dense granules accumulated in irregular cisternae when the cleavage phase of sporogenesis ended. The cisternae grew larger and were transformed into roughly spherical bodies while their contents became arranged into a "highly ordered ellipsoidal-hemispherical pattern." These became the gamma particles of the mature zoospore.

The second theory was advanced by Cantino and Mack (1969) to account for "gamma-like" figures, observed in amoeboid zoospores, which seemed to originate from the double membranes of the nuclear cap and nucleus in regions where these membrane pairs were close to one another. Accordingly, formation of gamma particles occurred when these membranes evaginated either into the cytoplasm or into mitochondrial cavities and then fused together. Cantino and Mack did not view the two methods of gamma

formation as mutually exclusive, but did propose that this second mechanism might be a means for production of additional gamma particles by spores.

The present observations on the breakdown of gamma particles may shed some light on these theories about their formation. Perhaps the most important fact is that the small vesicles which come from the electron dense matrix during the breakdown of gamma measure about 80 mu in diameter. From the electron photomicrographs in Lessie's and Lovett's paper, I have estimated that the dense granules believed to form the gamma matrix are also about this size. They first appear in cisternae, then accumulate in somewhat spherical vesicles, and finally condense to form the gamma matrix. The 80 mu vesicles are released from the gamma matrix, move into roughly spherical vesicles, and then appear in smaller vesicles (MVB) or cisternae--almost exactly the reverse process! Although Lessie and Lovett speak of granules where I speak of small vesicles, I have found that by using their methods of fixation and staining, and by varying the section thickness, the vesicles can look like the granules.

On the other hand, there is no similarity between gamma decay and the second theory of gamma formation in amoeboid spore (as contrasted to sporogenesis) advanced by Cantino and Mack. If a second class of gamma particles exists, it has not identified itself as a variant in the breakdown pattern. Conceivably, the sheets of membranes incorporated into the gamma matrix could, if they were not "decomposed", be transformed again into the vesicles that the gamma particles eventually liberate; unfortunately, the present observations neither refute nor support this notion. However, it must also be emphasized that the membranes contained in the electron dense matrix are extremely difficult to resolve,

and any changes that they might have undergone could easily have passed unnoticed. Furthermore, such changes could have occurred at a time prior to the germination stages studied here.

If there is a parallel between gamma breakdown and gamma formation, then the gamma particles apparently break down into the units of their construction. In this sense the gamma particle and the nuclear cap are similar, each functioning as a storage organelle--the nuclear cap preserving previously synthesized ribosomes ready for protein synthesis (Lovett, 1963) and the gamma particles preserving previously synthesized materials presumably necessary for rapid cyst wall formation. As would be expected on this basis, the existence of both organelles is uniquely limited to the zoospore stage in the ontogeny of B. emersonii.

4. The gamma particle as a universal organelle among fungal zoospores.

Encystment is a universal process among the zoospores of aquatic fungi. If gamma particles play an integral role in this phenomenon, they may also belong to some class of organelles that occurs universally in zoospores.

When the appearance of a gamma particle is defined in general terms, i.e., as an electron dense particle contained in a vesicle, then similar structures do indeed occur. Cytoplasmic inclusions conforming to this general form can be identified in seven species of fungi, which represent five orders and three classes of Phycomycetes: Allomyces (Blondel and Turian, 1960; Fuller and Calhoun, 1968; Moore, 1968), Monoblepharella (Fuller and Reichle, 1968), Rhizidiomyces (Fuller and Reichle, 1965), Rhizophlyctis (Chambers and Willoughby, 1964), Nowakowskiella (Chambers et al., 1967), and Phytophthora (Reichle, 1969). Although none of the structures are morphologically identical to gamma particles, variation within this diverse assemblage of fungi is not unreasonable. Among them,

Allomyces is the relative nearest to B. emersonii, and its zoospores contain the particles that most nearly resemble gamma particles. Recent investigations of B. Britanica (Cantino and Truesdell, 1971) have shown, surprisingly, that this close relative does not contain structures that could be described in the general terms used above. Yet it does contain a class of electron dense (stain with OsO_4, UO_2^+, or Pb^{++}) cytoplasmic inclusions that are not surrounded by a membrane, and which contain a membranous network even more obvious than that of the gamma matrix. Preliminary investigations also indicate that during encystment they behave in a manner similar to gamma particles. Therefore, it is my opinion that the cytoplasmic inclusions of B. britannica should be considered as gamma-like particles.

The only fungal zoospores investigated to date which do not contain inclusions similar to gamma particles, are those of Olpidium brassicae (Temmink and Campbell, 1969a, b; Lesemann and Fuchs, 1970). But these spores contain multivesicular bodies in which varying amounts of an electron dense substance are found. Their similarity to gamma particle breakdown products is striking.

After encystment, "lomosome-like" bodies are found along the cell surface of Olpidium. These appear to be the results of fusions of the MVB with the plasma membrane. Thus it may be said that the MVB in Olpidium are functioning as gamma particles in B. emersonii. One might view the MVB as incompletely formed gamma particles. It is certainly true that many other "conglomerate" structures present in B. emersonii are not formed in Olpidium, although many components of such structures are present in a less organized state; e.g., the ribosomes remain scattered throughout the cytoplasm (Temmink and Campbell, 1969a) and do not aggregate into a nuclear cap, and the many small mitochondria never fuse into a

single large mitochondrion (Lessie and Lovett, 1968). Therefore, why not entertain the thought that, in Olpidium, formation of a gamma-like particle begins but stops at a less-developed stage than it does in B. emersonii?

An investigation of the cytoplasmic inclusions of various zoospore species, in my opinion, would supply some valuable information on the universal mechanisms of encystment. I must agree with Bartnicki-Garcia's observation that "Solutions to some of the most important problems of fungal morphogenesis probably depend on...answers to the following questions: where are cell wall structural polymers synthesized? Are they polymerized in some intracellular site...and somehow transported in an orderly way to...the cell wall?

VII. MACROMOLECULAR SYNTHESIS DURING GERMINATION

Only a few macromolecular components in the spores of B. emersonii have been studied. These include some detailed investigations (Camargo et al., 1969) of the regulation of glycogen synthetase. It was concluded that glycogen synthesis is regulated by intracellular concentrations of glucose-6-phosphate, which stimulates synthetase activity 90-fold in zoospores but only 4-fold in 3-hour plantlets.

Extensive studies on regulation of nucleic acid and protein synthesis have come from Lovett's laboratory at Purdue University. Not until spores have germinated and produced germ tubes do measurable amounts of RNA begin to accumulate. (Lovett, 1968, in the system used, the RNA synthesis begins ca. 40-45 minutes after encystment.) This is followed 30-40 minutes later by synthesis of protein and, about 40 minutes thereafter, DNA. Thus, during the early stages of germination there is no measurable net increase in either RNA or protein, but pulse labeling experiments with C^{14}-uracil and C^{14}-leucine showed that synthesis of RNA and protein does begin, at very low rates, about the time of encystment. Actinomycin D, at 25 ug/ml, inhibits detectable uracil incorporation in germinating spores (Lovett, 1968). Nonetheless, spores in contact with the antibiotic encyst and develop up to the time when the directly measureable increase in RNA should begin; then they stop growing. They are not noticeably different from those formed in the absence of the drug in that (a) the nucleolus fails to show the increase in size that normally accompanies germination,

and (b) the shape of the primary rhizoid changes somewhat. Since early protein synthesis is not affected significantly by inhibition of RNA synthesis (assuming lack of uracil incorporation does in fact denote this), it would seem that the ribosomal-, transfer-, and messenger-RNAs necessary for it are all present in ungerminated spores.

Cycloheximide, at 20 ug/ml, inhibits protein synthesis in germinating zoospores. Spores encyst normally, nuclear cap membranes fragment, and ribosomes scatter throughout the cytoplasm as usual, but the germ tube does not form and the retracted flagellar axoneme does not disappear. Although germination does not proceed as far as it does in the presence of actinomycin D, the structural changes of encystment apparently require only the protein and RNA found in a non-germinating zoospore. One obtains the impression that the spore has all the necessary ingredients for encystment packaged and waiting, and that some signal is needed for it to start using them.

In summary, Lovett's experiments (and, more recently, those of Soll in Sonneborn's laboratory at Wisconsin; Soll, 1970) suggest: first, the spores of B. emersonii can consummate the structural changes associated with encystment (Section III, D) without apparent protein synthesis; and, second, neither the foregoing events nor subsequent ones essential for germ tube formation require concomitant synthesis of RNA.

Lastly, let us come to the interesting question: how is protein synthesis suppressed in the non germinating spore? Schmoyer and Lovett (1969) investigated some factors responsible for regulation of protein synthesis in germinating zoospores. When ribosomes isolated from nuclear caps were combined with cell-free protein synthesizing systems derived from young germlings, no synthetic activity was detected; when

such inactive ribosomes were mixed with active ones (obtained from growing cells), the latter were rendered inactive. However, inactive ribosomes were activated by washing with KCl. From the KCl extracts, a fraction with inhibitor properties was isolated. Its behavior on gel columns resembled that of an inhibitor fraction isolated from a non-nuclear cap portion of the spore. It was concluded that the spore possesses a protein-synthesis inhibitor located in its cytoplasm and bound to nuclear cap ribosomes, and that inhibition is probably not due to background nuclease activity.

VIII. ENVIRONMENTAL INFLUENCES ON ZOOSPORES

A. EFFECTS OF LOW TEMPERATURES

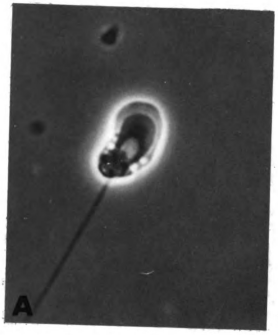
The behavior of the zoospores of B. emersonii at low temperature (ca. 0-1°C) differs markedly from that at slightly higher temperatures. This is not only of theoretical interest but also of practical significance because some routine procedures (Lovett, 1967a; Cantino et al., 1969) include manipulation of zoospores in an ice-bath. It appears that ca. 4°C may be the transition temperature for the behavioral change. Oxygen consumation is not detectable at 4°C (Cantino et al., 1969), but above this point, it increases linearly with temperature. Thus, below 4°C those energy producting functions which consume oxygen must shut down.

Soll and Sonneborn (1969) report that spores maintained in an incubator at 3-4°C can eventually germinate, although it takes much longer than at higher temperatures (e.g., 15°C). However, in an ice-water bath at 0-1°C, I do not observe encystment; in fact, spores that remain sufficiently long under these conditions lyse.

1. Morphological changes during incubation at 0-1°C

During incubations at 0-1°C, spores eventually swell two to three fold (compare Figure 28, C and 28, D) and become spherical. Small cytoplasmic particles exhibit a rapid random (presumably Brownian) motion not observed in swimming and amoeboid zoospores. Evidently, water uptake during swelling appreciably lowers cytoplasmic viscosity. The single

Figure 28. Comparison of normal and swollen zoospores. A. elongate amoeboid zoospore. B. Encysted zoospore. C. Cold swollen zoospore with flagellum. D. Cold swollen zoospore shortly after flagellum has been absorbed. Arrow points to swollen mitochondrion. All pictures at same magnification, 2,300X.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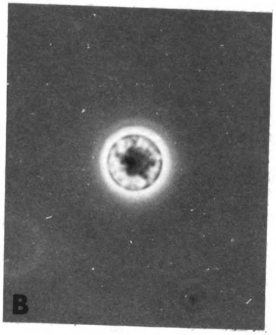
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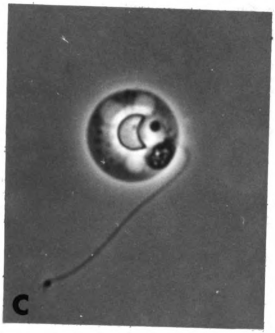
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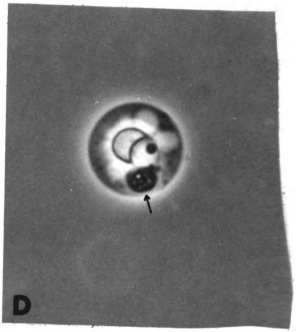

D

mitochondrion enlarges somewhat and may become globose. Both light micrographs (Figure 28, C, D) and electron micrographs (Shaw and Cantino, 1969; Cantino et al., 1969) also indicate that the mitochondrion is swollen, the latter pictures also showing that the backing membrane can break and that some of the lipid bodies may become dispersed throughout the cytoplasm.

At first, spores in this swollen state can swim and (if brought back to room temperatures) are initially 95-100% viable (Cantino et al., 1968; Deering, 1968). Eventually, however, flagellar activity becomes increasingly erratic at 0-1°C and finally stops. The flagellum straightens out and extends directly away from the spore. Then, at the base of the flagellum, the membrane sheath pulls away from the axoneme, the flagellum wraps around the spore, and it is absorbed. The time required for complete absorption may vary from a few seconds to a few minutes for different spores in the same suspension; this seems to be directly related to the rate at which a spore is swelling. The axoneme can usually be observed within the spore, pushing against the plasma membrane and distorting its spherical contour momentarily. As the spore continues to swell, its spherical shape is regained; the swelling continues until the spore bursts. The cytoplasm is discharged quite violently into the suspending medium, and the flagellar axoneme uncoils from its position within the spore to become readily visible. It is my impression that the amount of swelling is limited by the amount of membrane contributed from the flagellar sheath; if the flagellum were not absorbed, the spore would not enlarge as much and would burst sooner.

A spore that is removed from the ice-water bath after flagellar absorption follows one of three possible paths. It may continue to swell

and eventually burst, as it would have had it remained in the ice-water bath; it may reduce its volume and encyst (minus flagellar retraction, of course); or, in rare instances, it may decrease in volume and assume amoeboid characteristics. In the latter event, the axoneme remains inside the spore, but begins to move, producing bump-like distortions in the contour of the cell.

The method of flagellar retraction described above for chilled swollen spores is similar to one described (and labeled "wrap around") by Koch (1968) for non-chilled zoospores of B. emersonii. Koch also portrayed two other types of flagellar retraction displayed by non-chilled spores--"body twist" and "vesicular"--which I, too, have observed in chilled swollen spores. The pictures provided by Koch (1968; Figure 20-28) to illustrate these three methods of absorption invariably show his non-chilled spores to be highly swollen. However, his pictorial evidence for the method of flagellar retraction described earlier (Section IV), which involves rotation of the nuclear apparatus, shows spores that are not swollen.

Zoospores of B. emersonii can swell for a variety of reasons besides cold shock, e.g., overheating, changes in osmolarity, even the kind of paper used to wipe a microscope slide! Flagellar absorption associated with such swollen spores differs importantly from flagellar retraction in non-swollen spores: in the former, the nuclear apparatus does not rotate, the spore increases in volume, and the processes of flagellar absorption and zoospore encystment are not intrinsically associated; in the latter, the opposites are true.

2. The influence of low temperature incubation on encystment.

Increasing the duration of cold incubation of a spore population increases the percentage of spores encysting after the population is

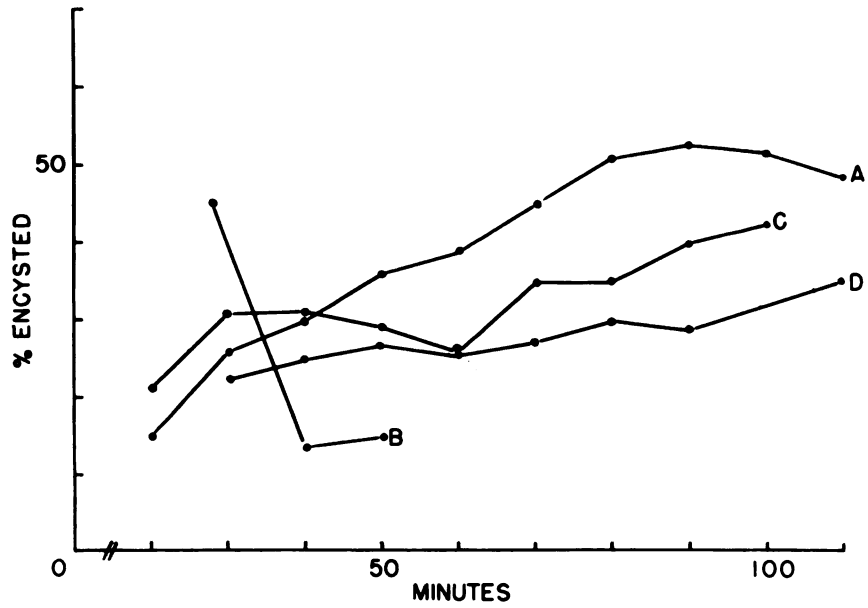
removed from the cold (Cantino et al., 1969). This behavior is partially dependent on the nature of the medium. In Figure 29, curve C represents a spore population suspended in a PIPES-buffered medium (see legend for details). Spores are very stable in this system and display no lysis during the entire 110 minutes in the cold. After an initial rise, there is no further increase in encystment capacity (as measured at 22°C) until spores have been chilled for 70 minutes.

When spores are cold incubated in medium GC (Figure 29, curve A, see legend for composition), the encystment percentage increases immediately and continues rising to a maximum at ca. 90 minutes, after which it declines. Spores begin to lyse around the time of maximum encystment, and continue to do so thereafter.

While they are in the cold, there is no way of distinguishing spores that have been triggered to encyst from those that have not; therefore, it is impossible to determine if the former are the ones that are lysing. In other words, I cannot determine if cell lysis is the direct cause of decreased encystment. Yet, it is tempting to suppose that for each spore, the triggering of encystment always precedes lysis. In a typical population, this event would occur asynchronously to the extent that, while one spore is being induced to encyst, another may be lysing. In this manner, maximum encystment would be limited by the synchrony of the particular population under consideration. In populations displaying a highly synchronous response to a cold incubation, 100 per cent encystment would be expected.

When glutamate is omitted from medium GC (Figure 28, compare curve B with curve A), spores become very sensitive to low temperatures. Lysis ensues within the first 40 minutes and is accompanied by a substantial decrease in the population's encystment capacity. Almost all cells lyse by 60 minutes.

FIGURE 29. Influence of cold incubation on encystment capacity. Encystment percentage after washed spores are transferred from 0-1°C to 22°C. Curve A: medium GC, containing 0.5 mM Na phosphate (pH 7.8), 0.2 mM $CaCl_2$, 5 mM KCl, and 1 mM Na glutamate. Curve B: medium GC minus glutamate. Curve C: medium GC with 2/5 of its KCl replaced by 2 mM piperazine-N-N'-Bis (2-ethane sulfonate) at pH 7.0 (PIPES; Good et al., 1966). Curve D: Na morpholinopropane sulfonate (MOPS) buffer (a GOOD buffer; Calbiochem, Los Angeles) at pH 7.8. Population density for curves A - #, 5 x 10^6 spores/ml.


50
% ENCYSTED
0
50
100
MINUTES
A
B
C
D

Although such lability evidently results from omission of glutamate in medium GC, work with other media demonstrates that glutamate *per se* is not essential for maintenance of zoospore integrity at low temperatures.

Spores suspended in media composed entirely of MOPS buffer (Figure 29, curve D) are very stable; there is no lysis throughout the cold incubation. Spores swell more gradually and survive longer than in medium GC; suspensions have been kept in this medium for up to 3 hours with less than 1% lysis, and they can probably be kept up to 6 hours or more without much additional breakage.

Spores suspended in Na or K phosphate buffers (1-4 mM, pH 6.0, 6.8, 7.8) display behavior patterns similar to that obtained in medium GC without glutamate; lysis usually begins after 30-40 minutes in the cold.

It is evident that the behavior of cold incubated spores is greatly dependent on the medium in which they are suspended. It is not yet possible to make generalizations about the influence of individual compounds or ions on either spore viability in the cold or cold induced encystment. However, the function of glutamate should be examined further, for it does appear to control lysis in medium GC. In this connection, it is of interest that in another medium composed of 2 mM sodium MOPS (pH 7.8), 1 mM KCl, and 0.1 mM glutamate, spores are not very stable and lysis begins after only 70 minutes of cold incubation. This is unexpected, since spores are stable in medium GC, which contains both KCl and glutamate, and they are very stable in MOPS buffer alone. These results point to the interesting possibility, consistent with all available data thus far, that the presence of either glutamate or phosphate can lead to instability in the cold, while the presence of both together yields cold-stable spores.

Such an interaction might be directly related to the metabolic mechanisms associated with spores at low temperatures.

B. SELF-INHIBITION IN SPORE POPULATIONS

While examining the effect of cold incubation on encystment, it became evident that the population density of the suspension was influencing the number of cells encysting. This was investigated further. When a population of spores was kept in medium GC (see Figure 29, Curve A for composition) at 0-1°C for 90 minutes and then brought to 22°C, its encystment percentage was inversely related to population density (Cantino et al., 1969). Such "self-inhibition" is readily apparent between 10^6 and 10^7 spores/ml., but it has not been precisely determined at what density it is first detectable. However, Soll and Sonneborn (1969) have noted slight interactions (e.g., increased asynchrony of encystment), in their system, at concentrations as low as ca. 6×10^5 cells/ml.

These observations have some obvious implications: (a) the density of a spore suspension is a variable that must be rigidly controlled to obtain reproducible results in germination experiments; (b) the population density can be regulated so as to yield populations of either almost wholly encysted or wholly non-encysted spores; and (c) of more theoretical interest, any insight into the mechanism of self-inhibition might reveal important information about the mechanism underlying control of encystment. For these reasons, it was desirable to look more closely at "self-inhibition" of encystment. The results of the following experiments give additional insight into the phenomenon.

1. Inhibition of encystment by cell-free supernatants from spore suspensions.

The supernatants of high density spore suspensions were examined for inhibitory properties. A "Cold Supernatant" was obtained from a suspension

maintained at 0-1°C for 90 minutes; and, a "Warm Supernatant" was obtained from the same cold-incubated suspension, but after a secondary incubation at 22°C for an additional 20 minutes. The supernatants were tested for inhibitory properties by diluting them to either 50 or 90% of their original concentration with a suspension of 10^7 "assay" spores/ml. A complete description of the procedures is given in the methods section.

The results of two experiments are given in Table 2; each value for the percent encysted is an average of three assays.

In the first experiment, the "warm supernatant" displayed an inhibitory effect over the GC control at both 50% and 90% concentration levels (Table 2; compare line 1 with line 3, line 4 with line 6). In the second experiment, the warm supernatant inhibited only at the 90% concentration, and to a lesser extent than in the first. This difference in relative inhibition can be partially, and, perhaps, totally explained by the fact that the controls of the second experiment had a considerably lower level of encystment than would be expected under these conditions at this population density (see Cantino et al., 1969; Figure 3A). I was unable to determine the course of this drop in encystment, but such unexplained variability is not uncommon in spore assays.

Within experimental error, all values for the percent encysted in the warm supernatant assays are essentially the same (Range 29.1-38.0%), regardless of the supernatant concentration level (50% or 90%), or the population density of the suspension from which the supernatants were obtained (8.8×10^6 spores/ml. and 1.2×10^6 spores/ml., respectively, for experiments 1 and 2). If this is coincidence or experimental fact will have to be determined from further experiments. Why all samples

TABLE 2. INHIBITION OF ENCYSTMENT BY CELL-FREE SUPERNATANTS

Experiment Number [a]	Assay Spores (ml.)	Medium GC (ml.)	Cold Supernatant (ml.)	Warm Supernatant (ml.)	Final Population Density (spores/ml.)	Percent Encysted[b]	Amount of Inhibition[c] (%)
1a	2.5	2.5	---	---	5×10^6	45.3	---
"	"	---	2.5	---	"	43.9	1.4
"	"	---	---	2.5	"	29.1	16.2
1b	0.5	4.5	---	---	10^6	93.0	---
"	"	---	4.5	---	"	91.5	2.5
"	"	---	---	4.5	"	34.9	59.1
2a	2.5	2.5	---	---	5×10^6	29.9	---
"	"	---	2.5	---	"	32.5	-2.6
"	"	---	---	2.5	"	34.0	-4.1
2b	0.5	4.5	---	---	10^6	66.1	---
"	"	---	4.5	---	"	76.2	-10.1
"	"	---	---	4.5	"	38.0	28.0

[a] Supernatants for Exp. 1a, 1b derived from suspensions of 8.8×10^6 spores/ml.; for Exp. 2a, 2b, 12.0×10^6 spores/ml.

[b] Average of three assays.

[c] Percent encystment in experimental less percent encystment in control.

should possess the same inhibitory properties is a moot point. One could speculate that ca. 30% encystment is the lower level to which inhibition may be forced under these experimental conditions, and that each contained enough inhibitor to reach this limit. If cold incubation induces rounding, perhaps it does so irreversibly and no leverl of inhibitor activity is able to stop the induced cells from encysting.

The Cold Supernatant, unlike the Warm Supernatant, does not suppress encystment (compare lines 1 and 2, 4 and 5, 10 and 11). Therefore, the imparting of inhibitory properties upon the suspending medium must take place after spores are removed from the cold. Presumably, it could result from either release of inhibitory substances into the medium or utilization of substances present in the medium in limiting quantities and required for encystment. The latter alternative seems unlikely; there is no evidence for the presence of specifically required substances except oxygen (Cantino et al., 1968), and the latter would not have been limiting in the Warm Supernatant because there was ample time and agitation for it to reach saturation levels before the assay. Changes in pH probably were not limiting because supernatants were within 0.2 pH units of one another, and encystment is unaffected by pH over the range 6.0-9.0 (Soll, et al., 1969). I hypothesize, therefore, that an encystment inhibitor is released by spore suspensions of B. emersonii.

2. The influence of environmental factors on self-inhibition.

Self-inhibition of germination among other fungi is well known (see review by Sussman, 1965; also, Blakeman, 1969; Garrett and Robinson, 1969; Fletcher and Morton, 1970; and references therein); its occurrence during encystment in B. emersonii seemed sufficiently important to warrant its further characterization. The influence of selected salts, temperatures, and pH levels on encystment at various population densities was therefore

examined, not with a view toward doing a comprehensive survey but, rather, to finding some appropriate set of conditions that would produce minimum or maximum inhibition and establish patterns for the effect of specific variables on self-inhibition. Nine different systems (combinations of variables) were investigated (Table 3).

Spore suspensions were harvested, washed once, and resuspended in the medium under investigation. The suspensions were maintained in the cold for only 20 minutes (measured from the time of filtration; see methods); this allowed the testing of media in which spores were labile at low temperatures. Samples of the washed and resuspended spores were placed in prechilled assay flasks (see methods; shaking flask assay) and diluted with ice cold suspending medium to obtain a series of suspensions at different population densities. Encystment was initiated by transferring the spores (in their assay flasks) to a constant temperature water bath at either 15°C or 22°C (hereafter referred to as the "secondary incubation"). Within minutes after the temperature had been raised, spores began encysting; after 30 minutes of secondary incubation, almost all spores which were to encyst did so (30 minutes is not necessarily adequate for maximum percent encystment in other systems; see Section IX; Soll et al., 1969; Soll and Sonneborn, 1969).

The data displayed appreciable scatter; therefore, straight lines corresponding to Equation 1 were fitted to the data by linear regression (Goldstein, 1964). The correlation coefficients and confidence intervals were also determined (Goldstein, 1964).

$$(1) \quad R = a + bp$$

In Equation 1, R is the decimal fraction of encysted spores in the suspension; p is population density in spores/ml. ($\times 10^{-6}$); and a and b are constants determined by the regression techniques. The absolute value

of b is referred to as rate of inhibition because it is the rate at which encystment decreases as population density increases; specifically, it is a measure of the decrease in R for every increase of 10^6 spores/ml.

Curves corresponding to Equation 2 were also fitted to the data by

$$(2)\quad R = a' + b' \ln p$$

regression techniques.

The correlation coefficients for the linear and logarithmic equations were similar. This may mean that the true relationship between percent encystment and population density is not given by either relationship but, rather, that both relationships are almost equally good approximations. Overall, the linear equation yielded a slightly better fit, and, therefore, will be used for the following comparisons among the test systems. The equations and their correlation coefficients (r) are tabulated in Table 3.

In every system examined, inhibition of encystment increases as the population density increases; this relationship is reflected in the fact that r is less than zero. However, there is always the possibility that this might have occurred by chance, i.e., that a population of unrelated (with respect to R and p) sample points might have fallen into a linear pattern. Thus, it was necessary to determine the significance of r, i.e., the probability that r does not equal zero. According to Goldstein (1964), when

$$(3)\quad t < \sqrt{\frac{r^2(N-2)}{1-r^2}}$$

where N equals the number of sample points and t is the random variable of a t-Distribution, r is significant with a confidence greater than that for the value of t.

As Equation 2 indicates, the significance of r depends only on the value of r and N. Since system 3a (Table 3) has both the lowest value

TABLE 3. SELF-INHIBITION IN DIFFERENT ENVIRONMENTAL SYSTEMS

System Number	Suspending Medium	Temperature °C	Sample Points	Regression Line $R = a + bp$ $R = a + b \ln p$	Correlation Coefficient (r)	r^2
1	GC[a]	22	21	R = 0.898 - 0.081p	-0.884	0.781
				R = 1.055 - 0.378 ln p	-0.898	0.806
1a	GC	15	26	R = 0.900 - 0.079p	-0.770	0.593
				R = 1.012 - 0.349 ln p	-0.774	0.599
2	GM[b]	22	18	R = 0.991 - 0.061p	-0.863	0.745
				R = 1.007 - 0.203 ln p	-0.800	0.640
2a	GM	15	29	R = 0.985 - 0.051p	-0.774	0.599
				R = 1.060 - 0.230 ln p	-0.761	0.579
3	Na Phosphate 1 mM, pH 7.8	22	18	R = 0.989 - 0.098p	-0.905	0.812
				R = 1.157 - 0.447 ln p	-0.912	0.832
3a	Na Phosphate 1 mM, pH 7.8	15	15	R = 0.885 - 0.079p	-0.752	0.566
				R = 0.929 - 0.295 ln p	-0.738	0.545
4	Na Phosphate 2 mM, pH 7.8	22	18	R = 0.702 - 0.057p	-0.946	0.895
				R = 0.807 - 0.267 ln p	-0.927	0.859
5	Na Phosphate 2 mM, pH 6.1	22	17	R = 0.769 - 0.048p	-0.762	0.581
				R = 0.835 - 0.216 ln p	-0.752	0.566
6	K Phosphate 2 mM, pH 7.8	22	26	R = 0.757 - 0.041p	-0.782	0.612
				R = 0.794 - 0.167 ln p	-0.759	0.576

[a]Medium GC is defined in legend to Figure 28.

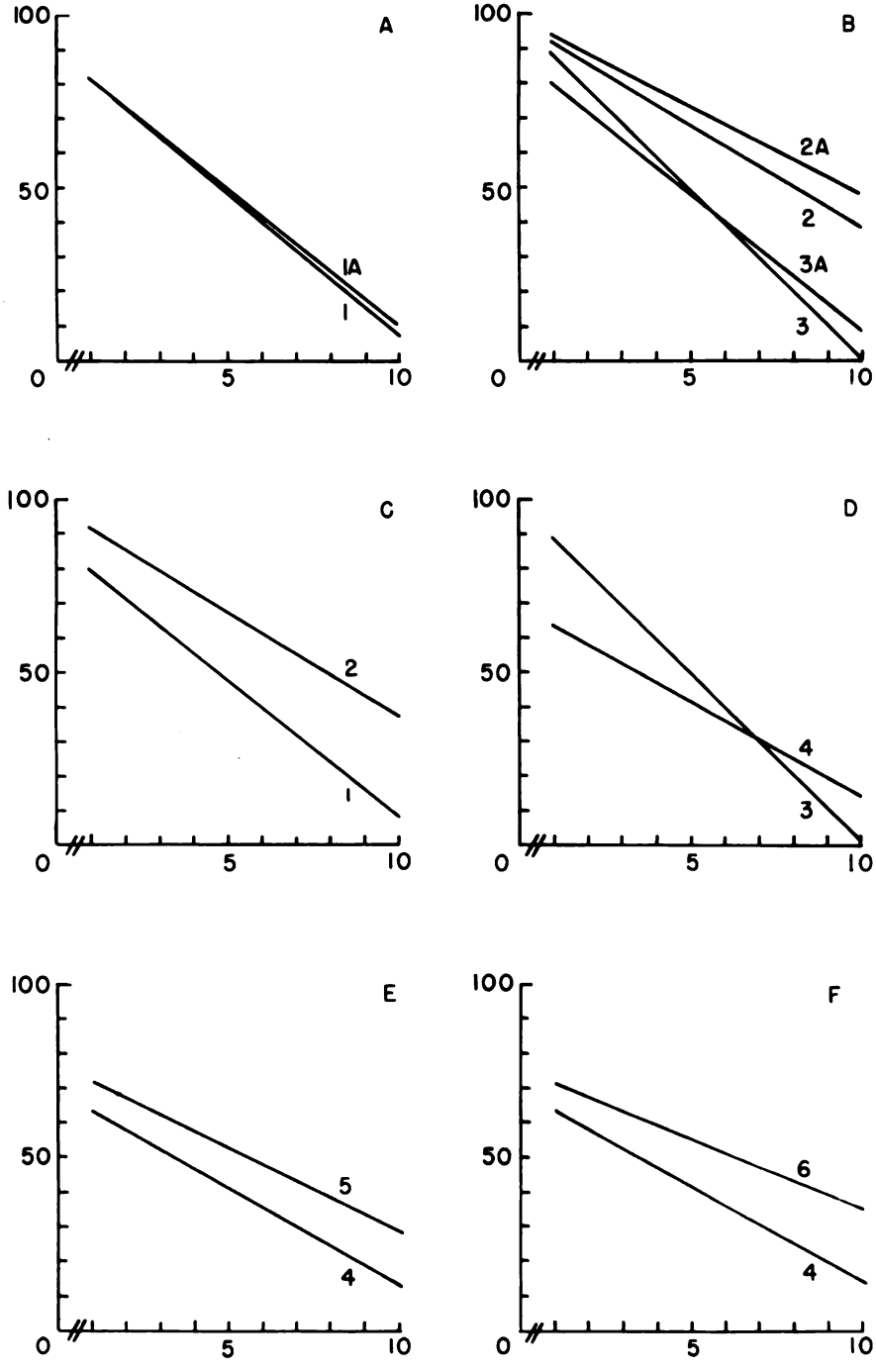
[b]Medium GM differs from medium GC in that Ca is replaced by an equivalent amount of Mg.

of r and N of all the systems tested, a significant value of r for this system implies a significant value of r for the remaining systems. Calculations made for system 3a, where r = 0.75 and N = 15, yield a value of 4.05, while t at a 97% confidence limit (as determined from a table of critical values for a t-Distribution; Goldstein, 1964) is equal to 3.01. Thus the correlation between inhibition of encystment and population density is highly significant, and apparently occurs under all conditions investigated (Table 3).

The regression lines for the nine systems investigated are plotted in Figure 29. In each graph (A, C, D, E, F), two systems are compared which differ with respect to only one variable (except B, in which two comparisons are made).

a. The influence of secondary incubation temperatures. Preliminary work had already suggested (Cantino et al., 1969) that the relationship between population density and encystment was not sensitive to the secondary incubation temperature. The influence of this secondary incubation temperature was further tested for medium GC, GM, and sodium phosphate by comparing systems 1 and 1a, 2 and 2a, and 3 and 3a, respectively (identified in Table 3). Two temperatures, 22°C and 15°C, were compared in each case. It is obvious (Figure 30, A, B) that, in all three media, the 7°C difference in secondary incubation temperature had little effect on the regression lines. However, it might also be argued that differences associated with changes in secondary incubation temperatures are reflected in the squared correlation coefficients (r^2) since they are significantly less for the lower temperatures (0.59 to 0.57 vs. 0.81 to 0.75). To a small degree, the difference may reflect increased scatter at 15°C, but most likely it results primarily from an alteration in the

FIGURE 30. Effect of environmental variables on self-inhibition in spore populations. Curve numbers correspond to system numbers in Table 2. Vertical axes: percent encystment; horizontal axes: spore/ml ($\times 10^{-6}$). Straight lines that best fit the data were established by standard regression technique; see text (Section VIII, B, 2) for details.


A
100
50
0
5
10
1A
1
B
2A
2
3A
3
C
2
1
D
4
3
E
5
4
F
6
4

functional relationship between R and p, i.e., a linear relation between R and p at 15°C is not as good an approximation of the actual relationship between these variables as it is at 22°C.

b. The relative effects of Ca and Mg. Since medium GC (systems 1 and 1a, Table 3) differs from medium GM (systems 2 and 2a) only in the substitution of Ca for an equivalent amount of Mg, the behavior of spores in these two media can be compared to determine the relative effects of Ca and Mg on self-inhibition. Regression lines for system 1 and 2 (Figure 30, C) are well displaced from one another with respect to percent encystment. A very small overlap of confidence intervals (not plotted) between these two systems supports the reality of this difference. It can be concluded with a fair degree of certainty, therefore, that less self-inhibition will occur with Mg than Ca for any specific population density between the limits of 10^6 and 10^7 spores/ml. A similar conclusion can be drawn from the comparison of systems 1a and 2a (not plotted; same media, but with a 15°C secondary incubation temperature).

c. The effect of different concentrations of Na phosphate. A comparison of systems 3 and 4 (Table 3, Figure 30, D) reveals the effect of doubling the concentration of sodium phosphate at pH 7.8. Although the rates of inhibition (slopes of regression lines) differ greatly, this difference tests with a confidence of only one 70% (test for non parallelism in Goldstein, 1964). However, the probability that the difference is real is strengthened by the fact that all three systems in 2 mM phosphate buffers (Table 3, systems 4, 5, and 6) have considerably lower rates of inhibition than the systems in 1 mM concentrations (Table 3, systems 3, 3a).

d. Comparison of phosphate buffers containing Na and K. In system 6, K is substituted for the Na in system 4. A comparison of regression lines for these two systems (Figure 30, F) shows significant displacement along the vertical axis, thus signifying consistently less inhibition with K (within the range 10^6 to 10^7 spores/ml.). In addition, the value r^2 changes from ca. 0.09 to 0.6 (Table 3); such a lowered value of r^2 indicates that the linear function (Equation 1) is not a very good approximation of the real relationship of R and p in the system containing K.

e. Comparison of Na phosphate at pH 7.8 and 6.1. In systems 4 and 5, sodium phosphate buffers at pH 7.8 and 6.1 are compared. Again, a shift along the vertical axis is noted (Figure 30, E). Although not as great as that obtained in a comparison of systems 4 and 6, it is nonetheless significant, displaying only a slight overlap of 90% confidence intervals. Thus, in Na phosphate, there is less self-inhibition at pH 6.1 than 7.8 At the lower pH, the value of r^2 is also smaller (ca. 0.6 vs. 0.9; Table 3). Thus, a linear relationship between R and p constitutes a relatively poor approximation for describing self-inhibition in the pH 6.1 system. Interestingly, the change from pH 7.8 to 6.1 yields a behavior pattern similar to that induced by substituting K for Na.

f. Summary. The results of the foregoing comparisons among nine systems examined for self-inhibition in the range 10^6-10^7 cells/ml. may be summarized as follows:

(1) In every system, inhibition of encystment increased as the population density was raised.

(2) In keeping with preliminary observations, the assay temperature (i.e., secondary incubation temperature) had surprisingly little effect on inhibition, although a small change was reflected in the comparative values of r^2.

(3) Substitution of Mg for Ca lowered self-inhibition.

(4) Doubling the concentration of Na phosphate from 1 mM to 2 mM lowered the rate of inhibition.

(5) The substitution of K for Na in 2 mM Na phosphate at pH 7.8 lowered self-inhibition and the value of r^2.

(6) A change of pH from 7.8 to 6.1 in a Na phosphate system lowered self-inhibition and the value of r^2.

C. ALTERNATIVE MEANS OF EFFECTING ENCYSTMENT

Thus far, two methods for regulating zoospore germination in B. emersonii have been discussed in some detail: cold treatment and control of population density. Below, additional ways of doing it are outlined briefly.

1. Effects of some inorganic salts.

The chlorides of K, Na, Rb, and Cs elicit encystment of zoospores under certain conditions (Soll and Sonneborn, 1969); Br^- and I^- are equally suitable as counter ions for K^+ and Na^+, but their Rb and Cs salts have not been tested. The K and Na salts of more complex anions such as $SO_4^=$ or $MoO_4^=$, which induce encystment to varying degrees, are not as effective as the halides (Soll, 1970). Neither LiCl nor NH_4Cl cause encystment; on the contrary, in every instance tested, LiCl strongly (but reversibly) inhibits encystment previously (or simultaneously) induced by other slats. $CaCl_2$ and $MgCl_2$ initiate encystment, but with inhibition of germ tube formation.

2. Effects of sulfonic acid azo dyes.

Sulfonic acid azo dyes such as Biebrich Scarlet and Methyl Orange trigger encystment quite effectively. For example, concentrations of Biebrich Scarlet as low as 0.05 mM induce 25% encystment in otherwise non-encysting spore suspension. The percent encystment, for a given quantity

of dye, can also be greatly increased by mixing the dye and suspension at 0-1°C, letting the mixture remain at these temperatures for 5-15 minutes, and then bringing it back to some higher temperature (e.g., 22°C).

Neither of the above dyes seems to stain markedly any particular spore structure or organelle. But, when added to Difco PYG agar media at concentrations sufficient to induce encystment, they inhibit growth after the early germling stage. Although a wide range of sulfonic acid azo dye structures apparently induce encystment, no attempt has yet been made to correlate structure with effectiveness. I can state, however, that the relatively simple structured Na benzene sulfonate is without effect, and that many cationic dyes cause swelling and lysis within a few minutes.

IX. KINETICS OF ENCYSTMENT

Soll and Sonneborn (1969) have made a detailed study of the influence of cellular and environmental variables on the germination kinetics of B. emersonii zoospores, and many of their results have already been cited in previous sections of this thesis. In their first paper (Soll et al., 1969), methods were presented for interpreting germination curves. Before considering them, we will discuss an alternative approach.

When induced by any of themethods heretofore described, encystment of spore populations follows a characteristic pattern of kinetics. If plots of encystment percentage vs. time are constructed, the curves are sigmoid, always display a slight lag before encystment is detected, and reach final encystment-percentage plateaus which are frequently below 100%. All evidence to date indicates that once a spore population reaches a plateau, there will be no additional encystment until the population is again induced to encyst. To illustrate: a typical spore population was induced with Biebrich Scarlet; encystment leveled off within 30 minutes, after which the plateau held steady for at least eight hours.

A. THE NORMAL DISTRIBUTION AS A MODEL

The sigmoid shapes of the curves described above indicate that the frequency of encystment may be normally distributed about a mean time. This hypothesis can be tested by plotting the data with modified coordinates such that encystment percentage is transformed (see, for example, Goldstein, 1964) into normal equivalent deviates (N.E.D.) or probits (N.E.D. + 5). Alternatively, the data can be plotted on normal probability paper.

In any event, sigmoid curves will now map linearly if encystment is normally distributed with respect to time. This is, in fact, what happens when encystment curves for spore populations which have attained 100% encystment are so plotted. However, for a population which exhibits a plateau under 100%, the mapping is not linear unless all encystment-percentage points are calculated relative to the final encystment percentage eventually attained by the population. In effect, this treats the encysting spores as if their behavior were independent of non encysting spores; the implications of this will be discussed shortly. It should be noted that the treatment of encysting spores as a separate population independent of non encysting spores is applicable to the limiting case where 100% of the spores encyst. Thus, this is the most general and comprehensive way by which to view the kinetics of encystment.

Since the normal distribution is an appropriate model for describing the time course of encystment of the zoospores of B. emersonii, the parameters which characterize a normal distribution are sufficient to characterize an encystment curve. There are only two such parameters, the mean and the standard deviation. The mean, in this instance, is the arithmetic average of the time it takes each encysting spore to encyst after induction; it will be referred to as the "mean time of encystment," $\bar{T}$. It also equals, as a result of the normal distribution symmetry, the time at which 50% of the encysting spores have encysted, and it may be read directly from encystment curves.

The other parameter, the standard deviation (s), can be used to completely and quantitatively represent the synchrony of an encysting population. It is a measure of the dispersion of individual spore-encystment times about the mean time of encystment. For a population of n

spores with individual encystment times T_i, it may be specifically defined as:

$$(4) \quad s = \sqrt{[1/(n-1)] \sum_{i=1}^{n} (T_i - \bar{T})^2}$$

However, it is not essential to determine s with this equation because the slope of a transformed (i.e., to linear form) encystment curve is equal to 1/s. Thus, both $\bar{T}$ and s can easily be determined from transformed encystment curves (see curve 6, Figure 31). With the aid of these two parameters, any information about the time course of encystment can be calculated. Several illustrations follow.

Suppose it is desired to determine how long it takes 97.5% of a spore population to encyst after induction. It is only necessary to look up 0.975 in a table of areas under the standard normal density function and read off the corresponding distance from the mean in terms of standard deviations (= 1.96). Therefore if, for example, s = 6.9 minutes (as in Exp. 6, Table 4), it would take 6.9 x 1.96 = 13.52 minutes to go from $\bar{T}$ to 97.5% encystment. Since $\bar{T}$ = 28.9 (Table 4), the total time required for 97.5% encystment = 28.9 + 13.5 = 42.4 minutes.

Alternatively, if it is desired to determine how long it takes a spore population to go from X% to Y% encystment, it is only necessary to establish the distance of each from $\bar{T}$ in terms of standard deviations as determined above, and then to calculate the interval in minutes.

The foregoing methodology for analyzing and characterizing germination curves offers some distinct advantages over that employed by Soll et al. (1969) in their recent interesting work with B. emersonii. While they recognized that encystment was normally distributed, they did not choose to exploit this fact; rather, they used the sigmoid curve per se to determine two basic parameters, zoospore "T_{50}" and zoospore "major slope."

The T_{50} was defined as the time necessary for 50% of the zoospores to become round cells (i.e., encyst). The major slope was the slope of the straight line used to approximate the "major rise (or fall) portions" of encystment curves, and was used as a measure of synchrony. However, in comparison with s, the major slope is an inferior measure of synchrony for three reasons: first, it rests on the assumption that encystment is uniformly distributed over a selected interval, which it is not; second, it can be greatly influenced by the magnitude of the specific interval about T_{50} through which the approximating line is drawn; and third, it is affected by the degree of uniformity with which data points are distributed within this specific interval. Such difficulties are further complicated by the fact that the combination of T_{50} and the major slope does not fully characterize the germination curves; to extract additional information from them, new parameters such as the initial lag period, T_{80}, and T_{90}, had to be utilized.

B. COMPARISON OF INDUCTION METHODS

In these studies of the kinetics of encystment, three inducers were used: cold incubation, sulfonic acid azo dyes, and KCl. The data were plotted and characterized by the standard methodology described in the previous section. Representative linear mappings, derived by transformation of the kinetics data, are delineated in Figure 31; additional examples, summarized in terms of $\overline{T}$, s, and maximum percent encystment, are listed in Table 4.

The tabular data reveal that neither mean time of encystment nor synchrony are dependent on the maximum percent encystment. This observation holds true regardless of the method by which maximum percent encystment is regulated--for example, for altering the time spores are incubated

FIGURE 31. Time course of encystment after different induction procedures. Curve 1, cold induction; Curve 3, Biebrich Scarlet combined with cold; Curve 6, KCl. The curve numbers correspond to Exp. no. 1,3,6 in Table 4. A method for determing $\overline{T}$ and s is illustrated with curve 6. Data have been transformed to linear mappings by using a normal probability scale.

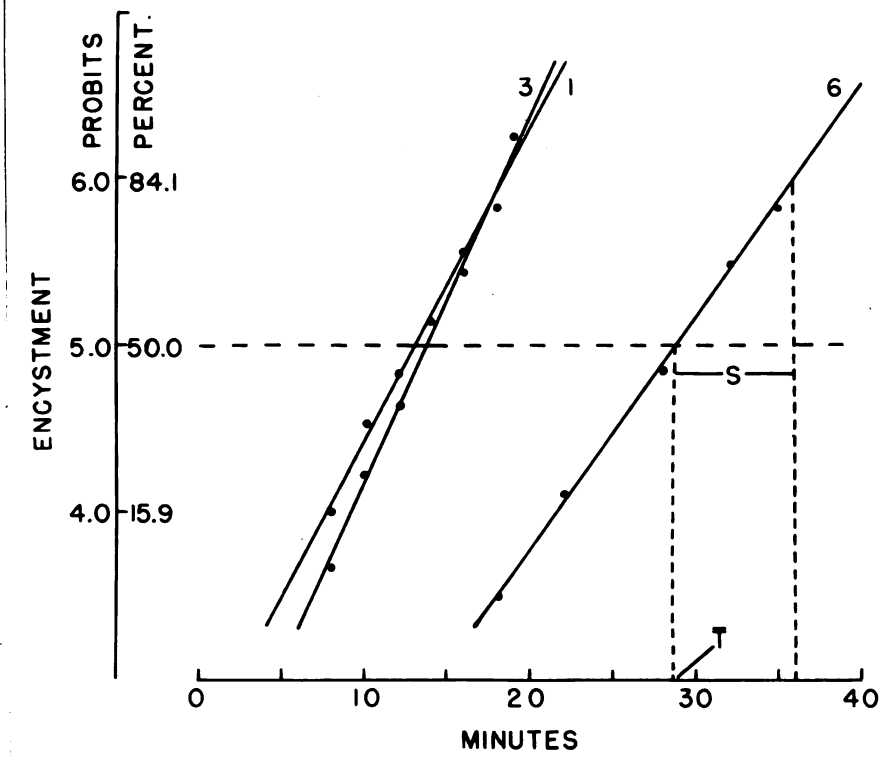

ENCYSTMENT
PROBITS
PERCENT.
6.0
84.1
5.0
50.0
4.0
15.9
3
1
6
S
T
0
10
20
30
40
MINUTES

TABLE 4. COMPARISON OF DIFFERENT INDUCTION METHODS

Experiment Number	Method of Induction[a]	Population Density (spores/ml. x 10^{-6})	Maximum % Encystment	$\bar{T}$ (minutes)	s
1	2.5 hr. at 0° - 1°C	4.04	31.4	13.1	5.4
2	2.0 hr. at 0° - 1°C	2.18	22.3	14.2	3.8
3	Biebrich Scarlet (1 mM) with 15 min. at 0°-1°C	1.9	100.0	13.7	4.5
4	Biebrich Scarlet (1 mM)	4.3	43.7	12.7	4.2
5	Methyl Orange (0.5 mM)	4.7	14.3	14.2	4.8
6	KCl (50 mM)	3.5	100.0	28.9	6.9
7	KCl (50 mM)	2.7	55.0	29.6	8.9
8	KCl (25 mM)	4.1	16.0	30.7	9.1

[a] For Experiments 1 and 2, the spore suspension was incubated at 0° - 1°C and then brought to 22°C (zero time); for Experiment 3, it was pre-chilled to 0° - 1°C, mixed with pre-chilled dye, and then brought to 22°C after fifteen minutes (zero time); for Experiments 4 - 8, the cold incubation was omitted (zero time measured from time of addition of dye or salt to spore suspension). In all cases, spore suspensions were harvested in Na MOPS buffer (2 mM, pH 7.8).

at 0-1°C for the cold-induced encystment; by including (or not including, as the case may be) a short cold-incubation (see Section VIII, C, 2) with the azo dye induction, or by substituting one dye for another (i.e., Methyl Orange for Biebrich Scarlet); or by varying KCl concentrations. It is obvious from these observations that the interplay between endogenous and exogenous factors which regulate the fraction of the spores in a population that encysts does not affect the time it takes a spore to encyst after induction.

If the process of induction is conceived of as a trigger for the succeeding processes of encystment, then for each specific methods of induction the trigger is (for each spore) an all-or-none event that does not affect the rate of encystment.

Within this conceptual framework, an additional observation must be rationalized. Spores induced to encyst by cold and sulfonic acid azo dyes have a mean time of encystment about half of that for spores induced by KCl. Apparently, the rate of the triggering process will not account for the differences in $\overline{T}$, for it has been determined (Soll, 1970) that as little as 30 seconds of contact with 50 mM KCl is sufficient to trigger complete encystment in a population of zoospores. Thus, the higher value of $\overline{T}$ for KCl-induced cells must result from differences in subsequent (i.e., secondary) events leading to encystment. One might speculate that the increase in $\overline{T}$ results from an inhibitory effect of KCl on these subsequent processes, but this does not seem likely because variations in KCl concentrations do not affect $\overline{T}$. The triggering of encystment with cold or azo dyes, as compared with the triggering by KCl, must increase the average rate and/or decrease the number of secondary processes which lead to cyst formation.

X. CONCLUDING REMARKS

The spore of Blastocladiella emersonii contains a highly ordered arrangement of membrane-bound organelles. During encystment, this subcellular assemblage is rapidly disorganized by a cascade of changes: the flagellum is retracted; the nuclear apparatus rotates; the cell becomes spherical, loses volume, and forms a cyst "wall". Substantial vesiculation accompanies the process.

This succession of events is temporarily coordinated and spatially integrated: Axonemal translocation and rotation of the nuclear apparatus results from structural-mechanical transformations requiring no special energy sources or mechanisms other than those needed for the processes associated with encystment. Slightly before and during cyst wall formation, the decay of Gamma particles generates vesicles that fuse with the plasma membrane and thereby alter it; presumably they bring enzymes and/or structural components to the cell surface to lay down the foundation for synthesis of the cyst wall. Moreover, vesicles fusing the plasmalemma may, on the one hand, contribute to the volume decrease associated with encystment; on the other hand, by depositing new cyst wall material, they may also be generating the surface forces needed for the change in cell shape and translocation of the axoneme.

Above all this there also stems the question: by what means are such events prevented in a non-encysting spore? There must be interlocking ways by which a zoospore keeps some things shut down. A simple on-and-off inhibitor-mediated process could underlie one of them, for Schmoyer and

Lovett (1969) provide direct evidence for an internal inhibitor that suppresses protein synthesis, and I have indirect evidence that a substance released by spores is capable of regulating encystment.

Yet, satisfying though it is to achieve simple answers to complex problems, the existence of such chemical agents stimulates new questions. One of them is especially meaningful: should the unidentified material which decreases percent encystment in a zoospore population be viewed as an encystment inhibitor or as a zoospore stabilizer? The significance of this question goes well beyond the problem in semantics that it poses. Suppose it could be shown unequivocally that the encystment "inhibitor" actually stabilizes zoospores in the sense of buffering them against adverse environments. For example, if the "inhibitor" prolonged the time that swimming spores could withstand some new external stress, or the proportion of them that lysed during or after the stress, then "stabilizer" might be the more appropriate term to use. Already there is some evidence to suggest that the stabilizer concept is preferable to the inhibitor concept, at least insofar as it applies to those properties of spore supernatants that prevent encystment. I find that spore populations most reluctant to encyst upon induction are also the ones that most frequently exhibit the greatest resistance against cold-induced lysis. Soll (1970) also reports that zoospores derived from plants grown under relatively crowded conditions, as compared to cultures at lower population density, are more resistant to KCl-induced encystment and survive longer in balanced salt solutions. Hopefully, these suggestive observations will stimulate more investigations in this direction.

In any case, returning to the question of causality, the zoospore's internal architecture may also be operating to prevent encystment; unfortunately, it is exceedingly hard to demonstrate. The notably compact nature

of the Gamma matrix could conceivably suppress any enzyme activity contained within. The GS membrane around it might also serve a regulatory function, i.e., *via* selective permeability. However, it is also clear that sub-cellular compartmentalization need not always provide a limitative function; the membrane surrounding the ribosomes in the nuclear cap is obviously not needed to inhibit protein synthesis, for this can be accomplished by way of the inhibitor. It may be, therefore, as Schmoyer and Lovett (1969) suggest, that the function of the nuclear cap membrane is to protect inactive ribosomes from degradation in a non-germinating zoospore of *B. emersonii*. But even this seemingly logical answer may not be correct, for there are other uniflagellate fungi (e.g., *Rhizidiomyces* and *Monoblepharella*; Fuller and Reichle, 1965, 1968) in which non-encysting zoospores carry around a cap-like ribosomal aggregare not "protected" by a surrounding membrane. In fact, in some zoospores (e.g., *Olpidium*; Temmink and Campbell, 1969a), ribosomes are evenly scattered throughout the cytoplasm. Perhaps the primary--if not the only--role of the nuclear cap membrane in the zoospore of *B. emersonii* is to provide an immediate source of endoplasmic reticulum for early protein synthesis.

This inquiry into the nature of encystment also calls for brief consideration of some aspects of the kinetics of encystment. Spores induced with low temperatures and sulfonic acid azo dyes display similar kinetics, while spores induced with KCl show a much greater mean time of encystment. The evidence suggests that the difference in behavior must result from an increase in the number and/or the average rates of encystment "processes" which ensue *after* triggering. When the reason for this difference is uncovered, an important aspect of encystment will have been resolved. But, in the meantime, a comparison of similarities among induction methods may also be fruitful. First, as far as I can tell, they all yield essentially

the same sequence of structural changes associated with encystment, from which it can be argued that all induction methods must affect pathways that converge at some common step or process. Second, they all involve the placement of a spore under great stress. During cold incubation, the cell eventually becomes balanced on the verge of lysis as it takes up water and approaches its "elastic limit." In sulfonic acid azo dyes, the effects are not so dramatic, but experience shows that spores in contact with these dyes are more labile to the effects of other environmental factors such as cold incubation, fixation, etc. In KCl, spores are also being pushed toward their limit; in 50 mM KCl, there is a little lysis, and at higher concentrations lysis is substantial.

One way of harmonizing these observations into a unified concept of the trigger mechanism is to view the induction step as a perturbation of the delicate balance of cellular controls in a spore. A momentary imbalance could evoke emergence of the new set of interrelated processes which comprise encystment. If this accurately represents what takes place, then it would be most enlightening to find answers to the question: what specific cellular controls will, when disturbed, lead to breakdown of other control mechanisms?

Diverse induction methods may disrupt different control mechanisms. Disengagement of some of them will be sufficient to induce encystment; disengagement of others will not, and may, instead, cause autolysis or cell death. For example, cold incubation may be the type of trigger that interferes in a non-discriminating fashion with control processes in the zoospore. If it interferes with those critical things that suppress encystment without tampering with those that cause cell death, the spore will be induced to encyst. It can also be supposed that when the different induction methods act on dissimilar controls, the results may lead to the breakdown

of others. Depending on what is first attacked, the sequence of subsequent disruptions will probably vary, as well as their rates. Thus, the first control mechanism to be attacked will establish the rate of subsequent events; this will be reflected in the value of $\overline{T}$ for the particular induction method used.

Finally, one may well ask, what does all this have to do with the real world of aquatic fungi, and the more "natural" agents that induce their motile propagules to encyst? Mycologists and phytopathologists have recognized for many years that the element of change plays an important--albeit a poorely understood--motivating role in germination. This knowledge is reflected in generalizations of the following sort: "Under circumstances which provide for prolonged motility...encystment can be readily induced, often quite quickly, by changing some existing environmental factor, or introducing a new one, be it physical or chemical" (Hickman and Ho, 1966). The fact that we have disturbed spores in the laboratory with treatments harsher than some of those generally encountered in nature should not mask the utility of these laboratory techniques for uncovering fundamental aspects of the encystment process. It was demonstrated for the three induction methods examined that the severity of the perturbation only affects zoospore stability (whether this be measured as the number of spores that encyst or the number that lyse); it has no demonstrable effect on the processes associated with encystment. Although, ideally, less drastic methods of induction might seem to be preferable, there are practical reasons for continuing to use these experimental methods. Primarily, less drastic methods do not yield the high levels of encystment or synchrony needed for certain kinds of work. Secondarily, the high population densities required for many biochemical and other kinds of experiments markedly inhibit encystment; more stringent methods of induction are therefore required.

In my experience with B. emersonii, what may be the most "natural" method of inducing encystment is simply to dilute a zoospore suspension to a lower population density. Unfortunately, this procedure is much less effective than the other methods used; furthermore, it results in spore suspensions of lower density. Some preliminary investigations indicate that its kinetics may be different than that for the other methods of induction. Further studies with this form of induction are certainly needed.

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APPENDIX

APPENDIX A

Methods

A. GROWTH AND HARVESTING OF ZOOSPORES

OC plants were routinely grown in the dark on Difco PYG agar (Bacto-Peptone, 1.25 g.; Bacto-Yeast extract, 1.25 g.; Bacto-Dextrose 3.0 g.; Bacto-Agar 20.0 g. per liter) at 22°C in 100 cm. diameter plastic Petri plates. The inoculum consisted of 1 ml. of suspension containing roughly 10^5 zoospores. This was sufficient to leave a thin film of liquid over the agar surface into which, after 21-24 hours growth, the mature OC plants would spontaneously discharge spores. The zoospores assumed elongate amoeboid characteristics and persisted for hours before encysting under these conditions. (In plates with a dry surface, spores may encyst within minutes after they are discharged.) Plates were flooded with 8-12 ml. of a medium (the composition of which depended on the specific experiment) after 25-75% of the plants had discharged. The resultant suspension was left on the plates for 15 minutes, then passed through filter paper (and collected). When concentration or washing of the suspension was required, it was chilled in an ice-water bath for five minutes and then centrifuged at 1,300 x G for three minutes at 0-1°C. The pellet was resuspended in ice cold medium. Any additional centrifugations were shortened to 2.5 minutes.

B. PHASE MICROSCOPY

Observations were made with a Wild M20 phase microscope using a Busch and Lomb ribbon filament light source. For photomicrography, the

microscope was fitted with a Kodak Pony II 35 mm. camera back. Photographs were taken through the 40X objective on Kodak panatomic X film exposed for 1/10 second with the light source set at highest intensity.

Temperature control of the speciman was achieved with an infrared filter placed between light source and microscope and, in instances where temperature regulation was especially important, with a controlled temperature stage. The latter consisted of a copper plate (11.5 x 6.5 x 0.1 cm.) soldered to a partially flattened U-shaped section of copper tubing (0.8 cm. ID). The microscope slide lay within the U of tubing and over a 1.3 cm. hold drilled in the plate to allow light transmission. The temperature of the apparatus was controlled by passing water, at the desired temperature, through the copper tubing. The temperature at any given position along the slide could be measured with a very fine copper-constant thermocouple.

C. THE DETERMINATION OF VOLUME CHANGES DURING ENCYSTMENT

Spores from one plate were harvested at room temperature in 10 ml. of distilled water and filtered. Encystment was induced by diluting the suspension 20 fold with a 2mM (each) NaCl-KCl solution. Samples of the diluted suspension were run through a Model B Coulter Counter equipped with a size distribution plotter (Coulter Electronics, Inc.) at 5 and 15 minutes after dilution to determine the distribution of spore volumes. Solution temperature was 22.5 C,and the Coulter Counter settings were: 1/AP = 0.707, 1/AM = 1, count interval = 4 seconds.

D. THE MECHANICAL DEFLAGELLATION OF ZOOSPORES

Spores from one plate were harvested in distilled water and resuspended in ca. 10 ml. of 1 mM each $MgCl_2$, $CaCl_2$, and NaCl, and 5 mM KCl. Half of the suspension was used as control and the other half was deflagellated in a 8 x 0.8 cm. test tube by aerating vigorously with air

passing through a Pasteur pipet. Samples from the control and deflagellated suspension were observed and photographed through the phase contrast microscope during encystment. Replicate samples were also plated onto PYG agar and scored for single plants and clones after one and three days growth at 22°C.

E. ENCYSTMENT ASSAYS

Two methods of assaying encystment, the slide assay and the shaking flask assay, were developed.

1. The slide assay: Five-hundredth ml. of spore suspension was pipetted onto the center of a (clean glass) microscope slide preincubated for at least 15 minutes at the assay temperature (new Thomas red label micro slides were used for all the experiments cited in this thesis and no additional cleaning was required). A paper box cover, ca. 1 inch high and saturated with water, was placed over the slide for the assay incubation period (usually 30 minutes), during which the cells settled to the slide surface and encysted. At the end of this time, a 10 ml. beaker, partially stuffed with paper wetted with either 2% OsO_v or 25% glutaraldehyde, were inverted over the (drops of) suspension to fix the cells. They were examined by phase microscopy at a convenient magnification (usually between 200 and 600X) and the number of encysted and non-encysted cells in a field scored. A sufficient number of fields were examined until more than 300 cells were counted.

2. The shaking flask assay: The routine procedure consisted of adding 3 ml. of suspension to a 25 ml. Erlenmyer flask mounted in a controlled temperature shaking platform water bath (Eberbach Corp.). The flask was slowly agitated (stroke, 2.5 cm.; 70 cy/sec) at the desired incubation temperature for 30 minutes, after which the cells were fixed by adding

either an equal volume of 4% glutaraldehyde containing 2 mM $CaCl_2$ or 0.6 ml. of 25% glutaraldehyde. The concentration of non-encysted cells was determined with a counting chamber (either a hemocytometer or an Eosinophil counting slide). A sample of original suspension (before incubation) was also fixed and counted similarly, and with this the percent encystment was calculated.

F. THE INHIBITORY EFFECT OF SUSPENSION SUPERNATANTS

1. Preparation of supernatants: Spore suspensions were harvested, washed, and resuspended in medium GC (0.5 mM Na phosphate, pH 7.8; 0.2 mM $CaCl_2$; 5 mM KCl; 1 mM Na glutamate) by the standard procedure, maintained in an ice-water bath for 90 minutes, then divided into two equal portions. One was immediately centrifuged for 3 minutes at 1,000 x G at 0°C, decanted from the pellet ot spores, and again centrifuged for 10 minutes at 9,500 X G at 0°C. to remove any spores that may have remained after the first spinning. The supernatant was stored in an ice bath until used (ca. 2.0 hrs.). It is referred to as the "cold supernatant."

The second aloquote was placed on a wrist shaker and slowly shaken for 20 minutes at room temperature (22°C) then removed, chilled quickly in an ice-water bath for 5 minutes, centrifuged by the same procedures used to isolate the cold supernatant, and stored in ice bath until used (ca. 1.75 hr.). This supernatant is referred to as the "warm supernatant."

2. Assay procedures: In order to examine the two supernatants for inhibitory properties, a new suspension of spores was harvested, washed, and resuspended in medium GC by the standard procedure. The population density was determined with a Coulter Counter and adjusted to 1×10^7 spores/ml. by the addition of cold GC medium. The suspension was kept at ice-water bath temperature for 90 minutes (measured from the time of filtration onto ice). Then, six different mixtures were prepared for assay.

Three of these contained 2.5 ml. GC medium, "cold supernatant," or "warm supernatant" (all maintained at ice bath temperature). The other three consisted of 0.5 ml. of spore suspension and were brought to a final volume of 5.0 ml. by the addition of 4.5 ml. of either GC medium, "cold supernatant," or "warm supernatant." The first three mixtures will be referred to as the 50% supernatant series, while the remaining three will be referred to as the 90% supernatant series, since the added supernatants (or GC medium in the control) have been diluted to 50% (and 90%) of their original concentrations. All of the above mixtures were assayed by the slide method within 2 minutes after preparation.

G. ELECTRON MICROSCOPY

1. Induction of encystment: Spores were grown and harvested by standard procedures and then caused to germinate by four different induction procedures (IP).

IP-1. Cold shock. Spore suspensions (2×10^6 spores/ml.) were filtered, chilled in an ice bath for two hours, and then transferred to a shaking water bath at 22°C (shaking rate, 88 cy./min.; stroke, 1.5") at which time germination commenced.

IP-2. Biebrich Scarlet. Ice cold biebrich scarlet (2 mM) containing either sodium phosphate buffer (2mM), pH 7.8, or sodium cacodylate (10 mM) was added to an equal volume of an ice-cold spore suspension (2×10^6 spore/ml.). After 15 minutes, the suspension was transferred to a shaker at 22°C as in (IP-1) above.

IP-3. Washing with salt solution. The spore suspension was washed once at 2°C with a solution containing 10 mM sodium cacodylate, 5 mM magnesium chloride, and 1 mM potassium chloride by centrifugations at 100 x g for 2.5 minutes as described previously (Cantino et al., 1968).

The washed suspension was kept in an ice bath (ca. 10 minutes) until adjusted to a population density of 10^7 spores/ml.; spores were then induced to germinate by adding 5 ml. of this suspension to 25 ml. of fresh washing solution at 25°C in a shaker as used in (IP-1) above.

IP-4. Salt effect. A Petri plate (10 cm. diameter) of discharging plants was flooded with 4 ml. of a solution of sodium cacodylate and calcium chloride, 10 mM each. Three ml. of the resulting suspension (3×10^6 spores/ml.) were removed, kept at ca. 23°C, observed with a prime microscope, and fixed after the spores started to clump; the acquisition of adhesive properties occurs just prior to flagellar retraction (Cantino et al., 1968).

In the foregoing procedures, the time of transfer to the shaking water bath was defined as zero time. Samples for fixation were usually taken at this point and at successive two or three minute intervals thereafter.

2. Fixation: The following solutions were used in the five fixation procedures listed below.

Solution A: 2% glutaraldehyde in 10 mM sodium cacodylate.

Solution B: 1% osmium tetroxide in 10 mM sodium cacodylate.

Solution C: 5 mM potassium chloride and magnesium chloride in 10 mM sodium cacodylate.

Solution D: 0.5% osmium tetroxide and 5 mM magnesium chloride in 10 mM sodium cacodylate.

Solution E: The supernatant from a mixture of 0.1 g uranyl acetate, 25 ml. water, and 2.5 ml. 10^{-1} sodium cacodylate after standing 24 hours at 6°C.

All spore suspensions were fixed at 0-4°C.

Fixation I. Equal volumes of Solutions A and B kept at 0-4°C were mixed immediately before use. To this was added an equal volume of spore suspension. After one hour, the spores were pelleted by centrifugation at 1,000 x g for 3 minutes, and washed three times during the next four hour period with 6 ml. volumes of salt solution C. The pellets were post fixed for 18 hours in Solution D and finally washed two times in Solution C before dehydration. This method was used with induction procedure (3).

Fixation II. To a 1:1 mixture of Solutions B and E, an equal volume of spore suspension was added. After 18-24 hours, the spores were pelleted and washed twice with Solution C at 1,000 x g. This method was used with induction procedures (2) and (3).

Fixation III. A fresh mixture of 1% glutaraldehyde, 0.1% osmium tetroxide and 10 mM sodium cacodylate was added to an equal volume of spore suspension. After 10 minutes, spores were pelleted at 1,000 x g for 3 minutes and fresh fixative added. After 2 hours, they were washed as in (1) above. This method was used with induction procedure (4).

Fixation IV. Glutaraldehyde followed by osmium tetroxide according to Lessie and Lovett (1968); this was used only with induction procedures (1) and (2).

Fixation V. Permanganate (2%) according to Shaw and Cantino (1969); this was used only with induction procedure (2).

Fixed spore pellets were dehydrated in ethanol and embedded in Epon according to Shaw and Cantino (1969). Sections were stained with lead citrate (Venable and Coggeshall, 1965) and/or uranyl acetate. Electron micrographs were made with Zeiss EM-9A and Phillips 100 electron microscopes. The electron micrographs chosen for display in this thesis were selected

from over 500 made during the study of encystment. They are not unique in what they demonstrate, but were chosen because they best represent the normal course of events.