BACTERIOLOGICAL REVIEWS, Dec. 1975, p. 345-404
Vol. 39, No. 4

Printed in U.S.A.

Growth and Differentiation of the Water Mold *Blastocladiella emersonii:* Cytodifferentiation and the Role of Ribonucleic Acid and Protein Synthesis

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INTRODUCTION

Analytical studies of development in eukaryotes by using the methods of cell and molecular biology have become commonplace. The primary stimulus for this has been the impressive progress achieved by the application of such methods to the study of bacteria and viruses. The development of higher, multicellular plants and animals involves large numbers of cells and in many cases the irreversible differentiation of specialized cell types. Such cell interactions and functional specializations seem certain to require much more complex regulatory mechanisms for their coordination than could be required in a single-celled bacterium, and the increased complexity in requirements is reflected in the larger amount and greater genomic complexity of the nuclear deoxyribonucleic acid (DNA) (131). Eukaryotic microorganisms with their smaller size, reduced developmental alternatives, and smaller genome, size (e.g., *Phycomyces* with a DNA content 6.7 times that of *Escherichia coli* [63], as compared with about 600 times for mammalian cells [131]) present experimental systems that, in theory at least, should be much more easily analyzed. The primary virtues of such simple organisms are their ease of culture, rapid growth at high cell densities in defined media, short and often manipulable life cycles, adaptability for mutant methodology, and, frequently, ease of producing synchronous differentiation in large cell populations. A number of fungi share many of the above characteristics, but in only a few has the potential been exploited at all extensively in the study of development. The unicellular yeast *Saccharomyces cerevisiae* fulfills the criteria for an optimal developmental analysis perhaps better than any other fungus and has been reviewed in detail elsewhere (100, 104, 248).

The nonfilamentous, lower aquatic fungi represent another group with considerable potential for developmental study (34). Many of these fungi are saprobes that grow on decaying organic materials in freshwater streams, ponds, and roadside ditches and can be isolated easily and maintained in pure culture (67, 230). Relatively few of these organisms have been studied in any detail, but some species have very simple growth requirements, requiring only an organic carbon source and inorganic salts for

growth; others may require organic nitrogen compounds, an organic sulfur source, and one or more vitamins (31). The majority of these fungi produce either of two sporangial types: thin-walled, ephemeral zoosporangia under conditions satisfactory for growth, and thick-walled, often pigmented resistant sporangia when subjected to unfavorable conditions. The uniflagellate zoospores released from these sporangia are uninucleate, but the growth-phase cells develop as multinucleate coenocytes, often sac-like in shape, with a number of branching filaments or rhizoids that grow outward to penetrate the organic substrate. Because of the limited rhizoid growth, such organisms frequently can be grown in suspension culture by using standard microbiological procedures and equipment (148). The variety of life cycles and morphology found in these lower fungi (class, *Chytridiomycetes*) and the methods used for their isolation and culture have been described by Emerson (67), Sparrow (230), and others (232).

The organisms in one order, the *Blastocladiales*, and, in particular, three genera that differ greatly in their growth forms and other characteristics have been studied extensively. *Blastocladiella emersonii*, a saprobe, grows as a simple saclike cell with basal rhizoids as described above and produces a single zoosporangium or resistant sporangium on separate thalli. *Allomyces* species, such as *A. arbuscula* and *A. macrogynus*, are also saprobic, coenocytic organisms but grow as branching mycelia on which both zoosporangia and resistant sporangia are produced in large numbers. They also have alternating haploid mycelial phases that produce male and female gametes. (For details of the variety of life cycles in this interesting genus, see Emerson [66].) The third genus, *Coelomomyces*, is an obligate parasite of mosquito larvae (51, 52) and has only recently been discovered to have the metazoan *Cyclops* as a second and apparently obligate host (266). Virtually nothing is yet known concerning the biochemistry of *Coelomomyces*, although it is of considerable interest due to its potential for the biological control of mosquitoes. Considerable work has been done with both *Allomyces* and *Blastocladiella*, but *Blastocladiella emersonii*, in particular, provides a developmental system with most of the desired characteristics for experimental study outlined earlier. Its only drawback is that techniques for genetic analysis are not yet available since it has no known sexual reproduction. *Blastocladiella* grows rapidly as a single-celled coenocyte, and the timing and type of differentiation can be manipulated with ease. Furthermore, any stage in the life cycle can be induced to occur synchronously by using suspension cultures in chemically defined media and without recourse to special or time-consuming procedures. The earlier work of Cantino and co-workers with *B. emersonii* was largely centered on the differences in enzyme activities of cells developing along the resistant sporangial and zoosporangial pathways and has been reviewed in detail (31, 34), as have recent studies on the form and function of zoospore gamma particles (38, 175). (A color film of the life cycle entitled "Pathways of the Water Mold *Blastocladiella emersonii*," prepared under the direction of E. C. Cantino, is available from BFA Educational Media, Santa Monica, Calif.)

Three areas of research with *Blastocladiella* that have been emphasized in our own laboratory but not previously reviewed are intracellular differentiation, ribonucleic acid (RNA) synthesis, and protein synthesis. The cytodifferentiation of *Blastocladiella* has now been examined in considerable detail through all stages of the life cycle with both light and electron microscopy, and the RNA and protein synthetic patterns and rates have been studied during the same stages. It thus seems worthwhile to review and integrate these two types of information, particularly because the most striking changes in synthetic rates are closely correlated with the periods of active cellular differentiation in the life cycle.

In the discussion an attempt has been made to integrate what is known about the regulation of RNA and protein synthesis during differentiation in *Blastocladiella* with the information available for other fungi and a few additional microorganisms. The emphasis on RNA and protein turnover in the discussion was stimulated by an earlier essay of Mandelstam (159) in which he emphasized the striking biochemical similarities found in a variety of microorganisms undergoing sporulation. The only fungal system considered by Mandelstam was yeast sporulation; however, as will become apparent, the conditions that induce sporulation and the subsequent biochemical events during differentiation of *Blastocladiella* and a number of other fungi also display many features in common with other microorganisms. The role of fungal RNA and protein synthesis in development will be discussed from this point of view.

GROWTH AND DIFFERENTIATION

The growing plant of *B. emersonii* can follow either of two basic developmental pathways, depending upon its environment. One of these leads to an ephemeral zoosporangial plant (also

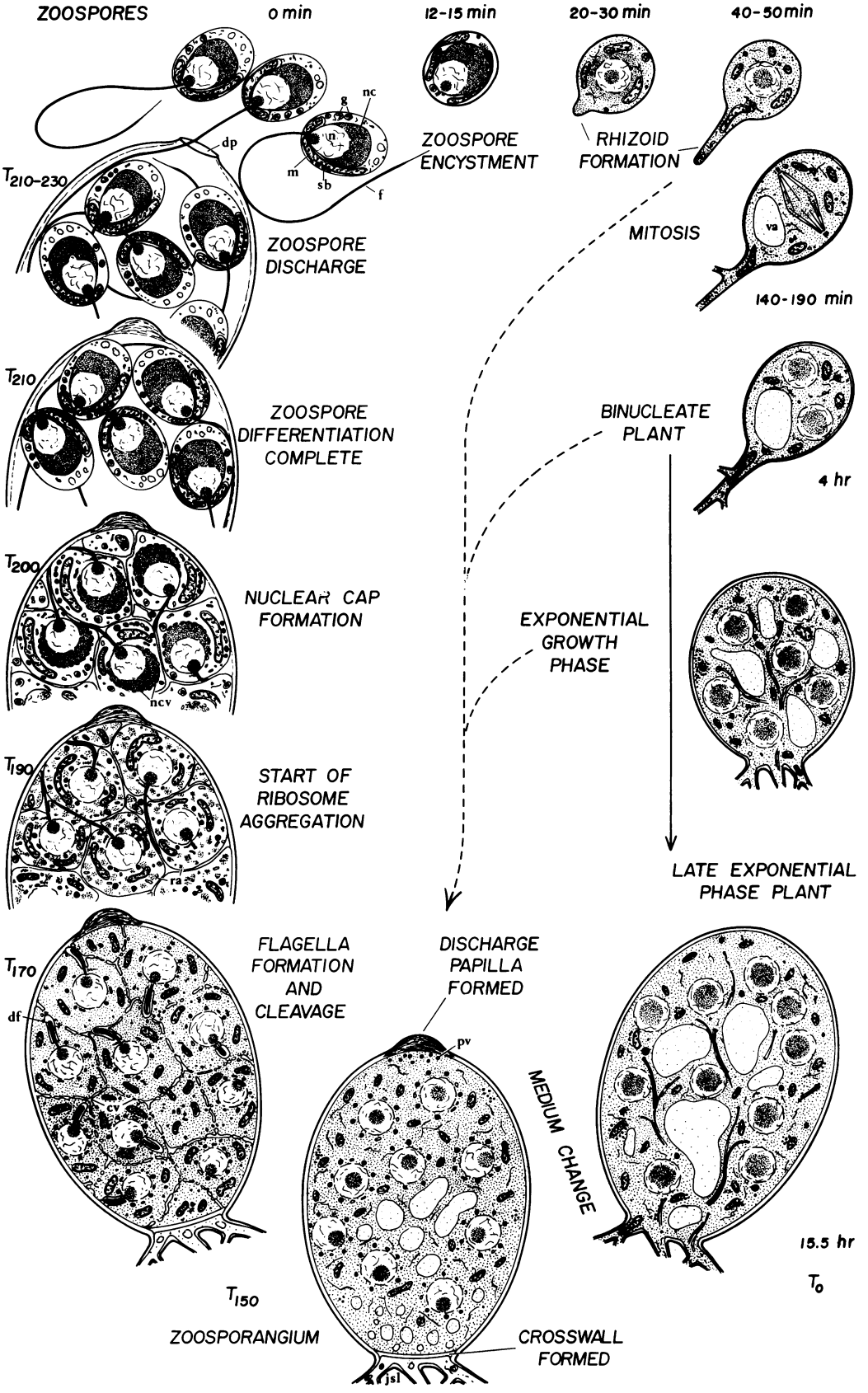
called an ordinary, colorless plant) that produces and discharges a large number of zoospores at maturity. The second results in a dormant, thick-walled, resistant sporangium that may persist as such for long periods but which also eventually differentiates and discharges zoospores (31, 34). The zoosporangial cycle normally occurs under conditions favorable for rapid, unrestricted growth, whereas resistant sporangium development represents the response of the organism to a variety of unfavorable conditions, such as crowding or high bicarbonate or cation concentrations. The early growth stages are morphologically quite similar in both cases, and a cell developing along either "track" can be induced to form the alternate sporangial type if the conditions are changed at any time up to an irreversible "point of no return" (34).

Although the resistant sporangia have been the subject of considerable study, very little of the work has dealt with RNA and protein synthesis (30), and none from the point of view taken in this review. We also know very little about the details of intracellular differentiation in this cell type. For these reasons the resistant sporangia will not be considered here, and the following discussion will deal exclusively with the zoosporangial cycle of rapid growth and differentiation (Fig. 1).

For convenience of discussion, the zoosporangial cycle can be divided into five, well-defined stages: the zoospore, zoospore encystment and outgrowth (germination), exponential growth, zoosporangium formation, and zoospore differentiation. The times indicated for each of these stages in Fig. 1 represent the averages obtained under defined growth conditions discussed later. It should be kept in mind, however, that the timing of many events can be significantly altered if the environmental conditions are changed; some experimentally useful examples of this will become evident in later sections.

Zoospores

Growth and differentiation of *Blastocladiella* begins with the zoospore, a nongrowing, asexual, motile unicell that serves to disperse and propagate the organism. The zoospore can encyst and begin germination within a few minutes of its release from the zoosporangium, or it can remain motile for many hours, depending upon the composition of the medium (225, 228, 237, 238, 251). It is a highly differentiated cell (Fig. 1) whose most prominent features under the light microscope are the long, flexible posterior flagellum, the large anterior nuclear cap surrounding most of the nucleus, and the "side body" complex. Electron microscope study (13, 15, 35, 36, 39, 41, 79, 142, 147, 193, 251) has revealed a wealth of additional subcellular detail. These features have been discussed extensively in published papers, and it will suffice here to summarize the most important features. The flagellum (about 15 μm long) is of the typical 9 + 2 eukaryotic type with its enclosing sheath a continuation of the flexible plasma membrane surrounding the body (about 7 by 9 μm) of the zoospore proper (Fig. 1, and 2–6) (35, 142, 251). The flagellum arises from a basal body whose proximal end lies adjacent to the spore nucleus. A second, small centriole lies next to the basal body in a shallow depression of the nuclear envelope. Nine sets of three microtubules extend from the proximal end of the basal body forward along the surface of the nuclear and nuclear cap membranes (80, 142, 175). A rootlet is also associated with the basal body, and its arms extend away at right angles from the basal body in channels surrounded by the single large mitochondrion that also envelopes the basal body. In addition to surrounding the flagellar apparatus and posterior nuclear region, the mitochondrion extends anteriorly along one side of the nucleus to form part of the side body complex. The side body consists of several lipid granules intermixed with an irregularly shaped, fused microbody complex lying against the outer side of the mitochondrion and surrounded on the outside by a double-layered membrane (Fig. 5) (40). The latter may or may not be a continuous sheet, is usually attached to the nuclear or nuclear cap membrane, and also frequently surrounds the anterior projection of the mitochondrion. The nuclear cap contains essentially all of the cellular ribosomes (147) surrounded by a double-layered, cytoplasmic membrane that is also continuous with the outer unit membrane of the nuclear envelope (Fig. 2, 3). Occasionally, small, secondary membrane-enclosed ribosome aggregates are found outside the nuclear cap proper. Nuclear pores occur within the nuclear cap region (Fig. 4) but have never been found in the small posterior portion of the envelope not enclosed by the cap membrane. Aside from some irregular small vesicles and the side body complex, the extracap cytoplasm contains glycogen granules, a few apical vesicles, and several γ particles (Fig. 6). The latter are membrane-enclosed, cup-shaped, dense bodies about 0.5 μm in diameter found only in the zoospore cytoplasm (36, 38). From this brief description, it should be clear that the zoospore represents a highly differentiated stage in the life cycle of *B. emersonii*.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T190
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LATE EXPONENTIAL PHASE PLANT
T170
df
FLAGELLA FORMATION AND CLEAVAGE
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ZOOSPORANGIUM
CROSSWALL FORMED
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FIG. 1.

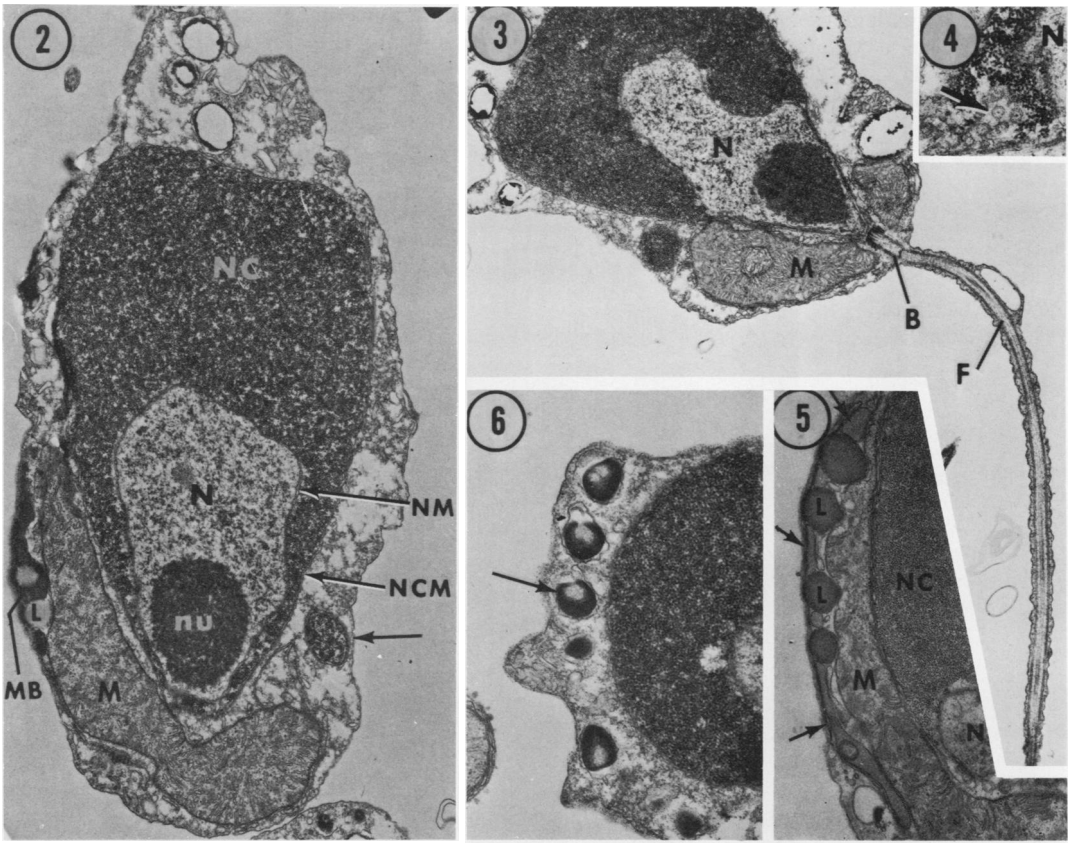


FIG. 2–6. *Zoospore ultrastructure.*

FIG. 2. *Long section through a zoospore to show the nuclear cap surrounding most of the nucleus, the single, large acentric mitochondrion, lipid droplets and microbody, and a membrane-enclosed satellite ribosome aggregate (arrow). ×10,944.*

FIG. 3. *Long section through a zoospore with the basal body of the flagella in a channel through the mitochondrion. ×10,260.*

FIG. 4. *Nuclear pores (arrow) in the portion of the nucleus surrounded by the nuclear cap. ×20,520.*

FIG. 5. *The lipid droplet, complex microbody (arrows), and mitochondrion of the zoospore side body complex. ×10,400. From Lessie and Lovett (142).*

FIG. 6. *Membrane-enclosed γ particles in zoospore cytoplasm (arrow). ×17,807. B, Basal body (kinetosome); F, flagella; L, lipid; MB, microbody; M, mitochondrion; N, nucleus; NU, nucleolus; NC, nuclear cap; NM, nuclear membrane; NCM, nuclear cap membrane. The procedures used for electron microscopy in these and subsequent figures are as described by Barstow and Lovett (14). (Fig. 2, 3, 4, 6; Barstow and Lovett, unpublished data).*

FIG. 1. *Zoosporangial cycle of Blastocladiella emersonii. The principle intracellular changes during growth and differentiation are shown, as observed by transmission electron microscopy. The early stages are disproportionately enlarged to show internal details, and the rhizoid systems are not shown in their entirety. The timing from 0 to 4 h indicates germination and early growth in synthetic medium (150); the timing from T_0 to T_{230} represents the time in minutes after the inducing medium shift starting at 15.5 h (142). The broken lines indicate that cells shifted to inducing medium at the respective stages will begin zoosporangium and zoospore differentiation but in proportionately smaller sporangia and with proportionately fewer zoospores produced, depending on the number of nuclei present (i.e., 1 to 256). cv, Cleavage vesicles and furrows; df, developing flagella; dp, discharge pore; f, flagella; m, mitochondrion; n, nucleus; nc, nuclear cap; ncv, nuclear cap membrane vesicle; pv, papilla vesicles; ra, ribosome aggregations; sb, side body granules of lipid and complex microbody; va, vacuole.*

Zoospore Germination

In the presence of 1 mM $CaCl_2$ and the absence of monovalent cations, zoospores can be maintained in a viable motile state for extended periods (225, 237). When diluted into medium containing KCl, they rapidly round up and encyst (Fig. 1, 7, 8) (150, 225, 251). The percentage of the cells that encyst will vary, depending upon the previous growth and manipulative treatment. The factors that influence encystment (or "round cell formation"), such as the ionic environment, cell density, and pretreatment, and the kinetics of the process have been studied extensively in the laboratories of Cantino (39, 237, 238, 251) and Sonneborn (225, 228). After a short lag, under optimum conditions nearly 100% of the zoospores will encyst

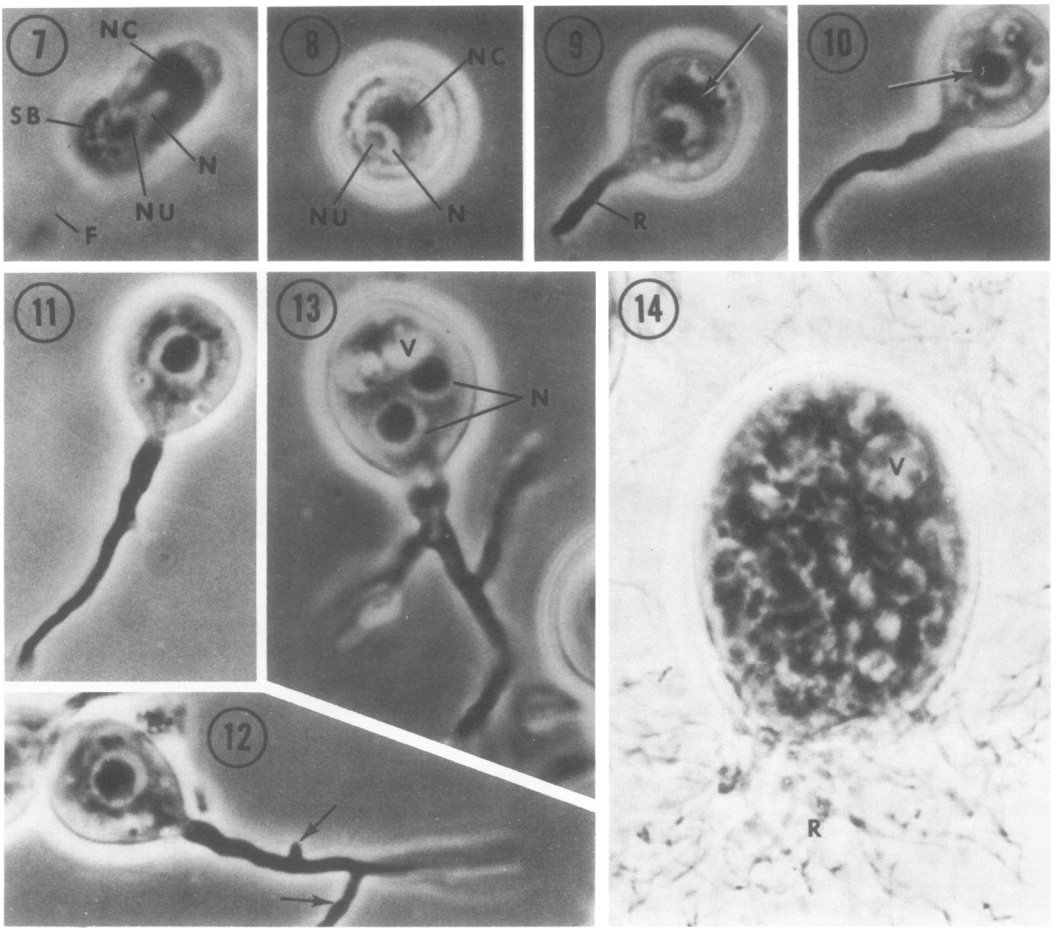


FIG. 7–14. *Germination and growth of Blastocladiella emersonii. The times given represent the interval after inoculation into PYG medium at 24 C. Fig. 7-13, ×2,100; Fig. 14, ×712.5. F, Flagella; NC, nuclear cap; N, nucleus; NU, nucleolus; R, rhizoid; SB, side body; V, vacuole. The phase photographs were prepared from unstained cells with a Zeiss Standard RA microscope equipped with phase optics. The cells of Fig. 7 and 14 were fixed and mounted as for electron microscopy (14); the cells for Fig. 8-13 were fixed 1 to 3 h in 2.5% glutaraldehyde in 1/2 DS and photographed as wet mounts.*

FIG. 7. *Motile zoospore.*

FIG. 8. *Encysted zoospore at 10 min. Note the nuclear cap and small nucleolus.*

FIG. 9. *Germling with rhizoid at 35 min. Many of the ribosomes are still concentrated next to the nucleus (arrow).*

FIG. 10. *Germling at 50 min when the nucleolus (arrow) has begun to enlarge.*

FIG. 11. *Early-growth-phase cell at 65 min with a fully enlarged nucleolus.*

FIG. 12. *Early-growth-phase cell at 65 min showing rhizoid branching.*

FIG. 13. *Binucleate growth-phase cell at 180 min with vacuoles and a well-developed rhizoid system.*

FIG. 14. *Multinucleate growth-phase cell at 15.5 h with numerous coarse vacuoles and an extensive, branched rhizoidal system.*

within a 10- to 15-min interval (224). The following changes are associated with this rapid event (150): flagellar axoneme retraction, loss of cytoplasmic microtubules, cell rounding and deposition of a thin cell wall, progressive disruption of the nuclear cap membrane, rearrangement of the mitochondrion and side body granules, and γ particle decay by production of vesicular material. The cell volume also decreases about 43% during this transformation (251). These and additional subtle changes have been reported in detail (41, 149, 150, 224, 226, 227, 250, 251), and it is perhaps worth reemphasis that nearly all of these striking alterations (called dedifferentiation in *Allomyces* [252]) require only about 60 s for completion in an individual cell.

The actual mechanism by which the changed ionic environment triggers encystment is not known, but the extensive studies of the system have clearly shown it to be an all-or-none event; once a zoospore is induced, it rapidly completes the process and proceeds to germinate, whether or not the conditions are suitable for subsequent growth. Since deflagellated zoospores encyst with normal kinetics (41, 225), flagellum retraction itself is not required for the process to go to completion.

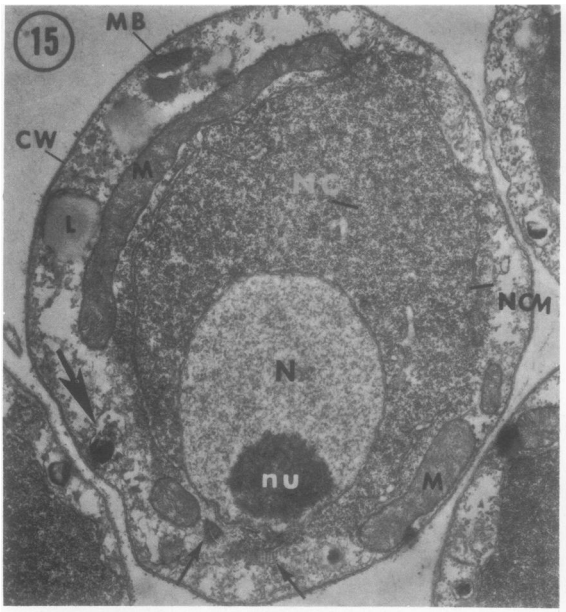
Cells at the above stage (Fig. 1, 12 to 15 min; Fig. 15) (round cell I) (224) continue their intracellular reorganization during the next several minutes. This includes the dispersal of the nuclear cap ribosomes throughout most of the cell and the appearance of rough endoplasmic reticulum (ER) elements; disappearance of the characteristic zoospore γ particles (Fig. 6), which are reported to produce multivesiculate bodies and may contribute to early wall formation (38, 250); loss of the cytoplasmic microtubules and flagellar axoneme fibers; organization of golgi centers (14); and sub-division of the large, previously single mitochondrion (26). Additional cell wall thickening also takes place during this period. The round cell II stage cells (224) then begin to "germinate" by a localized outgrowth of the cyst cell wall that by elongation becomes the primary rhizoid or germ tube (Fig. 1, 20 to 30 min; Fig. 8–11) (14, 150, 224).

Once the primary rhizoid is well established (Fig. 1, 40 to 50 min), the cells begin to grow rapidly and the cytoplasm largely resembles that of the growth-phase plants (Fig. 16). The most prominent changes are the enlargement of the previously compact nucleolus of the zoospore to the typical growth-phase size, increased amounts of rough endoplasmic reticulum, and the appearance of ribosome-free golgi equivalents containing numerous vesicles in association with membranes near the nuclear envelope (14). In addition, the primary rhizoid contains typical apical secretory vesicles, longitudinally oriented microtubules, and elongate mitochondria, as well as numerous ribosomes and occasional multivesiculate bodies (Fig. 19) (14).

Exponential Growth

In the single-generation cultures used for most contemporary experimental work, the growth phase represents the period between 1 and about 16 h (with cultures grown at 24 C). The actual extent of the growth phase is greatly influenced by both cell density and medium composition; growing plants can also be induced to differentiate into zoosporangia at any stage by a shift to an inorganic buffer solution (see Microcyclic Sporogenesis). *Blastocladiella* plants grow by enlargement of the original cyst body and the proliferation, branching, and elongation of the basal rhizoid system (Fig. 11–14). In semisynchronized cultures grown in synthetic medium, the first nuclear division (endonuclear mitosis) takes place between 140 and 190 min (150) (Fig. 1, 140 to 190 min). Cells grown in peptone-yeast extract-glucose medium (PYG) also begin the first mitosis at about the same time (107) and a second about 2 h later (254). If the subsequent divisions are synchronous, as they are in the water mold *Rhizidiomyces* (78), they should occur at about 1.75-h intervals to yield the average of 256 nuclei per plant at maturity (i.e., the cells contain 2 nuclei at 3 h and 256 nuclei at 17 h, when induced to differentiate at 15.5 h) (172). The time required to complete mitosis in any single cell is probably close to that reported for the related genus *Allomyces neo-moniliformis* by Olson and Fuller (186), who calculated mitotic periods of 10.1 min at 34 C and 33 min at 22 C. Thus, one would expect *Blastocladiella* cells grown at 24 C to require around 30 min to complete division since they require the same total time to go from 100% uninucleate to a 100% binucleate condition in cultures grown in a comparably rich medium (107). During the growth phase, mitosis is not accompanied by cytokinesis and the plants, thus, become coenocytic.

We have prepared electron micrographs of plants at intervals throughout the growth and differentiation phases (11, 12, 14, 142). During growth, the cytoplasm contains the normal assortment of nuclei, mitochondria, vacuoles, abundant ribosomes, and either scattered or localized aggregates of glycogen particles. The microbody granules originally observed as part of the zoospore side body complex (Fig. 5) (sb particles [142]) are present throughout the growth phase and nearly always have one side in close contact with a region of smooth endo-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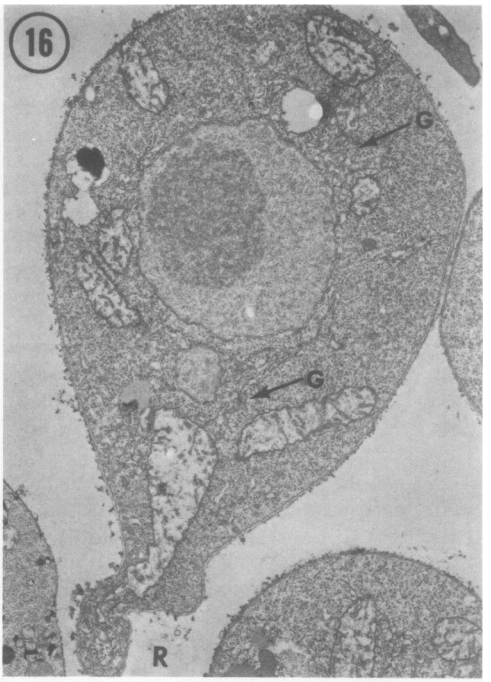
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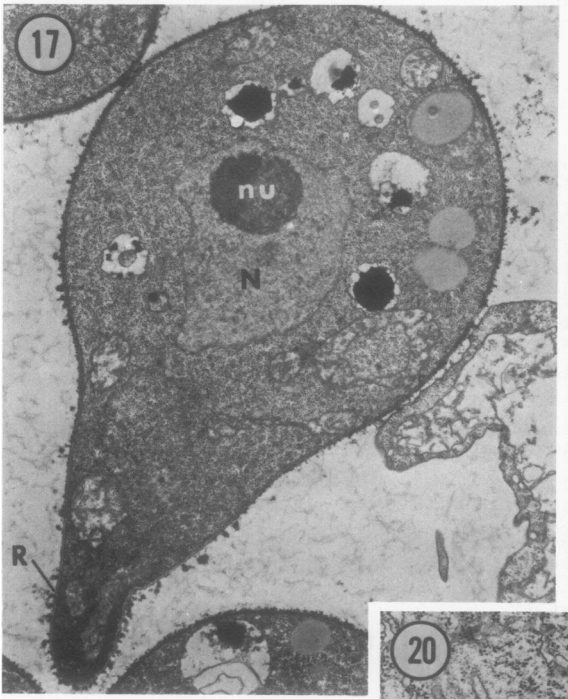
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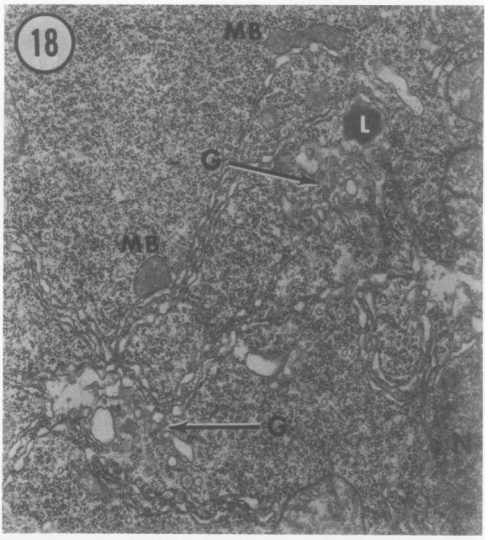
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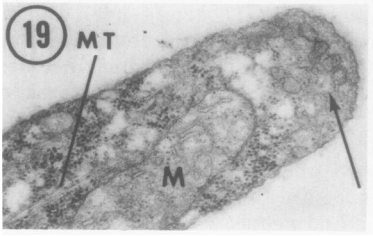
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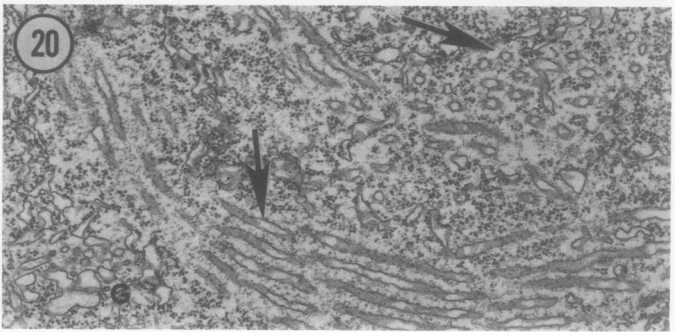
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plasmic reticulum (Fig. 18). These particles resemble typical microbodies in their association with the endomembranes, their finely granular appearance, frequent association with lipid droplets, and enzyme activity (60, 167). Fuller and Olson (81) proposed naming these particles "Stüben Bodies" because they are found in the side body complex ("Sitenkorper" of Stüben [234]) of both *Allomyces* and *Blastocladiella* zoospores. Mills and Cantino (167) have also recently referred to the fused stage in the zoospore side body as a "symphyomicrobody." However, since the particles clearly resemble microbodies in basic electron microscopic structure and enzyme content (catalase) and occur throughout the life cycle, a specialized name for the fused state in the zoospore seems unnecessary. Individual microbodies reappear during germination (14, 138; J. S. Lovett, unpublished data), which suggests that the fusion is a reversible process as it is for the single zoospore mitochondrion (26, 142).

Lipid droplets are less common than microbodies but are also usually associated with the endoplasmic reticulum. As a general feature, rough endoplasmic reticulum is notable for its scarcity in such rapidly growing cells; although it does occur, the great majority of the ribosomes have no direct association with the endomembrane system. Vacuoles appear in the cytoplasm at an early stage (about 60 min) and increase in size and number during growth. It is not known whether they contain lytic enzymes and are functionally equivalent to the lysosome-like vacuoles of *Neurospora* (160) and yeast (161).

One very conspicuous feature of the cytoplasm in mid-to-late log-phase plants is the presence of numerous tubular elements in the form of a branching network (11, 12, 142). The individual tubules are composed of a single unit membrane (about 0.87 nm in thickness) and have an outer diameter of 56.5 to 62.5 nm. Because of their large size relative to microtubules, they will be referred to here as "macrotubules" for convenience. Each macrotubule is surrounded by about a 35-nm ribosome-free zone and, when they occur in bundles of 2 to 30 or more, the tubules maintain a constant 90-nm center-to-center spacing. The result is often a series of rather regular hexagonal arrays within ribosome-free areas, as observed in cross section or parallel tubes in longitudinal section (Fig. 20). The origin and function of the macrotubules both remain to be established with certainty, but some pertinent information is available. No macrotubules have been found in the cytoplasm before 5 h. After that time they begin to appear in sections as single tubules and, as their numbers increase, they occur in bundles that extend throughout the cytoplasm in a complex, three-dimensional, branching network. Even at this stage, only an occasional section will include branching tubules, and interconnections with elements of the endomembrane system occur even less frequently. At the time when macrotubules first appear, numerous parallel arrays of vesicles are found extending outward from the localized vesicular zones, which we interpret to be the golgi apparatus equivalents in *Blastocladiella* (Fig. 18). Lipid droplets and microbodies are also associated with these vesicle-membrane systems, and the latter may, therefore, represent regions of active membrane synthesis. Since the first macrotubules are found in close approximation to these systems, it seems reasonable to propose that the linear membrane arrays represent a stage in macrotubule formation. If the macrotubules are accumulated membrane material to be used at a later stage (see Zoospore Differentiation), the exclusion zone surrounding the tubule could represent a mechanism to prevent association with ribosomes or other organelles until the time of their mobilization. The elaborate macrotubular system found in the late

FIG. 15–20. *Ultrastructure of the cytoplasm at encystment and during the growth phase. CW, Cell wall; G, golgi; L, lipid; M, mitochondrion; MB, microbody; MT, microtubule; N, nucleus; NC, nuclear cap; NCM, nuclear cap membrane; R, rhizoid (Fig. 16, 17, 18, 20; Barstow and Lovett, unpublished data).*

FIG. 15. *Encysted cell in basal salts medium at 15 min. Note the discontinuities in the nuclear cap membrane and ribosomes outside the membrane. A portion of the flagella basal body and rootlet are visible (small arrows), as well as a vesiculating γ particle (large arrow). ×13,338 (Abe and Lovett, unpublished data).*

FIG. 16. *Early-growth-phase cell at 60 min. Only a small part of the rhizoid lies in the section. ×8,892.*

FIG. 17. *Cell germinated in 20 μg of actinomycin D per ml and fixed at 60 min. The nucleolus remains small and compact, and the rhizoid has ceased elongation at a length equal to about one cell diameter. ×8,892.*

FIG. 18. *Cytoplasm of a growth-phase cell at 7 h. Two golgi centers are associated with the prominent paired arrays of membranes. Both the microbodies and lipid droplets are also next to the ER elements. ×10,552.*

FIG. 19. *Rhizoid tip to show the presence of apical vesicles (arrow), a mitochondrion, ribosomes, and microtubules. ×22,572. Reproduced from Barstow and Lovett (14).*

FIG. 20. *Macrotubules (arrows) in ribosome-free zones of a cell fixed at 14 h. ×19,437.*

growth phase later disappears rapidly during differentiation.

One other aspect of the growth phase merits comment. It is well established that the growth of filamentous fungi occurs at the hyphal tips and, further, that the deposition of new membrane and cell wall material is accomplished by secretory vesicle transport to the tip (89, 97, 164). Large numbers of such vesicles are found in the extreme tips of growing hyphae to the exclusion of other organelles. The growing rhizoids of *B. emersonii* contain similar clusters of irregular tip vesicles (Fig. 19), and identical vesicles occur individually in the subapical regions (14). It, thus, appears that these tubular structures grow by the same basic mechanism as the hyphae of filamentous fungi. The mechanism by which nonfilamentous fungi, such as *B. emersonii,* synthesize the cell wall of the thallus proper during growth has not been studied. Lacking experimental evidence, it might be presumed that this would more nearly resemble the process in higher plants, i.e., the continual deposition of new material in an expanding and stretching wall matrix. As might be expected if individual secretory vesicles moved to such a large surface area at random, we have observed no localized concentrations of secretory vesicles next to the plasmalemma at any stage during growth.

Conversion to Zoosporangia

When *B. emersonii* cultures are grown under our standard conditions (see Patterns of Biosynthesis), plants with discharge papillae begin to appear at 16 h, which coincides with the deceleration phase after the end of exponential growth. If left in the original growth medium such cells proceed to differentiate zoosporangia asynchronously over the next 2 to 3 h. On the other hand, when the cells are shifted to an inorganic salts medium at 15.5 h, the synchrony of sporangium formation can be greatly improved (148, 172). The two most obvious microscopic changes associated with the process are septation and papilla formation (Fig. 1), but these events are accompanied by numerous, less obvious intracellular changes. Between 16.5 and 17.5 h (T_{60} to T_{120}), frequent nuclear divisions are observed, and it is possible that all of the nuclei in a given plant divide once after induction to yield the final average of about 250 to 260 zoospores per plant. During the same interval, a cross wall forms to separate the upper multinucleate plant body from the lower rhizoid region. By the end of septation, the latter contains little more than vacuoles and membranes, and it is presumed that most of the contents are either withdrawn into the upper presporangial cell compartment or degraded.

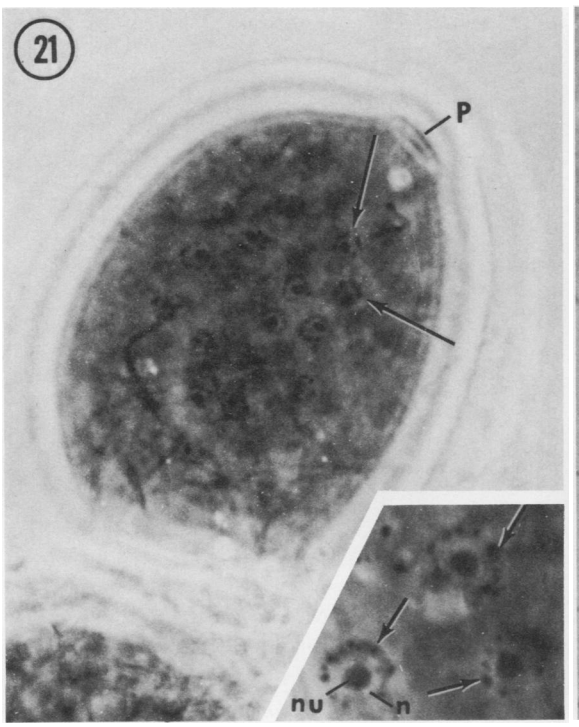
Between 17 and 18 h (T_{90} to T_{150}), a papilla is formed at the end opposite the rhizoids in a process whereby a localized region in the preexisting cell wall is replaced by a plug of amorphous papillar material (Fig. 1) (21). Large numbers of 150-rm diameter secretory vesicles crowd the cytoplasm near the plasma membrane at the site of papilla formation (Fig. 23). These vesicles are present only during papilla formation, contain material of similar staining density and texture to the papilla, and appear to release their contents into the papilla region by fusion with the plasma membrane. The vesicle contents give rise to the papilla, which appears first as a layer within the wall just outside the plasma membrane and then thickens outward to form the completed hyaline papilla plug (142). Papillae of the related genus *Allomyces* have been separately reported to contain pectin-like materials (216) and a mixture of glucose polymer and polypeptide (272). Partially purified papillae of *Blastocladiella* also contain an unidentified mucopolysaccharide-like material (68), whereas the cell wall they replace contains chitin and glucan as its primary constituents (D. R. Sonneborn, personal communication). To form a papilla, the cells must thus simultaneously secrete chitinases and glucanases in the papilla region to break down the preexisting cell wall polymers. The mechanism of transport for these enzymes is unknown, although they could be carried in the secretory vesicles along with the papilla material.

The appearance of the papilla vesicles coincides with the rapid formation of numerous, irregular, filled cisternae throughout the cytoplasm, which is another characteristic intracellular process associated with this stage of zoosporangium formation (12). However, the intracellular site of papilla vesicle production remains to be identified.

Soon after the medium is changed to induce differentiation (16 h), the previously well-organized bundles of macrotubules begin to lose their organized relationships to each other. At the same time, frequent connections are found between the tubules and elements of the endomembrane systems, including increased amounts of rough ER. The filled cisternae also appear in association with the increased rough ER and are interconnected with the macrotubules, which simultaneously lose their surrounding ribosome-free zones. The gradual disorganization and loss of the macrotubules

and their frequent connections to the increasingly prominent rough ER and filled cisternae clearly suggest the conversion of one into the other. Without direct evidence, this remains speculative. However, the effects of actinomycin D and cycloheximide on these conversions seem clearly consistent with such a hypothesis (W. E. Barstow and J. S. Lovett, unpublished data). Cells treated with either actinomycin D or cycloheximide at 16 h (just prior to these changes) block development as expected, but thin sections reveal that the macrotubules, nevertheless, do disappear. Instead of many cisternae forming, however, the cytoplasm contains extensive arrays of smooth ER-like membrane. When treated later (17 h, T_{90}), inflated cisternae are found but they contain few, if any, granules in contrast to sections from untreated control cells. Our present interpretation is that the membranes of the macrotubules are converted into the cisternal membranes and newly formed rough ER at this stage of intracellular differentiation. The tubules do not contain obvious contents, and it is probable that the granules of the filled cisternae are produced within the rough endoplasmic reticulum.

The large numbers of filled cisternae with 0.04-μm particles are a prominent feature of this stage in development, and one product of the cisternal contents has been identified as the membrane-enclosed γ particles of the zoospores (see next section). Although other potential products of the cisternal contents could be the papilla secretory vesicles described above and the cleavage vesicles (discussed in the next section), no evidence for this has been found. Implicit in our interpretation of the macrotubule conversions to cisternae and endoplasmic membranes is the intriguing idea that they are produced by the growth-phase plants as a reservoir of membrane material to be used on demand for differentiation of the zoosporangium and zoospores. Smith and Silverman (219) found the lipid content per cell to increase by only 13% during sporulation of cells induced to differentiate at 4 h. However, the formation of zoospores involves extensive membrane changes and



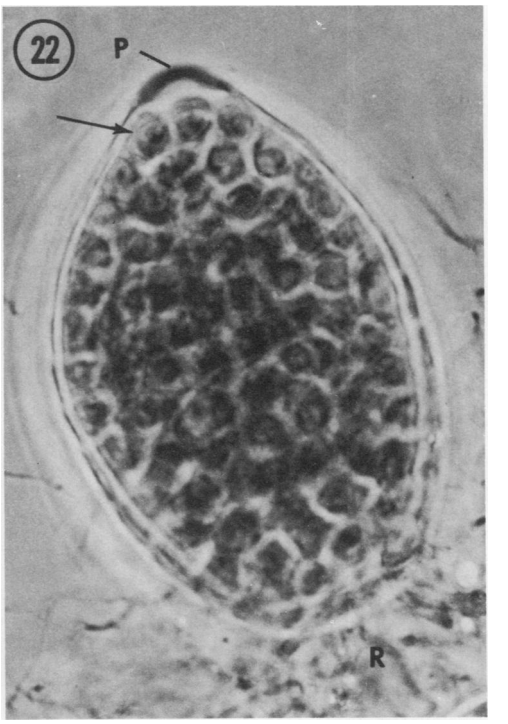


FIG. 21–22. *Lipid crown stage and predischarge zoosporangia. NU, Nucleolus; P, papilla; R, rhizoids. See legend to Fig. 7-14 for methods.*

FIG. 21. *Phase photomicrograph of a cell at the completion of zoosporangium differentiation with an apical papilla and nuclei surrounded by a halo of lipid droplets and microbodies (arrows) called the "lipid crown." ×920. Insert: an enlarged view of the nuclei and surrounding lipid and microbodies (arrows). ×2,300.*

FIG. 22. *Phase photomicrograph of a zoosporangium with differentiated zoospores just prior to release. The nuclear cap, nucleus, and nucleolus are visible in the zoospores (arrow) just beneath the papilla. ×920.*

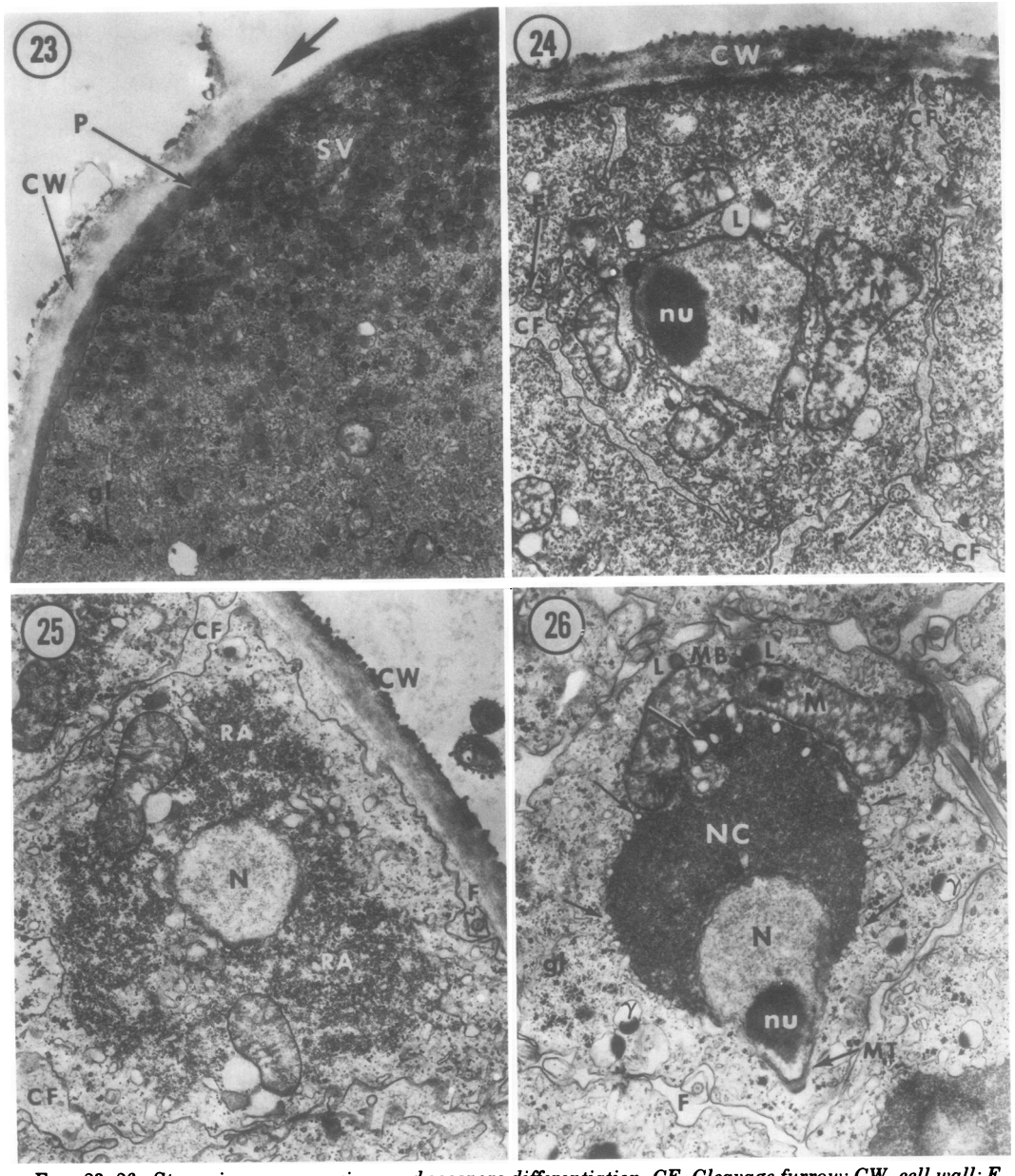


FIG. 23–26. *Stages in zoosporangium and zoospore differentiation. CF, Cleavage furrow; CW, cell wall; F, flagellum; GL, glycogen; M, mitochondrion; MT, microtubule; N, nucleus; NC, nuclear cap; NU, nucleolus; P, papilla; RA, ribosome aggregation; SV, secretory vesicles; γ, gamma particle. Reproduced from Lessie and Lovett (142).*

FIG. 23. *Papilla formation by secretory vesicle deposition of papilla material in the cell wall. Part of the wall has been "digested" away (large arrow) and is being replaced by the layer of amorphous papilla material. Numerous secretory vesicles are localized below the plasmalemma. ×7,658.*

FIG. 24. *Late-cleavage stage in zoospore differentiation. The completed flagella lie in the developing cleavage furrows, and each prezoospore nucleus is surrounded by mitochondria outside the lipid crown particles. The nucleolus is also condensed. ×9,590.*

FIG. 25. *Postcleavage aggregation of the ribosomes that will form the nuclear cap. ×9,900.*

FIG. 26. *Late stage of nuclear cap formation by fusion of vesicles (arrows) that surround the periphery of the ribosomal aggregation. At this stage, shortly before zoospore release, the mitochondrion is single, has the lipid and microbodies on its outer side, and has not yet assumed its position surrounding the flagellar apparatus. ×9,900.*

would require a minimum 60% increase in the total area of plasma membrane in cells induced at 15.5 h for which such preformed membrane could be utilized. By 18 h (T_{150}), the changes just discussed have converted the single-celled, growth-phase organism into one with a multinucleate, papillate zoosporangium subtended by a virtually empty basal cell and associated rhizoid system. Because each nucleus is also surrounded by a halo of lipid droplets (and microbodies in *Blastocladiella*) visible in the light microscope (Fig. 21), it has also been called the "lipid crown" stage (18). We have arbitrarily defined the completion of the papilla as the end of sporangium differentiation and the starting point for zoospore differentiation, even though in *Blastocladiella* one phase proceeds to the other without apparent delay. That it may, nevertheless, be a meaningful definition is suggested by a comparison with the related genus *Allomyces*; on a dry agar surface, the zoosporangia and gametangia of *Allomyces* cease development at the papillate lipid crown stage and only go on to differentiate zoospores or gametes if immersed in aqueous media (18).

Zoospore Differentiation

The earliest, clearly identifiable event in zoospore differentiation is the start of flagellum formation immediately after 18 h (Fig. 1, T_{150}). At the beginning of this process, the two unequally sized centrioles lie adjacent to each other in shallow depressions of the nuclear membrane. It is the larger centriole that becomes the zoospore basal body or kinetosome. The process by which the axoneme microtubules elongate from the outer end of the converted centriole has already been described in *Allomyces arbuscula* gametes by Renaud and Swift (192), and the sequence is essentially identical in *Blastocladiella* zoosporangia (142). When the axoneme fibers begin to elongate, characteristic "primary flagella vesicles" appear in the cytoplasm and, by fusion with additional "secondary vesicles," form an expanding tubular vesicle to enclose the elongating flagellar axoneme. These vesicles are readily identified by their finely reticulate contents and appear identical to the vesicles that coalesce to form the cleavage planes. The flagella initially elongate within individual, membrane-enclosed channels through the undifferentiated cytoplasm. However, cytokinesis also begins quite early with the result that the flagella soon lie free in the rapidly expanding cleavage channels as these interconnect with the channels surrounding the flagella. As indicated earlier, the growth-phase macrotubules have largely disappeared by this stage and have been replaced by numerous filled cisternae. Although it originally seemed that the cisternae might give rise to the many flagella and cleavage vesicles that suddenly appear in the same regions ([142] see Fig. 30, 32), we have found no evidence for such interconversions. Renaud and Swift (192) proposed that both the flagella and the cleavage vesicles were derived from the plasma membrane on the basis of their proximity and similar staining characteristics in *Allomyces*. Both types of vesicles finally give rise to plasma membrane of zoospores and would be expected to have the same composition. Thus, the similarity in staining is not very strong evidence for their derivation. Also, in *Blastocladiella* many of the flagella begin to form at a distance from the cell membrane, which would reduce the probability that we failed to detect direct blebbing from the plasmalemma.

Progressive fusion of the cleavage vesicles in a process requiring about 40 min (Fig. 1, T_{150} to T_{190}; Fig. 24) forms a three-dimensional network of cleavage planes delineating the area around each nucleus as a prespore unit complete with its flagellum lying free in the intercellular space. At this late cleavage stage, the lipid droplets and microbodies still surround each nucleus while the mitochondria are beginning to fuse together to form the single large mitochondrion of the zoospore. By this time, the electron-dense cisternal granules found at the papilla stage have changed significantly. By fusion in pairs and sequentially into larger numbers, the 0.04-μm diameter particles have formed 0.1-μm diameter aggregates in which the original electron-dense and transparent areas are still apparent. The larger particles have also coalesced into a smaller number of roughly spherical, 0.5-μm diameter cisternae (Fig. 27). By late cleavage, the 0.1-μm aggregates may be irregularly arranged or occur in distinct ellipsoidal patterns about 0.3 μm in diameter. During the next several minutes, the aggregate particles coalesce with the dense and transparent portions, rearranging to form the typical γ particles with their cup- or barrel-shaped profiles in sections (15). These particles, originally described by Cantino and Horenstein (33) at the light microscope level and by Cantino et al. (35, 36) by electron microscopy, are unique to the zoospore stage. We have found as many as eight profiles in a single spore section, and as many as 30 per cell (average, 8 to 15, depending on parent cell type) have been found in single zoospores by light microscopy (162). Membrane-enclosed particles of similar density have been found in the meiospores of *Allomyces macrogynus* (184) but are

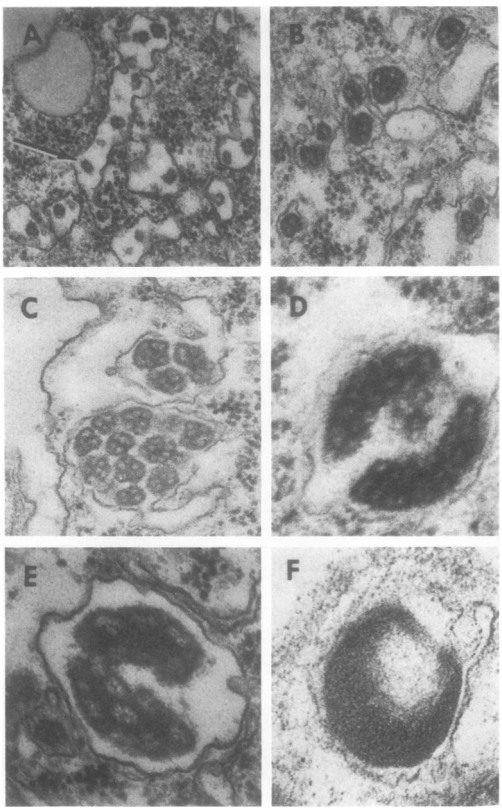


FIG. 27. *Stages in γ particle formation. (A) Electron-dense granules in cisternae at 18 h (lipid crown stage). The originally single 0.04-nm diameter granules with central electron-transparent zones (arrow) have begun to fuse in doublets and triplets. ×34,884. (B) A subsequent stage after further aggregation to yield about 0.1-nm diameter particles. ×68,970. (C) The accumulation of several 0.1-nm particles in a presumptive γ particle vesicle. ×46,170. Reproduced from Barstow and Lovett (15). (D–F) Further fusion and rearrangement of the electron-dense and transparent material that converts it into the characteristic 0.5-μm γ particle with its dense matrix surrounding an electron-transparent core. The mature γ particles are usually found during the period of nuclear cap formation (see Fig. 24–26). (D) ×68,970; (E) ×51,300; (F) ×67,180 (Fig. 27A, B, D, E, F; Barstow and Lovett, unpublished data).*

apparently lacking in *A. macrogynus* and *A. neo-moniliformis* zoospores (81).

Although Cantino and Mack (36) questioned the documentation for our original suggestion (142) that filled cisternae gave rise to all of the γ particles during zoospore differentiation, a recent reexamination of the process leaves little doubt that it was correct (15). The characteristic 0.04-μm, electron-dense particles with transparent cores retain their morphology throughout the entire process and can be traced from the cisternae connected to the macrotubules through all the intermediate stages. Furthermore, since virtually all of the numerous 0.04-μm cisternal particles produced at 15.5 to 18 h (T_0 to T_{150}) appear identical, it seems probable that this obvious burst of synthetic activity is directed entirely to γ particle precursor formation. The function of the γ particles remains to be established (see Zoospore Germination), but the recent progress in their isolation and analysis by Cantino and Myers (38) suggests that rapid progress should be made in the near future. The purified particles have been reported to contain DNA of unique buoyant density (174), 4S RNA (175), and chitin synthetase (37). The reader may refer to Myers and Cantino (175) for a detailed review of the structure and possible functions of the γ particles.

Another important change observable at the end of cleavage (18.7 h, T_{190}) is that the ribosomes occur in irregular clumps with areas largely lacking ribosomes between them. This irregular distribution is in sharp contrast to the densely packed ribosomes found in the growth-phase cytoplasm (Fig. 18). An important part of this change must result from a decrease in ribosomes caused by the significant RNA degradation that occurs throughout differentiation. On the other hand, it seems probable that actual ribosome clumping also begins at this stage since by 18.8 h (T_{200}) considerably larger aggregates of ribosomes are evident and the outer periphery of the prespore cytoplasm is devoid of such particles (Fig. 25).

Once cleavage and the associated changes are complete, the final events of zoospore differentiation, including nuclear cap formation, are completed very rapidly (15 to 20 min). Although the terminal stages are not as well documented and sequenced as we would like, it is clear that the ribosomes move to surround about two-thirds of the nucleus, with the thickest accumulation exactly opposite the basal body of the flagellum. At this stage the mitochondrion, lipid granules, and microbodies have moved to the outer area of the cell, and the latter granules lie at the outer side of the mitochondrion. Even before the ribosomes have become "packed" with the density found in the complete nuclear cap, vesicles begin to appear at intervals around the periphery. By the time the ribosomal aggregate has reached its final density and shape (crescent shaped in optical long section, Fig. 1), it is surrounded by a layer of vesicles (Fig. 26), which then immediately coalesce and flatten to form a continuous, double-layered membrane. The fusion of the nuclear cap membranes with the outer layer of the nuclear envelope apparently occurs at the

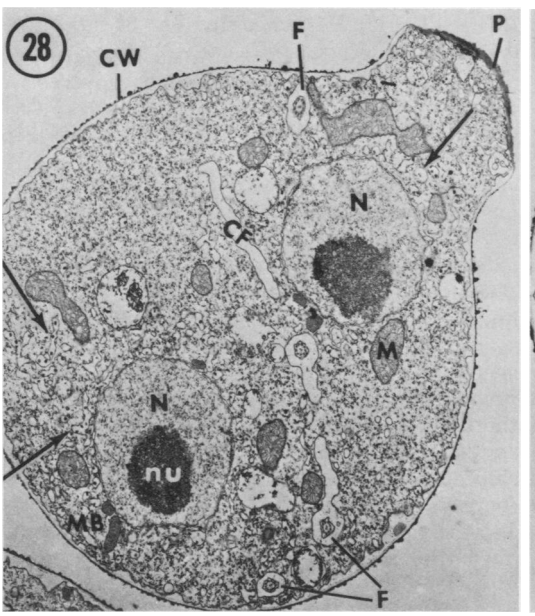
same time. The number of ribosomes enclosed in the nuclear cap have been estimated by direct counts on thin sections (32), and these results agree reasonably well with estimates based on the cap RNA content (138).

The last event in differentiation of the zoospore is the reorientation and coalescence of the large mitochondrion around the basal body-rootlet apparatus and the enclosure of the side body granules within a backing membrane. The backing membrane also surrounds part of the mitochondrion and is fused with the nuclear cap or nuclear membrane. The several separate microbodies also fuse at this time to form the single, large, irregularly shaped one found in the fully differentiated zoospore (Fig. 5) (40).

Microcyclic Sporogenesis

Hennessy and Cantino (107) have shown that very young germlings of *Blastocladiella* can be induced to differentiate zoospores if they are shifted from PYG growth medium to dilute phosphate buffer. Uninucleate cells can be induced to do this by a shift as early as 30 min after inoculation (equivalent to the 40- to 50-min stage in Fig. 1) and produce only a single zoospore. Hennessy and Cantino referred to the process as "lag-phase sporogenesis." However, microcyclic sporulation has been defined as the immediate recapitulation of sporogenesis following spore germination in bacteria (264) and the direct production of conidiophores and conidia by *Aspergillus niger* conidia in submerged culture (6). Similar terminology seems appropriate for the same phenomenon in *Blastocladiella*.

If germlings are induced after 1.5 h in growth medium, an increasing percentage of the plants produce two zoospores, the nuclear division resulting in a delay of about 30 min as compared with plants induced at 1 h. The zoospores produced by microcyclic zoosporangia display normal viability and appear typical when examined by electron microscopy (107). We have confirmed the above observations and found that cells in Spinner flask cultures can be induced to differentiate zoospores with good synchrony by shifting them to phosphate buffer (or 1/2 dilute salts [1/2 DS] solution) at any stage of the growth phase (Fig. 28, 29) (11, 13). Lodi and Sonneborn (145) and Silverman and Epstein (212) have also induced differentiation after 6 h of growth at 27 C. Cells induced at 3 h are advantageous be-



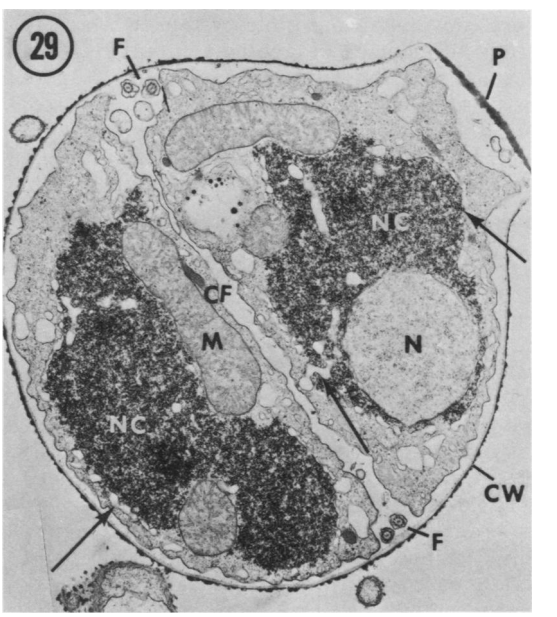


FIG. 28–29. *Minicycle sporogenesis. CF, Cleavage furrow; CW, cell wall; F, flagella; MC, microbodies; M, mitochondria; NC, nuclear cap; N, nucleus; NU, nucleolus (Barstow and Lovett, unpublished data).*

FIG. 28. *Binucleate zoosporangium 3.5 h after induction of sporogenesis. The apical papilla and basal septum (not in section) are complete; the flagella are also fully formed and cleavage furrow formation is well advanced. The precursors for γ particle assembly are prominent as filled cisternae (arrows).* ×7,729.

FIG. 29. *Binucleate zoosporangium near the end of zoospore differentiation. Cleavage is complete, the ribosomes have aggregated next to the nuclei, and the nuclear cap membrane vesicles (arrows) have begun to fuse to form the cap membrane.* ×7,729.

cause, unlike the microcycle cells, they produce two zoospores per sporangium and complete all of the normal events of zoosporangium and zoospore differentiation, including cytokinesis. The "minicycle" has provided a system that enabled us to test a possible requirement for the presence of macrotubules as a prerequisite for normal differentiation (i.e., as a source of γ particle and other membranes) since macrotubules are not normally produced until about 5 h. In cells induced at 3 h, no macrotubules were found, but in all other respects the intracellular changes that accompany normal sporulation were virtually identical to, and appeared in the same sequence as, those reported for cells induced in the late log phase (13; Barstow and Lovett, unpublished data). These changes include cross wall and papilla formation to produce the lipid crown stage zoosporangium, the numerous filled cisternae, flagella formation, cleavage, γ particle differentiation, and the organization of nuclear caps. From these results it is evident that the macrotubules are not an obligatory intermediate. On the other hand, the lack of reserve membrane and other materials could be the cause for the longer time required to complete the process (4 h versus 3 h in mature plants). The binucleate minicycle seems to provide a very convenient and rapid system in which to study developmental problems. It only requires 7.5 h to complete the cycle from spore to spore at 25 C; adequate cell densities can be used; and the small cell size permits preparation of electron micrographs of thin sections through entire cells at reasonably high magnifications.

PATTERNS OF BIOSYNTHESIS DURING THE ZOOSPORANGIAL CELL CYCLE

Before considering the significance of regulation during specific stages in the growth and differentiation of *Blastocladiella*, it will be worthwhile to review the general patterns of synthesis occurring during the life cycle. The growth of a single generation from zoospores to fully differentiated zoosporangia with zoospores at 19 h represents a dry weight increase of over 400-fold. This has necessitated the use of very different culture densities for the study of germination and growth during the first few hours as compared with late-log-phase and post-log-phase sporogenesis. We have routinely used 1×10^6 to 3×10^6 cells per ml in Spinner flasks for studies of germination and early development in a complete medium. This chemically defined medium contains 19 amino acids, glucose, thymine, thiamine, and inorganic salts (150), and cultures grown in it have a dry weight doubling time of 125 min ($\mu = 0.333$/h). Sonneborn et al. (224) have used a somewhat similar medium with 14 amino acids (DM-2), and an unpublished modification of it supports a growth rate and final yield equivalent to the standard Cantino PYG (Difco) broth (0.125% peptone, 0.125% yeast extract, 0.3% glucose, 0.005 M phosphate buffer [pH 6.8]). The effects of cell density on germination kinetics have already been discussed, and the densities we use are considerably higher than those used by Sonneborn et al. (0.9×10^5 to 9.5×10^5/ml). Cell densities of 1×10^6 to 3×10^6/ml represent a compromise between an adequate yield of plant material for extraction or fractionation and reasonable developmental synchrony; low-density plate and Spinner flask cultures provide excellent synchrony but low cell yields, whereas densities greater than 3×10^6/ml lead to increasingly poor synchrony (225, 237, 251). In large scale PYG cultures at 24 C, exponential growth with a dry weight doubling time of 130 min ($\mu = 0.406$/h) continues for at least 12 h at 1×10^6/ml and for 14 h at 5×10^5/ml (1). However, such high-density cultures undergo metabolic changes in the deceleration phase and do not differentiate synchronously (4). For optimal synchrony at this stage, we have regularly used Spinner flask cultures grown at 1.34×10^4/ml in PYG medium (T = 81 min; $\mu = 0.515$/h), which are shifted to 1/2 DS at 15.5 h (3, 172).

Motile zoospores respire actively [$Q_{O_2} = 9.4$] and slowly metabolize endogenous materials (238) but do not synthesize measurable amounts of macromolecules. Soll and Sonneborn (226), for example, found the barely detectable rate of [^{3}H]leucine incorporation in zoospores to be only 0.1% of that in cells after germination. The significance of such a low rate (63 counts/min per 10^2 cells per h) is uncertain, but it does seem apparent that the metabolism of the zoospores is channeled primarily toward energy production for motility and that their biosynthetic pathways are effectively repressed. Despite the ability of zoospores to swim for considerable periods of time under appropriate conditions, when diluted into either an inorganic germination solution or growth medium they begin to germinate after only a few minutes. This brief period of motility contributes to the lag period between inoculation of zoospores into growth medium and the beginning of a dry weight increase after 60 min (Fig. 30) (150). Subtracting the time to encystment (5 to 10 min, depending on cell density) gives a true lag in dry weight of about 50 min during which a small decrease in weight is observed. Part of the dry weight loss during the lag phase

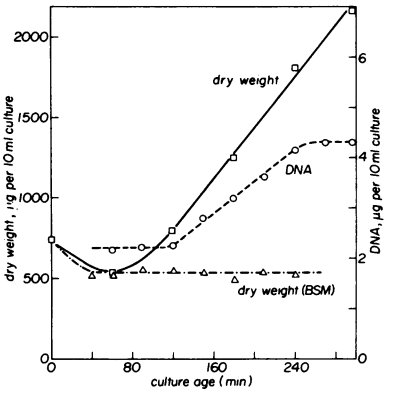


FIG. 30. *Changes in dry weight and DNA content of cells during germination and growth in synthetic medium and in inorganic salts medium (BSM). Modified from Lovett (150).*

may be attributable to the secretion of materials during encystment (41) and the rapid utilization of glycogen (237).

In synthetic medium a net accumulation of RNA per cell begins at 40 min and of protein at 80 min (Fig. 31). The preferential synthesis of RNA between 40 and 80 min leads to an increase in the RNA/protein ratio from 0.32 in zoospores to the value of 0.4 found for growth-phase cells (Fig. 32) (138). The amount of DNA per cell doubles between 120 and 240 min (Fig. 30) whereas most of the plants undergo nuclear division between 140 and 190 min (Fig. 1). The overlap indicates a lack of mitotic synchrony if we assume nuclear division to require 30 min or less, but it also implies that the DNA must be be replicated soon after mitosis is complete and that the G_1 phase is very short in *Blastocladiella* as it is in *Schizosaccharomyces* (20) and *Physarum* (180). The rate of RNA and protein accumulation do not decrease during the period of mitosis and DNA synthesis in synthetic medium (150).

If *B. emersonii* zoospores are placed in a totally inorganic growth medium (basal salts medium [150] or germination solution [227]), they germinate normally. They do not increase in dry weight (Fig. 30) and gradually decrease in RNA and protein content (Fig. 31) while increasing in size and producing a well-developed primary rhizoid with normal branching. However, mitosis does not take place. This limited development is presumably supported at the expense of endogenous reserves and/or turnover.

The main growth phase of *Blastocladiella* has not been examined in detail, but the data available (4, 87, 163, 172) all clearly indicate balanced exponential growth. This conclusion is based on measurements of cell volume, dry weight, nucleic acid, soluble protein, and glycogen content. Cell wall polysaccharides have not been measured during growth of the zoosporangial plants, but by analogy with the log-phase growth of resistant sporangial plants (152) the cell wall polysaccharides would also be expected to increase exponentially during balanced growth. DNA has only been measured following germination and during the late exponential phase where it, too, was found to increase exponentially (172).

The time of growth deceleration varies with temperature and cell density as indicated earlier. With 1.34×10^7 cells/liter growing in well-aerated PYG medium at 24 C, this occurs between 15 and 15.5 h in Spinner flask cultures.

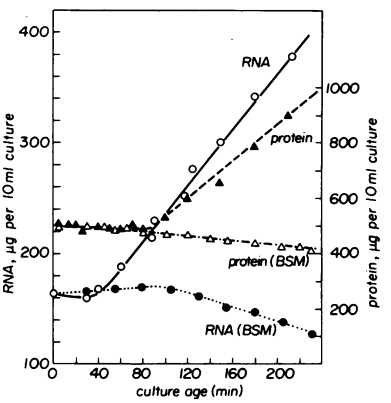


FIG. 31. *Changes in RNA and protein content of cells during germination and growth in synthetic medium and in inorganic salts medium (BSM). Modified from Lovett (150).*

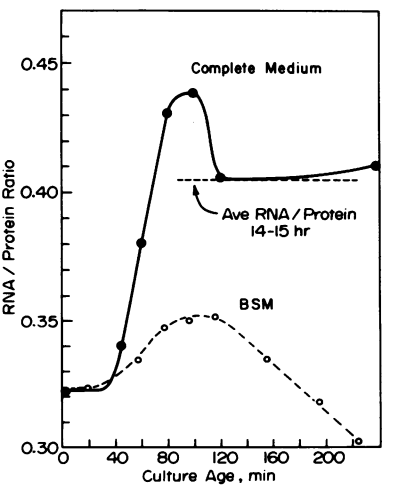


FIG. 32. *RNA/protein ratio of cells germinating in synthetic medium or in an inorganic salts medium (BSM). Modified from Leaver and Lovett (138).*

In uninduced PYG cultures, 5 to 10% of the plants form discharge papillae by 16 h, and during the subsequent 3 h all of the plants eventually produce papillae and discharge zoospores, but the synchrony in such cultures is very poor. If the plants are shifted to a 1/2 DS (172) medium at 15.5 h, before papillae form, the subsequent synchrony is excellent (see Growth and Differentiation). The data in Fig. 33 illustrate the changes in several cellular constituents after the medium shift to induce differentiation. The dry weight, RNA, DNA, and protein content continue to increase for a period after the shift but all at considerably reduced rates. Each reaches a maximum at a specific time, and then all but DNA show a steady decrease during the period of differentiation. The significance of these changes will be taken up in later sections.

From the discussion up to this point, it is obvious that major alterations in the rates of RNA and protein accumulation occur at the beginning and end of the growth cycle, and in each instance the change is correlated with a period of rapid cell differentiation. It is a widely held notion that cellular differentiation is to a considerable degree determined by the transcription and translation of specific messenger RNA (mRNA) molecules. To the extent that this is true of *Blastocladiella*, it must take place against a background of substantial change in the overall rates of biosynthesis. This poses two important questions with regard to the analysis of regulation during differentiation. The first and obvious one relates to the kinds of specific new activities that lead to differentiation. The second concerns how the new activities (or events) are related to the concurrently changing rates of RNA and protein synthesis. That is, are the bulk rates controlled by specific regulatory mechanisms associated with differentiation, or are the changes in these activities merely a reflection of a changed emphasis in cellular activity to intracellular reorganization rather than growth? At the end of the growth phase, this can be characterized as a possible shift in both the quality and quantity of mRNA transcribed. The qualitative change would be differentiation specific, whereas the quantitative shift could either be directly coupled to differentiation or be an independently regulated phenomenon. It is not a simple matter to distinguish between these possible alternatives because the known methods to induce zoospore differentiation also cause profound changes in the synthetic rates. To be able to distinguish among the numerous possible relationships between specific mRNA and bulk RNA synthetic rates and to understand the role of specific mRNA's in differentiation, it seems essential to know how *Blastocladiella* regulates bulk protein and RNA synthesis during the growth phase. With such information one can more intelligently assess whether the regulation of these processes is inextricably coupled to the regulation of differentiation or whether the changes in ribosome synthesis, for example, merely represent normal growth-phase type controls.

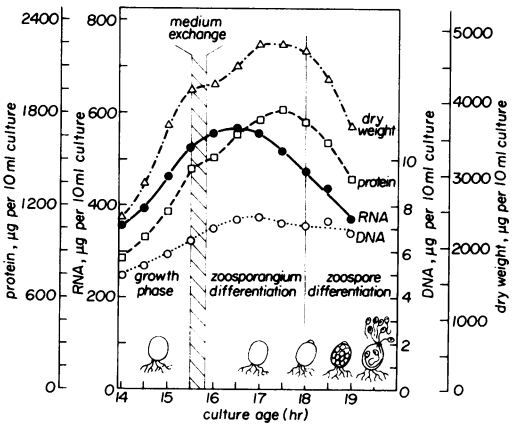


FIG. 33. *Changes in the dry weight, protein, RNA, and DNA content of cells before and after induction of zoosporogenesis. The cells were shifted from PYG growth medium to 1/2 DS at 15.5 to 15.83 h. Redrawn from Murphy and Lovett (172).*

REGULATION OF RNA AND PROTEIN SYNTHESIS DURING THE GROWTH PHASE

The ribosomes obtained by conventional bulk isolation procedures from growth-phase cells display the expected requirements for in vitro polypeptide synthesis with polyuridylic acid and phenylalanine (208). In high-density cultures ($\geq 5 \times 10^8$/liter) the cell-free specific activity rises to a maximum at midlog phase and then decreases, reaching nearly zero when the plants enter the stationary phase at 14 h (Fig. 34) (4). Similar results have been reported for the yeast *Saccharomyces cerevisiae* (155). In low-density cultures, on the other hand, a sharp decline to near-zero activity is not observed until after the cultures are induced to differentiate. The RNA extracted from ribosomes obtained by bulk isolation procedures from high-density, stationary-phase or postinduction-phase cultures of *Blastocladiella* is extensively degraded when examined by gel electrophoresis; on the other hand, whole-cell RNA carefully extracted at the same time shows no sign of degradation, and good

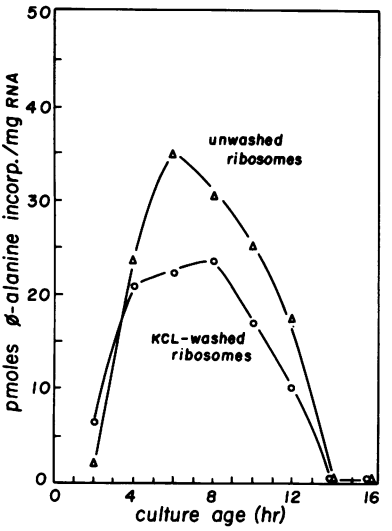


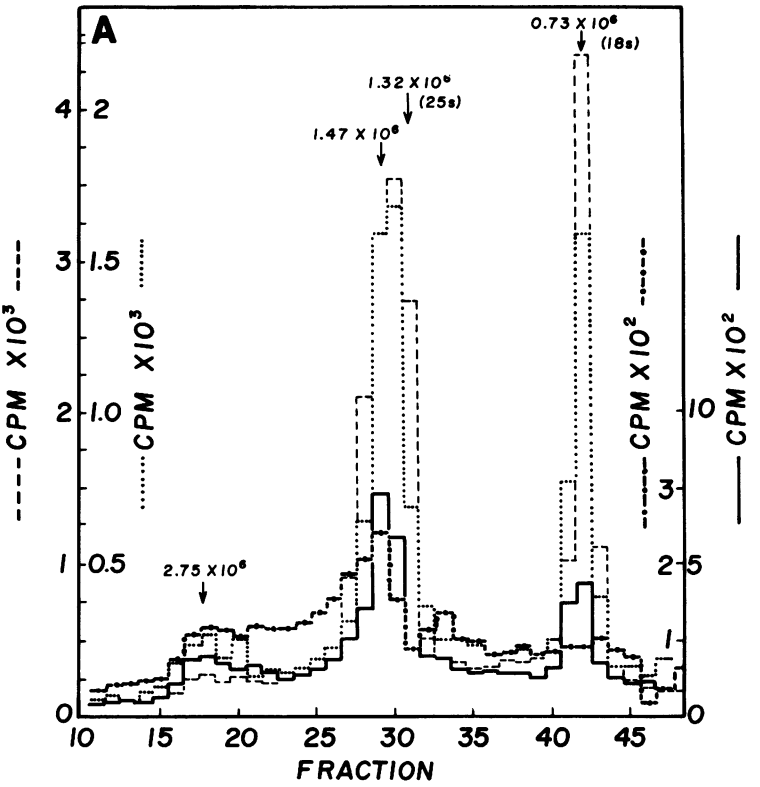
FIG. 34. *In vitro polyphenylalanine synthesis with poly(U)-mRNA and ribosomes isolated from Blastocladiella cells in the growth and stationary phases. The culture densities in cells per liter were: 2 h, 2 × 10⁹; 4 h, 1.25 × 10⁹; 6 h, 1.6 × 10⁹; 8 to 12 h, 1 × 10⁹; 14 to 16 h, 5 × 10⁸. Reproduced from Adelman and Lovett (4).*

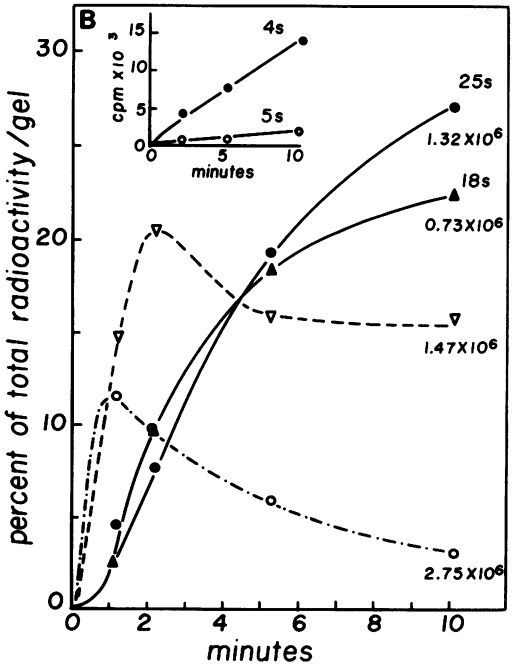
polysomes can also be isolated from the same stages by working rapidly on a small scale with special precautions to prevent nuclease activity (4). It seems evident from this that the stage-dependent reductions in in vitro activities are to a large extent artifactual and occur during ribosome isolation. The defective ribosomes must result from changes in the physiological state of the plants whereby the ribosomes are much more susceptible to degradation at the time of cell rupture. It seems reasonable to suggest that specific ribonucleases are produced or activated in response to the reduced rates of protein synthesis and that they may be localized in the vacuole fraction of the intact cell (160).

In our experience partial degradation of extracted ribosomal RNA (rRNA) almost always represents nuclease activity during the isolation procedure, primarily at the moment of cell breakage. For example, whole-cell RNA, carefully extracted from stages when substantial RNA degradation is known to take place, shows little or no evidence of breakdown when examined by gel electrophoresis (see Fig. 38A, T_{72}). Such a result makes considerable caution imperative when interpreting the significance of in vitro ribosome activities. It also leads us to conclude that the majority of the cytoplasmic ribosomes are engaged in polypeptide synthesis throughout the exponential growth period, despite the changes in activity indicated by in vitro assays.

Before examining the relationship between protein and RNA synthesis, it would be useful to summarize what is known about RNA synthesis in *B. emersonii*. The 60S and 40S subunits of the 80S ribosomes contain rRNA's with molecular weights of 1.32 × 10⁶ (25S) and 0.73 × 10⁶ (18S) (153). Nuclear fractionation procedures that would permit the isolation and characterization of the typical eukaryotic nucleolar rRNA precursors (56) are not yet available for *Blastocladiella*. Short-term labeling experiments with [³H]uridine have, nevertheless, indicated the existence of rRNA precursors (p-rRNA), even in whole-cell RNA preparations fractionated by gel electrophoresis (Fig. 35). The first high-molecular-weight precursor of about 2.75 × 10⁶ daltons is nonconservatively cleaved to yield about a 1.47 × 10⁶-dalton piece and the 0.73 × 10⁶-dalton 18S rRNA in a step that requires only 2 min (Fig. 35A). An additional 1.57 × 10⁶-dalton stage may also exist between the initial 2.75 × 10⁶-dalton precursor and the 1.47 × 10⁶-dalton intermediate. The conversion of the 1.47 × 10⁶-dalton p-rRNA to the 1.32 × 10⁶-dalton 25S rRNA is clearly a rate-limiting step and results in an accumulation of this precursor (Fig. 35A, B). Our estimates for the processing times agree well with independent measurements of the time required for [³H]uridine to enter the 25S and 18S rRNA in cytoplasmic ribosomes isolated on gradients. Radioactivity in the 18S rRNA appears in the cytoplasmic 40S subunit between 3 and 5 min after the addition of label, whereas activity in the 25S rRNA only appears after a further delay of 5 to 7 min. As discussed below, the processing of the 1.47 × 10⁶-dalton p-rRNA is also sensitive to conditions that affect the overall rate of rRNA synthesis.

It is not yet entirely clear how tight a coupling exists between the rates of protein and RNA synthesis in fungi (58, 133, 196, 236). Our results with *Blastocladiella*, if representative, certainly indicate that the rate or rRNA synthesis responds rapidly to changes in the rate of protein synthesis. To assess the regulatory responses of *B. emersonii* growth-phase plants, we have subjected them to conditions that place a sudden restriction on amino acid availability or have inhibited protein synthesis with cycloheximide. In these experiments the percentage of ribosomes in polysomes, the rates of protein and RNA accumulation, and in some cases the rates and patterns of [³H]uridine incorporation have been measured. The medium shifts, all done at a culture age of 2 h, have been (see Patterns of Biosynthesis During the Zoosporangial Cell Cycle): (i) from complete synthetic medium to an inorganic salts medium (the 1/2 DS used to induce differentiation [172]), (ii) from complete


A
0.73 X 10⁶ (18s)
1.32 X 10⁶ (25s)
1.47 X 10⁶
2.75 X 10⁶
CPM X 10³
CPM X 10³
CPM X 10²
CPM X 10²
FRACTION


B
4s
5s
25s
1.32X10⁶
18s
0.73X10⁶
1.47X10⁶
2.75X10⁶
cpm X10³
minutes
percent of total radioactivity/gel
minutes

synthetic medium to a minimal medium containing only the two essential amino acids, methionine and glutamic acid, and (iii) from complete medium with high arginine (2×10^{-4} M versus 1×10^{-4} M for other amino acids) to complete medium with very low arginine (2×10^{-6} M) (Lovett, manuscript in preparation).

The results obtained with the intermediate shift (ii) from complete to minimal medium are illustrated in Fig. 36 where it can be seen that the accumulation of both protein and RNA ceases immediately after the shift. The percentage of polyribosomes, reflecting the rate of protein synthesis, declines rapidly to one-half of the prestep value. After a delay of about 50 to 60 min, protein accumulation resumes at one-fourth of the prestep rate, whereas the RNA content decreases by 7%. The retention of half of the polysomes in the absence of any protein accumulation is consistent with protein turnover to provide amino acids for synthesis of the newly required biosynthetic pathways. The resumption of net protein accumulation at the new reduced rate should represent the completion of this process. At this lower synthetic rate, the prestep ribosome content would be in excess, and this is reflected by the only partial recovery of the polysome content to about 50 to 55%. The loss of RNA suggests that the reduced rate of protein synthesis also leads to the degradation of some of the previously formed ribosomes that are in excess after the step-down. Analysis of pulse-labeled rRNA before and after the minimal medium step-down has given results that agree well with this interpretation; i.e., the incorporation of [^{3}H]uridine decreases by almost 90% immediately following the step-down, and precursor synthesis and processing are both strongly inhibited (Lovett, manuscript in preparation).

The initial response of *B. emersonii* plants to step down (i) from complete to 1/2 DS medium was essentially identical to the minimal medium step. However, after the same delay of about 50 min, both the protein and RNA content of the cells decreased. As described earlier, these plants later produce zoospores, and further discussion will be deferred until we con-

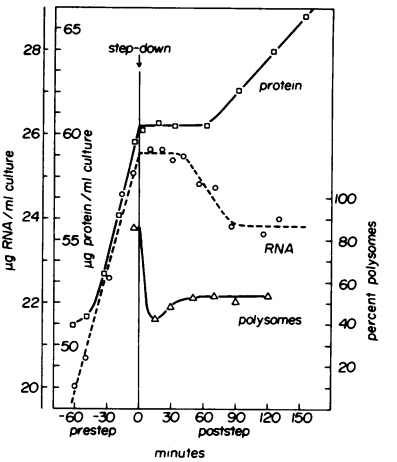


FIG. 36. *Effect of a step-down from complete to minimal medium on the rates of biosynthesis and cellular polysome content. Cultures were grown at 2 × 10⁶ cells/ml in complete medium (150) with 19 amino acids. Before and after the medium shift, samples were withdrawn at the times indicated for the analysis of RNA and protein, as described by Lovett (150), and for polysomes, by the procedure of Leaver and Lovett (138). The cells were shifted to the minimal medium (6.8 × 10⁻⁴ M glutamate, 1 × 10⁻⁴ M methionine, and glucose, thiamine, and salts as in prestep medium) by rapid concentration without suction in a large Sargent no. 500 filter, rinsed twice with the step-down medium, and resuspended to the prestep volume in the same. The entire procedure required 3 min ± 15 s. Control experiments (not given) have shown that the shift procedure itself does not decrease the rates of synthesis. The percentage of polysomes equals the absorbance under the polysome region of gradients (10 to 35%, exponential) over the total ribosome absorbance × 100 (Lovett, manuscript in preparation).*

FIG. 35. *Kinetics of [^{3}H]uridine incorporation into ribosomal and soluble RNA by early-growth-phase cells. A culture was grown for 4.5 h in synthetic medium (1 × 10⁶ cells/ml; 24 C); the cell density was then increased fourfold in fresh medium and pulsed with 5-[^{3}H]uridine (100 μCi/55 ml; specific activity, 20 Ci/mmol). At 1, 2, 5.25, and 10 min, 12.5 ml of cell suspension was removed and added to crushed ice containing carrier uridine and 0.001 M NaN_3 to prevent further incorporation. The labeled RNA was extracted, separated by polyacrylamide gel electrophoresis, scanned with a Gilford Instruments Linear Transport, sliced, and counted as described by Leaver and Lovett (138) (Lovett, unpublished data). (A) Radioactivity distributions after 4-h electrophoresis in 2.2% gels (two 1-mm gel slices per vial). The arrows mark the positions of the 2.75 × 10⁶-dalton and 1.47 × 10⁶-dalton precursors and the 1.32 × 10⁶ dalton (25S) and 0.73 × 10⁶-dalton (18S) rRNA determined with rRNA standards. Symbols: +++, 1-min pulse; ——, 2-min pulse; ·····, 5.25-min pulse; – – –, 10-min pulse. (B) Kinetics of appearance of [^{3}H]uridine in different RNA fractions. For each fraction the curve represents the percentage of the total counts per minute on each gel in the respective fraction versus time, with data derived from part A. Insert: 4S and 5S radioactivities were calculated as the total counts per minute under the respective peak versus time, corrected for differences in the total RNA per gel (Lovett, unpublished data).*

sider zoospore differentiation. The 100-fold step-down from high to very low arginine concentrations (iii) in complete medium was designed to repress the pathway for arginine synthesis and then suddenly make arginine the limiting factor. After this shift, the rate of protein accumulation remains unchanged for some 30 min, decelerates temporarily, and then returns to the original rate by 60-min poststep. The polysomes only decrease by 7% during the same period. In sharp contrast, the rate of RNA accumulation drops to zero just as rapidly as it did with the more stringent step-down experiments. However, at 60-min poststep it then begins to rise again at a rapid rate, along with the rate of protein accumulation. The kinetics of [^{3}H]uridine incorporation into rRNA again reflect the changes in total RNA (Lovett, unpublished data).

Cycloheximide is an effective inhibitor of whole-cell amino acid incorporation in *Blastocladiella* (150, 226, 227) and has been reported to inhibit one of the RNA polymerases in vitro (115). We have also found that, at 100 μg/ml, cycloheximide reduces whole-cell [^{3}H]uridine incorporation by about 47% and inhibits both synthesis and processing of the first p-rRNA. It has not yet been determined whether this is a primary effect on RNA polymerase, as the Horgen and Griffin results might suggest, or is a result of its inhibition of polypeptide synthesis on polysomes, where its effects are well documented. The important observation is that its immediate effect is a severe reduction in the rate of RNA accumulation, followed by complete inhibition only after 70 to 80 min. Both cycloheximide and the step-down to minimal medium cause an accumulation of the first p-rRNA, which suggests an initially more rapid block in processing than in transcription.

The differences in the response of *B. emersonii* to a shift in amino acids and to the inhibition of protein synthesis by cycloheximide provide a suggestion concerning the signal that controls the rate of bulk RNA synthesis. Cycloheximide arrests translation by blocking the translocation step on ribosomes (105, 211). Since there is no evidence that it affects amino acid uptake in *Blastocladiella* (227), cycloheximide should cause no change in pool sizes or, if it does, it should result in a transient increase by eliminating the "sink" via withdrawal for protein synthesis. The step-downs, on the other hand, should all cause a rapid depletion of the internal pools of one (arginine step-down) or many amino acids (minimal medium step-down). In the case of *Blastocladiella*, it seems evident that the depletion of even a single amino acid causes a much more rapid inhibition of RNA synthesis than simply blocking protein synthesis, as proposed by Roth and Dampier (196) for yeast. This kind of regulation is already well documented in bacteria and indicates a stringent control system in which the response is most rapid to changes in the availability of free amino acids (i.e., via the level of aminoacyl-transfer RNA [aminoacyl-tRNA]) (65). More work needs to be done to reveal the details of the mechanism for these growth-phase controls, but *Blastocladiella* seems to be a favorable organism for such experiments.

PROTEIN AND RNA SYNTHESIS DURING CONVERSION TO ZOOSPORANGIA

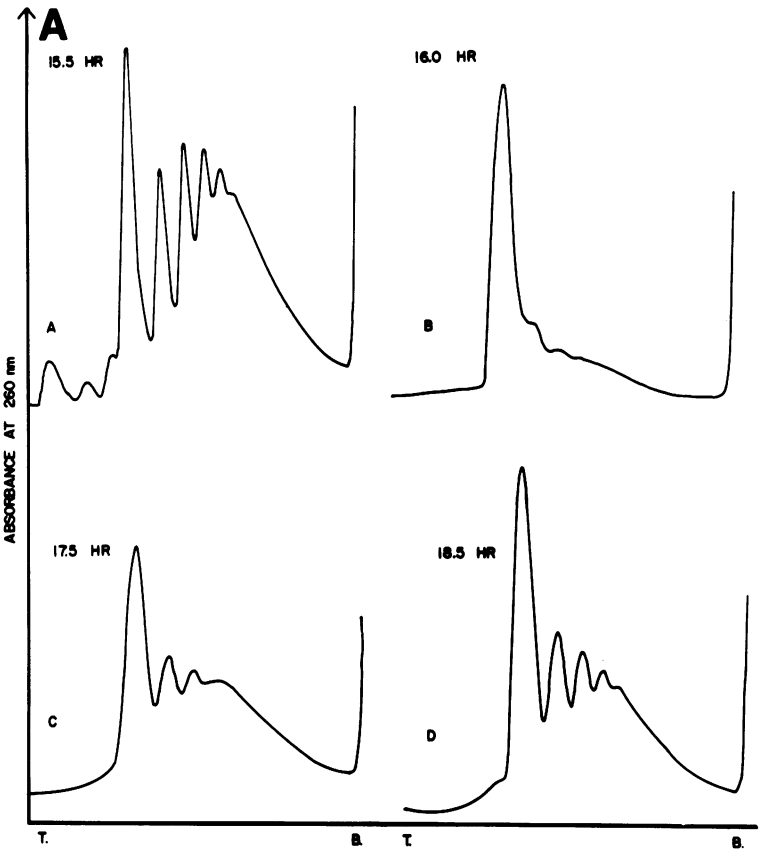
Immediately after the medium change to induce zoosporangium differentiation, the rates of increase in dry weight, RNA, DNA, and protein are first sharply curtailed and then cease after a period that is specific for each component (172). The limited synthesis at low rates after the inducing medium shift (Fig. 33) probably results from the small amount of original growth medium (6%) remaining after the exchange. The lipid content has been found to increase throughout sporulation in cells induced at 4 h (219), leading to a 13% increase per cell and a decrease in the ratio of phospholipid versus neutral and glycolipid. An additional observation by Silverman and Epstein (212) is that the cyclic guanosine 5'-monophosphate (GMP) level in cells increases by 50- to 100-fold between 60 and 150 min after induction. Most of this rise appears to occur during the conversion to zoosporangia (cyclic adenosine 5'-monophosphate [AMP] does not increase at this stage). Neither the cause nor the effect of this shift has been determined, but the known and suspected regulatory roles of cyclic nucleotides in other organisms suggest that it may be of significance.

The sharp 60% reduction in the rate of protein accumulation after induction is accompanied by a rapid runoff of the polysomes to less than one-half of their preshift level by 16 to 16.5 h (Fig. 37 A, B; Fig. 1, T_{60}). This seems entirely consistent with the response of growth-phase cells to a reduced amino acid supply that was discussed earlier. Between 16.5 and 17.5 h (T_{60} to T_{120}), the percentage of ribosomes in polysomes then returns to 65% (or 85% of the preinduction value), and the amino acid incorporation rate also rises rapidly at the same time (3, 172). The rise in apparent amino acid incorporation is largely due to the increased specific activity of the amino acid pools when cells are pulse-

labeled in the shift-down medium (1/2 DS) as compared with the PYG growth medium. Nevertheless, the increase in incorporation parallels the increased uptake of [^{3}H]leucine by the cells after the medium shift, and the radioactivity incorporated during 2-min [^{3}H]leucine pulses is associated with the polysomes during differentiation, even at the point of their lowest percentage (Fig. 38, 16.5 h). After 17 to 17.5 h (T_{90} to T_{120}), both the whole-cell uptake of leucine and its incorporation decrease rapidly, whereas the distribution of ribosomes between monosomes and polysomes remains virtually constant. A significant decline in the total protein per cell begins at the same time and continues until the end of differentiation. This degradation could provide a continuing supply of amino acids for the internal pools and explain the steadily decreased incorporation of externally applied amino acids. Lodi and Sonneborn (145) have also recently reported proteolysis to be extensive during sporulation in *Blastocladiella* cells induced after 6 h of growth. In the presence of carrier, they observed release of cold trichloroacetic acid-soluble radioactivity from uniformly labeled cells at a rate of 12%/h. The 35% loss of prelabeled protein is in agreement with our reported 25% net loss in total protein (172). It is particularly interesting that Lodi and Sonneborn found that the sporulating cells release a protease characteristic of the growth phase (pH optimum, 7 to 10) into the medium and replace it with a protease characteristic of the zoospores (pH optimum, 5.5). Most of the pH 7 to 10 protease release actually occurs during zoospore differentiation (145). The proteins degraded by the proteases are not known, but one fraction may represent ribosomal structural proteins since nearly 35% of the total RNA is also lost during differentiation (Fig. 33), and this must represent the destruction of many ribosomes. The persistence of polysomes throughout all but the last stages of the degradative interval (17.5 to 19 h; T_{120} to T_{210}) serves as evidence for new protein synthesis via turnover, as does the susceptibility of the differentiating cells to cycloheximide inhibition (e.g., macrotubule conversions, cleavage, etc.). A small amount of newly synthesized protein becomes associated with the ribosomes as late as 18 h, apparently by exchange since it is not removed by procedures that release nascent polypeptides (2).

In contrast to protein, the rate of RNA accumulation drops by 92% after the medium shift and, after only an 8% increase, ceases altogether at 16.5 h (T_{60}). This is immediately followed by a progressive loss of RNA, which continues throughout differentiation. Except for the small increase after induction, the initial responses of the RNA synthetic rates closely resemble those to a step-down in the growth phase, including a 45- to 50-min lag before the onset of degradation. The slower initial shutdown of synthesis is probably due to the slower and less complete change in the medium. The rate of [^{3}H]uridine incorporation drops rapidly to near zero by 16.5 to 17 h (T_{60} to T_{190}), with progressively more of the label being found in the precursor regions (2.75×10^6- and 1.47×10^6-dalton p-rRNA) and a virtual loss of 18*S* rRNA (Fig. 39). This clearly suggests a rapid shutdown in both the synthesis and processing of rRNA with turnover of the small amount of new rRNA that is produced. Since precursor uptake also decreases sharply, it is difficult to calculate the actual amounts of synthesis that such pulse-label data represent. However, experiments with uniformly prelabeled cells and with cells whose pools were preloaded just prior to this stage (172) have convinced us that bulk RNA synthesis and processing are shut down soon after the inducing medium shift; certainly the reduced uridine uptake could not explain the precursor accumulation patterns or the loss of newly synthesized 18*S* RNA. The close correlation between the cessation of uracil and uridine incorporation into rRNA, the loss of 18*S* rRNA label, and the start of net RNA decrease also suggest a sharp metabolic shift. The activation of nucleases could account for the turnover, net degradation, and reduced uptake of RNA precursors (by flooding the monophosphate pools). The latter is, in fact, accompanied by the release of radioactivity from cells prelabeled with [^{14}C]uracil (172).

Some RNA is, nevertheless, made after the shutdown in bulk synthesis (172), but the turnover problem has prevented measurement of the actual amount. It is tentatively assumed that this represents the formation of newly required mRNA. The evidence for mRNA synthesis at this stage is fragmentary and inferential being based, as it is, on the effects of actinomycin D added to the cells at different times after induction. The results are at least consistent with an mRNA hypothesis although this will require direct verification. Actinomycin D added at 16.5 h (T_{60}) completely blocks papilla formation but, if added just prior to the appearance of visible papillae (17 h, T_{90}), it fails to prevent their assembly (172). Another example comes from the inhibition of specific stages in macrotubule conversion to the cisternal precursors of γ particles described earlier. The insertion of the new mucopolysaccharide papilla in


A
15.5 HR
16.0 HR
17.5 HR
18.5 HR
A
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C
D
ABSORBANCE AT 260 nm
T.
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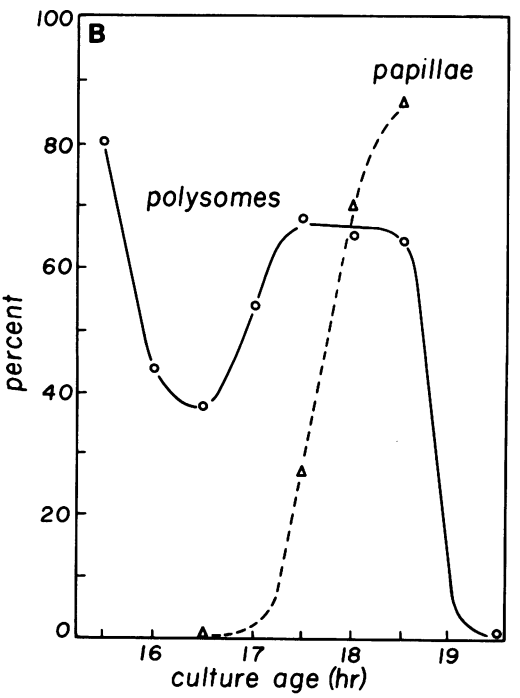
B
papillae
polysomes
percent
100
80
60
40
20
0
16
17
18
19
culture age (hr)

the cell wall might be expected to require the stage-specific production of new enzymes and should provide a good experimental system in which to test a requirement for mRNA synthesis; this is now being examined.

SYNTHESIS DURING ZOOSPORE DIFFERENTIATION

With either the minicycle or induced mature cells, the changes just described take place over a period of about 2 h while the coenocytic cell is being converted into a zoosporangium with a basal cross wall and apical papilla. At this point it is poised on the threshold of the short period of rapid intracellular differentiation that serves to convert the sporangial contents into zoospores. Since this differentiation takes place against a background of continuous and rapid loss in dry weight, RNA, and protein content per cell (Fig. 33), the evaluation of new synthesis is difficult. The failure of the cells to take up significant amounts of RNA precursors after 16.5 h (T_{60}) was alluded to earlier. Amino acids, on the other hand, enter the cells readily throughout most of both zoosporangial and zoospore differentiation. However, large changes in amino acid uptake after the inducing medium shift and possible pool compartmentalization (as shown for other fungi [239, 268]) preclude measurement of the synthetic rates unless the in situ specific activities are known.

Despite these difficulties, some information

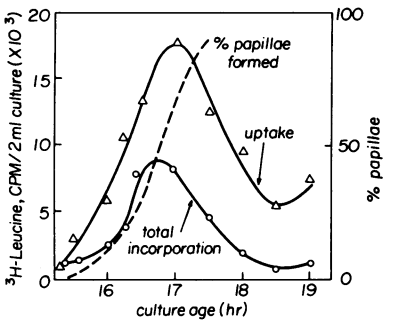

FIG. 38. *Uptake and incorporation of [^{3}H]leucine by Blastocladiella cells induced to differentiate at 15.5 h. Samples of cells were removed from the culture and pulsed for 2 min at each time point. Reproduced from Adelman and Lovett (3).*

indicating a requirement for mRNA synthesis in zoospore differentiation can be put forward. First, some RNA synthesis does take place as measured by the slow, linear [^{14}C]uracil incorporation rate throughout differentiation as described above; second, the zoospores produced contain a significant amount of presumptive mRNA [poly(A)-associated RNA] (Lovett and Wilt, 154); and third, the completion of differentiation is blocked by actinomycin D if it is added at 18 h (T_{150}). Until greater resolution is obtained, we can only conclude from these observations that some RNA synthesis is required, even though the numerous changes that take place imply the necessity for a variety of new materials, e.g., flagella and flagellar rootlet formation, cytoplasmic microtubules, cleavage membranes, γ particles, mitochondrial fusion, and ribosome "packaging" in the nuclear cap. It is, of course, possible that some or all of the newly required materials are synthesized during sporangium formation and that zoospore differentiation, per se, represents largely reorganization and assembly of such preformed materials; certainly a significant part of it involves membrane transformations.

Three experimental observations point to a continuation of protein synthesis between 18 and 19 h (T_{150} to T_{210}), at least at a minimal rate. Leucine uptake and incorporation continue to take place, although at a steadily decreasing rate; a significant and nearly constant percentage of polysomes persist (4, 172), and the new pH 5.5 protease appears (145). The synthesis and exchange of ribosomal proteins have been demonstrated at 18 h (T_{150}) after net ribosome synthesis has ceased, and this activity may continue after T_{150}, particularly if it is associated with changes in ribosome conformation and activity (2). The final decrease in [^{3}H]leucine uptake and incorporation between 18 and 19 h (T_{150} to T_{210}) is correlated with a 50% decrease in the free internal leucine pools (all other amino acid pools also decrease after 18 h; some begin earlier [171]). Since the total protein content also decreases, we do not know to what extent the amino acids are re-utilized, metabolized, or excreted.

The surprisingly stable 65% level of the polysomes throughout the interval from 17.5 to 19 h

FIG. 37. *Changes in the polysome content of cells before and after inducing zoosporogenesis. (A) Sucrose gradient absorbancy profiles of cytoplasmic ribosomes. Late-log-phase cultures were induced by a shift to inorganic medium (1/2 DS), as indicated in the legend to Fig. 33. The ribosomes were then extracted, separated on 10 to 35% exponential sucrose gradients, and pumped through a flow cell to measure the absorbance at 260 nm. (B) Change in polysome percentage versus time of differentiation. The percentages were calculated from the gradients in part A as described in the legend to Fig. 36. The dashed curve indicates the percentage of the plants with discharge papillae, a morphological control for developmental synchrony. Reproduced from Adelman and Lovett (4).*

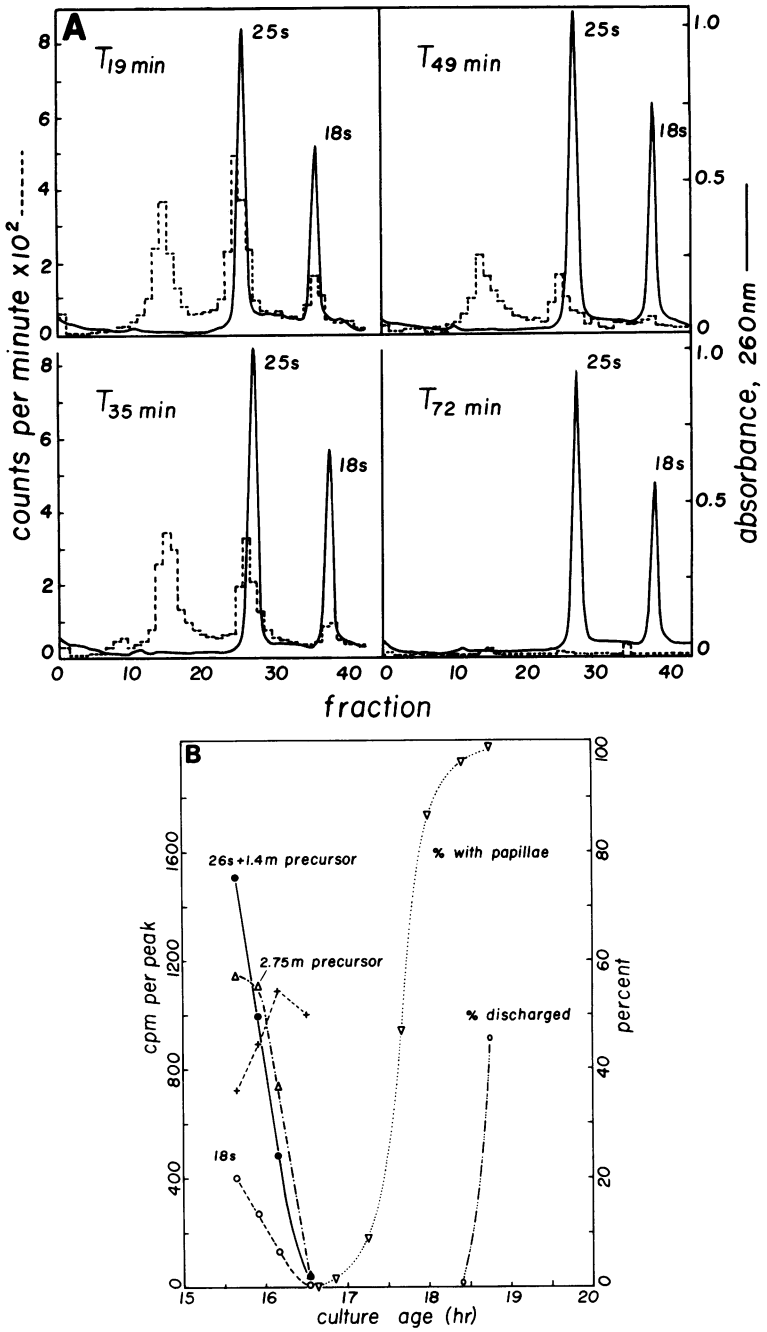

FIG. 39. *Shutdown of rRNA synthesis during induced zoosporogenesis in Blastocladiella. A culture was grown and induced to differentiate zoosporangia and zoospores at 15.5 h (T_0) by the method of Murphy and Lovett (172). At the times indicated, 100 ml of culture was removed, concentrated to 25 ml, and pulsed for 10 min with 24 μCi of [³H]uridine (specific activity, 36 Ci/mmol). The RNA was extracted and separated by gel electrophoresis (2.2% gels were run 4 h at 6 mA per gel), and the gels were scanned, sliced and counted, as described in the legend to Fig. 35. (A) Absorbancy and radioactivity profiles of RNA labeled in 10-min pulses. The times given are for the midpoint of each pulse period (T_{19}, 15 h, 49 min; T_{35}, 16 h, 5 min; T_{49}, 16 h, 19 min; T_{72}, 16 h, 42 min). After the first pulse interval, it is clear that very little of the 1.47 × 10⁶-dalton precursor is processed into the 1.32 × 10⁶-dalton 25S RNA, and the new 0.73 × 10⁶-dalton 18S RNA is lost. (B) Kinetics of rRNA synthesis and processing after induction. The radioactivity under the peaks in part A has been plotted versus time after induction. Symbols: +, percentage of the total counts per gel in the 2.75 × 10⁶-dalton precursor; ▽, percentage of plants with papillae; ○···—···○, percentage of plants that have discharged zoospores. The shutdown of rRNA synthesis is completed during the first half of zoosporangium differentiation, and the small amount of new synthesis during this interval is not processed into 18S and 25S rRNA species. (Lovett, unpublished data).*

(T_{120} to T_{200}) deserves comment. One might, on the face of it, conclude that the decrease in total protein and intermediate polysome level signals extensive and rapid protein turnover between 18 and 19 h (T_{150} to T_{210}) as a result of proteolysis and resynthesis. The net loss of protein is clear-cut, but the implication of significant new synthesis by turnover requires cautious interpretation. In actively synthesizing growth-phase plants, the level of polysomes is consistently ≧85%, whereas the level drops to 40 to 45% after a step-down to decrease the amino acid supply. The intermediate level of polysomes during differentiation could result from a depletion of the amino acid pools and/or a restricted supply of aminoacyl-tRNA or tRNA. It could also be due to a reduced rate of mRNA synthesis, a reduced rate of translation, or some combination of these. Although the amino acid pools do decrease in size, we have no direct reason to consider them limiting. The existence of high levels of aminoacyl-tRNA and aminoacyl-tRNA synthetase activity in the zoospores (see next section) also suggests that the amino acid supply is probably adequate. At present, a combined reduction in mRNA availability and the appearance of a translation inhibitor seem the most probable explanation (3).

The distribution of ribosomes over the polysome size range does not differ significantly between the 17- to 18-h (T_{90} to T_{180}) polysomes and the growth-phase polysomes; this is in contrast to an increased proportion of small multimers in the postinduction pattern with 40% polysomes at 16 h (T_{30}) when the cells are reacting to the drop in the external amino acid supply. The recovery to the 65% level by 17.5 h (T_{120}) could reflect an adjustment of the amino acid supply and a reduced concentration of mRNA molecules. If the rate of initiation and aminoacyl-tRNA availability were not restricted, the level of polysomes could be set by saturation of all the mRNA chains with ribosomes (assuming some minimal or average spacing). These polysomes could produce protein at the normal rate per ribosome, but since fewer function the actual rate per cell would be less. If, in addition to proteolysis and a reduced mRNA supply, the rate of translation were slowed by the buildup of inhibitor as we propose, one might expect a significant level of polysomes with either a constant amount or a decrease in total protein synthesis, as observed.

A reduction in the rate of peptide bond formation without a correspondingly reduced rate of initiation would rapidly lead to a saturation of the mRNA, with little net synthesis. Our evidence for a ribosome-associated translation inhibitor in zoospores will be disussed in the next section. It is relevant to note here that after induction this activity rises very sharply to reach a high level at 17 h (T_{90}) (Fig. 40); and, further, the inhibitor is found in KCl extracts of ribosome pellets obtained with procedures that yield good polysomes (Fig. 37); it seems probable that the inhibitor functions to reduce the translation rate during this stage. If our hypothesis is correct, the average synthesis time or transit time for protein synthesis on the inhibited polysomes should be much longer than during the preinduction growth phase. This has been shown to be the case during early germination (138).

The persistence of polysomes as late as 18.5 to 19 h (T_{180} to T_{210}) may seem inconsistent with the formation of nuclear caps between 18.8 and 19 h (T_{200} to T_{210}) (Fig. 1) (142). This results from two factors: the way polysomes are estimated and the degree of synchrony. There is very little decrease in the polysome percentage between 17.5 and 18.5 h but a large 29% decrease in the total RNA. The slight, apparent change in polysome percentage actually masks a real decrease in total polysome-bound ribosomes per cell of some 31% over this interval; the magnitude of the decrease is hidden because the percentages were measured with respect to the recovered ribosomes at each time point. Furthermore, because the changes are rapid as the zoospores near completion, any lack of synchrony in the culture greatly magnifies the overestimation of polysomes (i.e., some lagging plants and some already discharging). The nearly constant polysome percentage between 17.5 and 18.5 h (T_{120}

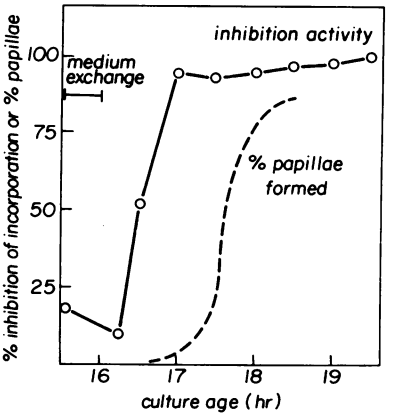

FIG. 40. *Appearance of a ribosome-associated inhibitor of amino acid incorporation after the induction of zoosporogenesis in Blastocladiella. This inhibitory activity was obtained in a 0.5 M KCl wash of ribosomes at different time points and assayed for inhibition of poly(U)-dependent [^{3}H]phenylalanine incorporation in a standard assay. Reproduced from Adelman and Lovett (3).*

to T_{180}) does indicate a proportionate decrease in both ribosomes and mRNA during differentiation. The extent to which new mRNA is produced has not yet been assessed. By the time the nuclear caps have formed at 19 h (T_{210}), both RNA and protein synthesis are virtually undetectable, and the zoospores are ready for release from the parent plant.

Protein and RNA synthesis have not been studied throughout microcylic sporogenesis, but some data are available for the period through the midpapilla stage for cells induced in phosphate at 1 h (107) and for cells induced in 1/2 DS at 2 h (Lovett, unpublished data). In both cases there is little, if any, further increase in either protein or RNA per cell after the medium change, and both constituents decrease after a 40- to 60-min lag. The plants grown for 2 h in synthetic medium and shifted to 1/2 DS show a sharper cutoff in RNA and protein accumulation, and both constituents also show greater decreases after the lag. Hennessy and Cantino (107) found that the total dry weight increased for the first hour after the change and then began to decline again after 1.5 h. It is significant that all of these changes are basically the same as those that occur in mature plants induced at 15.5 h by a similar medium shift. Such similarities reinforce the conclusion drawn from electron microscopy that the primary cytological and physiological events of differentiation are identical in cells induced to sporulate at different stages in the grown phase; the only significant variable seems to be one of scale.

The production of zoosporangia and gametangia by induced cultures of the related genus *Allomyces arbuscula* has also been examined (28, 72, 73, 235). The formation of gametangia is inhibited by actinomycin D, but the differentiation of gametes is insensitive if the drug is added after the papillate, lipid crown stage (72, 235). This is consistent with our observations with *Blastocladiella* (172), including the differential stage dependency of the effects of actinomycin D inhibition on septation and papilla formation. Fahnrich (72) and Stumm and Croes (235) concluded that gametogenesis required mRNA transcription prior to the completion of gametangia but not during gamete differentiation. Burke et al. (28), on the other hand, reported that both zoosporangial and zoospore differentiation by *A. arbuscula* were insensitive to actinomycin D. As a result they proposed that any required mRNA was already available before the growth-phase mycelia were induced to sporulate. This difference seems surprising and needs further examination. Both *Allomyces* systems were sensitive to cycloheximide, indicating a need for some protein synthesis throughout the production of gametangia and zoosporangia and their respective motile cells.

Stumm and Croes (235) found no significant change in the level of polysomes during the 3-h period after gamete induction in *A. arbuscula;* $^{32}PO_4$ label was also incorporated into the monosome and polysome regions on gradients at a decreasing rate throughout the same period. They concluded from slight shifts in the distribution of the counts that mRNA synthesis increased but that rRNA synthesis also continued during the entire process. Since both their polysome and $^{32}PO_4$ incorporation data were obtained with whole mycelia-plus-gametangia extracts, it is impossible to know what proportion of the polysomes and $^{32}PO_4$ incorporation actually represented gametangial versus mycelial contributions. However, Fahnrich (73) examined the RNA from gametangia labeled with $^{32}PO_4$ during gamete differentiation after detachment from the mycelia and found only the tRNA to be labeled. This suggests that the whole mycelia preparations of Stumm and Croes (235) represented mainly mycelium activity and that rRNA synthesis is repressed in differentiating gametangia, as it is at the same stage of *Blastocladiella* zoosporogenesis. The results of Fahnrich (73) present good evidence that the mRNA transcription required for gametogenesis occurs during the differentiation of gametangia. Although perhaps different in detail, the results with *Allomyces* and *Blastocladiella* suggest that differentiation of motile cells in these related genera share many common features.

REGULATION OF SYNTHESIS IN ZOOSPORES

As described earlier, the free-swimming zoospores germinate rapidly when placed under appropriate conditions, but as long as they are motile they effectively suppress any measurable synthesis of macromolecules while generating energy for motility. The functional significance of the highly ordered internal structure of zoospores may lie in the need to satisfy two basic requirements: a separation of the catabolic, energy-yielding pathways and products from the anabolic, energy-utilizing systems for RNA, protein, and other macromolecular synthesis, and, a conservation of the biosynthetic systems in a suspended state that can be reactivated to support synthesis with little delay at the start of germination. The intimate association of the mitochondrion with the flagellar basal body, rootlets, and lipid droplets could ensure the localized availability of adenosine 5'-triphosphate (ATP) for flagella movement (238). The surrounding endomembranes, as

well as the continuous nuclear cap and extra-cap nuclear membranes, may further serve to compartmentalize and isolate the biosynthetic systems for RNA, protein, and DNA. Only traces of uracil and amino acid incorporation are detectable in zoospores (150, 226), and the significance of these low levels is uncertain.

To find out how synthesis is prevented in this stage, the condition of the protein biosynthetic system in zoospores has been studied in some detail (3, 4, 147, 150, 208). The enclosure of the ribosomes by membranes to form the nuclear cap results in two cytoplasmic compartments: one contains the ribosomes in continuity with the nucleus via the nuclear pores (Fig. 2, 4), whereas the second, extra-cap compartment normally lacks continuity with either the ribosomes or the nucleus. The ability to isolate intact nuclear caps (Fig. 41) (147) has made it possible for us to study the intracellular distribution of various factors required for protein synthesis, particularly with regard to the potential for the cap organelle to either prevent ribosome function or to conserve the ribosomes during the inactive period.

The cap ribosomes are not packed in ordered arrays and, when the caps were isolated and the ribosomes were released by homogenization or mild lysis, only 80S monomeric ribosomes and a small fraction of subunits were detected on gradients (208). With improved extraction conditions, we have now found evidence for a low level of polysomes (about 10 to 11%) in whole zoospore preparations directly fractionated on gradients (Fig. 42); they do not function in vivo, however (C. S. Gong and J. S. Lovett, unpublished data). The isolated nuclear cap ribosomes can be dissociated into 40S and 60S subunits by dialysis in 10^{-4} M $MgCl_2$ and have an RNA/protein ratio of 1.73 (2, 147). As expected from the distribution of the ribosomes, all of the 5S RNA is found in the nuclear cap fraction, whereas the 4S tRNA is distributed in both compartments (Fig. 43; nuclear cap, 61%; extra cap, 39%). Aminoacyl-tRNA synthetase activity also occurs in both the cap and extra-cap fractions but, with the exception of the phenylalanyl- and methionyl-tRNA synthetases, have not been examined individually. About 70% of the unfractionated charging activity was found in the extra-cap fraction and 30% within the nuclear caps (208). However, estimates of the methionine- and phenylalanine-specific synthetases after purification have shown the distribution for these enzymes to be quite different, with less than 10% of the total activity in the nuclear cap fraction (Gong and Lovett, unpublished data). The tRNA from both the cap and extra-cap regions is more highly charged with amino acids (87 to 100%) than is the tRNA from growth-phase cells (55%) (208). Elongation factors EF-1 and EF-2 are present in the extra-cap cytoplasm of zoospores, with activities equal to about 34 and 62% those of growth-phase cells, respectively (Gong and Lovett, unpublished data). Less than 10% of the expected EF-1 and EF-2 activities are found in the zoospore nuclear cap fraction, which suggests that most, but not all, of these factors are excluded by the cap membrane (unless all of the cap activity represents cross-contamination during fractionation). Since even 1/10 of the normal elongation activity should support some synthesis, this does not provide very strong evidence for a lack of elongation factors as the primary block to synthesis in vivo (i.e., via compartmentalization).

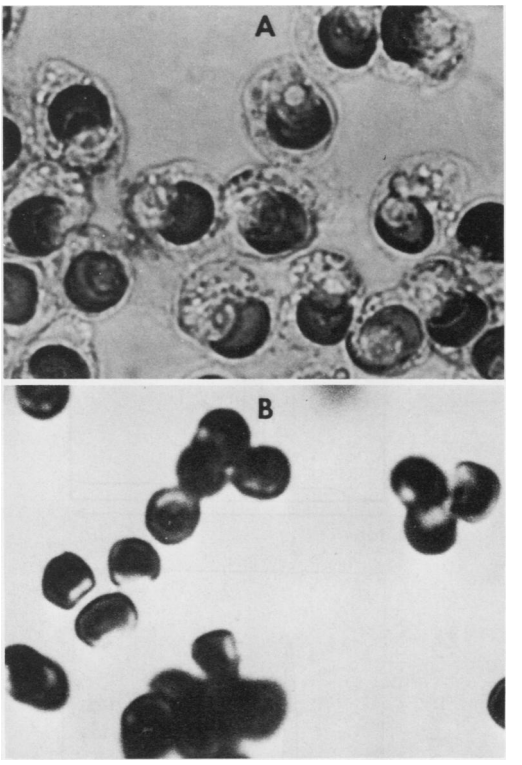

FIG. 41. *Appearance of zoospore nuclear caps before and after isolation. (A) Zoospores swollen in hypotonic $MgCl_2$ (0.001 M), fixed in osmium tetroxide fumes, and stained with toluidine blue. ×1,684. NC, Nuclear cap; N, nucleus; NU, nucleolus; SB, side body; F, flagellum. (B) Nuclear caps isolated from zoospores by the procedure of Lovett (147) and stained with toluidine blue as in part A. ×1,474. Reproduced from Lovett (147).*

A most interesting observation has been that cap ribosomes isolated in low-salt buffers [0.01 M tris(hydroxymethyl)aminomethane(Tris)-hy-

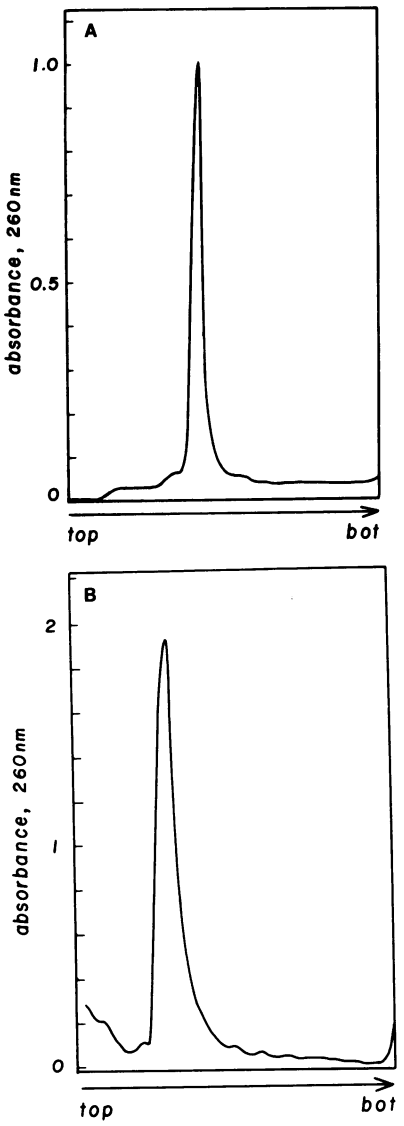

FIG. 42. *Ribosomes isolated from purified nuclear caps and whole-zoospore homogenates of Blastocladiella. (A) Zoospore nuclear caps were isolated and lysed by the procedure of Adelman and Lovett (3). After a low-speed spin at 10,000 × g for 10 min, the ribosomes were pelleted through a cushion of 35% sucrose in 10 mM Tris-hydrochloride (pH 8.5), 10 mM $MgCl_2$, 10 mM KCl, and 0.5 mM β-mercaptoethanol, suspended in the same buffer to 2 absorbancy units (260 nm), and centrifuged through a 5-ml 0 to 32.5% linear-log gradient at 190,000 × g for 45 min. The gradient was pumped through a flow cell to record the absorbancy profile at 260 nm. (B) Zoospore suspension was sonically treated for 10 to 15 s (Branson Sonifier, microtip, setting 1) in 20 mM Tris-hydrochloride (pH 8.5), 250 mM NaCl, 50 mM $MgCl_2$, 25 mM EGTA [ethylene-bis(β-aminoethyl ether) N,N'-tetraacetic acid] and made 0.22% with*

drochloride, 0.01 M $MgCl_2$, 0.01 M KCl (pH 7.2)] are virtually inactive when substituted for growth-phase ribosomes in cell-free incorporation assays (3, 208). If the same ribosomes are washed with high-salt buffer (i.e., 0.2 to 0.5 M KCl), their activity is comparable to that of plant ribosomes in poly(U)-dependent, [^{3}H]-phenylalanine incorporation assays (using growth-phase cell supernatants) (Table 1). The 0.5-M KCl buffer wash and the extra-cap, high-speed supernatants both contain material that will bind to and inhibit the activity of either KCl-washed cap ribosomes or growth-phase ribosomes. However, if an S-23 fraction is prepared in, and dialyzed against, 0.5 M KCl to remove the inhibitory activity, cell-free amino acid incorporation can be obtained with zoospore preparations in the absence of added growth-phase supernatant (Table 2). This re-

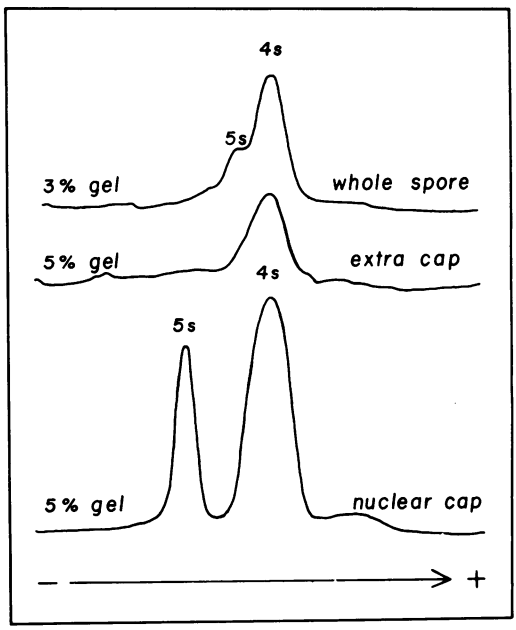

FIG. 43. *Distribution of 5S rRNA and tRNA in zoospores. RNA was extracted from zoospores, the extranuclear cap fraction, and purified nuclear caps and separated by polyacrylamide gel electrophoresis. The gels were scanned in a Gilford Linear Transport at 260 nm. The 3% gel was run for 1.5 h at 6 mA/gel, and the 5% gels were run for 2 h at 6 mA/gel. Only the low-molecular-weight region of the gels are shown, and the different areas under the peaks reflect different amounts of RNA applied to the gels. Reproduced from Schmoyer and Lovett (208).*

Triton X-100. After a low-speed centrifugation (10,000 × g, 10 min) 4.8 absorbancy units (260 nm) were centrifuged at 190,000 × g for 40 min and scanned at 260 nm as described above. (Gong and Lovett, unpublished data).

TABLE 1. *In vitro incorporation activity of nuclear cap ribosomes washed in buffers with different KCl concentrations*

Source of ribosomes	Molarity of KCl wash[a] (M)	Ribosomal protein[b] (counts/min per mg)
Nuclear caps	0.01	0
Nuclear caps	0.05	13,480
	0.5	23,120
Nuclear caps	0.5	67,040
−Poly(U)		600
−Enzyme		0
Growth-phase cells	0.5	95,540
−Poly(U)		16,380
−Enzyme		7,060

[a] Concentration of KCl used with 10 mM Tris-hydrochloride (pH 7.2) and 10 mM $MgCl_2$ in buffer used to wash the ribosomes before assay.

[b] Assayed with enzymes from growth-phase cell supernatants in a poly(U)-dependent phenylalanine incorporation assay. From Schmoyer and Lovett (208).

sult suggests, but does not prove, that all of the necessary protein factors are present in zoospores. Some reservation is necessary with this conclusion because the initiation factors have not yet been examined in zoospores and the incorporation could be due to polypeptide elongation without new initiations on the small fraction of polysomes already present. (These polysomes do not appear to function in vivo; Gong and Lovett, unpublished data). The effectiveness of the initiation inhibitors aurintricarboxylic acid and pactamycin (Table 2), nevertheless, does indicate that at least some initiation activity is present in zoospores.

The zoospore protein synthesis inhibitor does not prevent poly(U) binding (at 10 mM Mg^{2+}) but does inhibit aminoacyl-tRNA binding and peptide bond formation by ribosomes (208). The chemical nature of the inhibitor has not yet been established, but our present evidence points toward a low-molecular-weight compound, such as a nucleoside, nucleotide, or small oligonucleotide (3; Gong and Lovett, unpublished data). It is not phenylalanine or dimethylguanosine that has been reported as the spore germination inhibitor in *Dictyostelium discoideum* (8). It is not labeled when [^{3}H]nucleosides are added to cultures immediately after inducing zoospore differentiation, and it may, therefore, arise by modification of some preexisting material. We interpret the action of the inhibitor, its rapid appearance after the induction of zoosporangium and zoospore differentiation (Fig. 40), its presence in the zoospores, and its disappearance during germination (see next section) to mean that it functions to modulate or repress protein synthesis during the zoospore phase. Whether it may also have other functions remains to be established.

An earlier observation that zoospores could germinate and produce a small primary rhizoid in the absence of new RNA synthesis indicated that the zoospores contained stored mRNA to support the first essential protein synthesis (150). It has now been found that the zoospores contain significant levels of poly(A)-associated RNA (2.5 to 3% of the total RNA) and that 80% of this RNA is stored in the ribosomal nuclear cap, apparently as free ribonucleoprotein particles (154). Although this RNA fraction has not been fully characterized, the cytoplasmic poly(A)-associated RNA of other eukaryotes has been identified as mRNA (25), and we assume that it is the stored messenger fraction in zoospores.

The presence and location of the initiation factors and the initiation-specific $tRNA_F^{met}$ (as well as its charging level) remain to be analyzed. Even so, it seems nearly certain that all of the necessary protein factors are conserved in

TABLE 2. *Amino acid incorporation by whole-zoospore cell-free extracts*

Assay system	Sp act (counts/min mg of protein)	Percentage of control
Complete system[a]	35,843	100
Complete − S-23 fraction	0	0
Complete − GTP	14,105	39
Complete + aurin-tricarboxylic acid (0.1 mM)	5,147	14
Complete + pactamycin (0.1 mM)	8,961	25

[a] The S-23 zoospore fraction was prepared by 15-s sonic treatment (Branson Sonifier model S125, microprobe, setting 1) of 2 ml of packed zoospores suspended in 5 ml of extraction buffer (20 mM Tris-hydrochloride (pH 7.5), 500 mM KCl, 1 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid, 5 mM dithiothreitol), followed by 15-min centrifugation at 23,000 × *g* and dialysis of the supernatant (S-23) against the same buffer with 100 mM KCl. Incorporation activity was measured by 30-min incubation at 30 C in a 0.5-ml volume containing S-23 (455 μg of protein); Tris-hydrochloride (pH 7.8), 30 μmol; $MgCl_2$, 2 μmol; KCl, 25 μmol; ATP, 0.5 μmol; GTP, 0.025 μmol; phosphoenol pyruvate, 1.25 μmol; pyruvate kinase, 20 μg; yeast tRNA, 50 μg; reconstituted ^{3}H-labeled yeast protein hydrolysate, 2 μCi; amino acids not in the hydrolysate, 10 mmol each; and dithiothreitol, 2.5 μmol. Radioactivity was measured as hot trichloroacetic acid-insoluble material as described earlier (3; Gong and Lovett, unpublished data).

the zoospores along with the ribosomes, tRNA, and mRNA. Despite this, the activity and distribution data for all of the components will be needed if we are to identify the specific mechanism(s) that blocks active protein synthesis in the spore stage. The data do suggest that the ribosome inhibitor may prevent the association of ribosomes with the cap mRNA to initiate polypeptide synthesis, by preventing elongation or even initiation; an additional restriction due to the physical exclusion of required factors (i.e., initiation factors, $tRNA_F^{met}$, and some synthetases) still remains possible as well. A further potential function of the cap to protect the ribosomes from turnover during the period of inactivity remains to be studied.

RNA and protein metabolism have not been studied in the motile cells of *Allomyces*. It is of considerable interest, however, that Smith and Burke (218) reported about 20% polysomes in zoospore extracts from *A. macrogynus* and that Stumm and Croes (235) found an equivalent level in a mixture of gametes and motile zygotes of *A. arbuscula*. Both of these cell types contain nuclear caps similar to the zoospores of *Blastocladiella* and might be presumed to have a similar metabolism.

REACTIVATION OF RNA AND PROTEIN SYNTHESIS DURING ZOOSPORE ENCYSTMENT AND OUTGROWTH

As described earlier, no increases are detectable in the dry weight, DNA, RNA, or protein content of cells during early zoospore germination. This 45- to 50-min lag period, is, nevertheless, a time of active intracellular reorganization and includes a rapid concomitant reactivation of both protein and RNA synthesis. Net synthesis is negligible, but inhibitor experiments have shown some new proteins to be essential for further development of the encysted cells (150). The rapid activation of protein metabolism can be detected by both amino acid incorporation and the appearance of polysomes. Active incorporation begins at about 15 min after inoculation, whether measured by pulse or continuous labeling with radioactive amino acids (Fig. 44) (138, 150, 213, 226, 227). Cycloheximide (0.1 to 20 μg/ml), which blocks development beyond the encystment stage, also suppresses amino acid incorporation but does not prevent amino acid uptake (227). In addition, exposure of the cells to the specific initiation inhibitor 2-(4-methyl-2,6-dinitroanilino)-*N*-methylpropionamide (265) at a concentration of 5×10^{-5} M delays the start of amino acid incorporation by 10 min; it also reduces the incorporation rate by more than 88% and prevents germ tube formation in most cells (138). The results obtained with both inhibitors thus indicate a genuine requirement for protein synthesis.

Actinomycin D fails to block the formation of a short rhizoid or inhibit [^{14}C]leucine incorporation at the early stages (150), but it subsequently leads to a progressively severe inhibition of incorporation if the cells are exposed to it from the time of inoculation (213, 227). The extent of this inhibition has varied in the reports of three laboratory groups, each of which used different conditions, but in all cases at least one-third or more of the earliest amino acid incorporation between about 10 and 45 min was resistant to actinomycin D. Since this antibiotic inhibits uridine and uracil incorporation by more than 98%, we can conclude that the activation of the protein biosynthetic system can be achieved by the encysted cells in the absence of new RNA synthesis.

The postencystment rise in amino acid incorporation is preceded by a very rapid shift of the nuclear cap 80*S* ribosomes into the polysome fraction. Under the conditions that we use

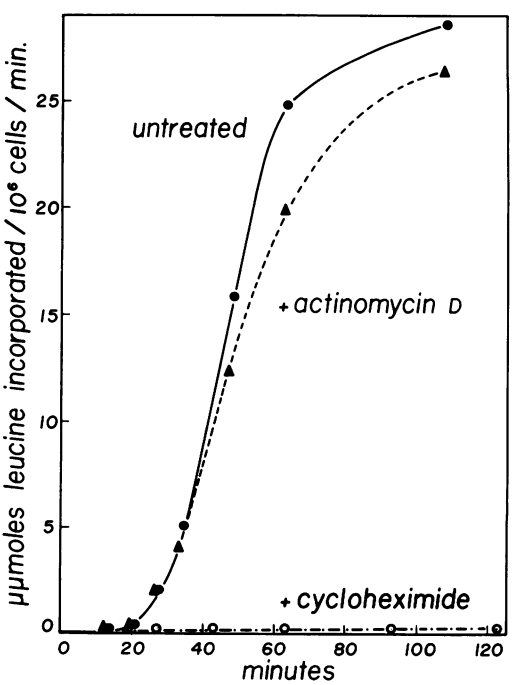

FIG. 44. *Rate of 1-[^{14}C]leucine incorporation during germination and early growth of Blastocladiella. Cells were germinated in complete medium minus leucine, and 5-ml aliquots were removed and pulsed for 5 min with and without 20 μg of cycloheximide per ml and 50 μg of actinomycin D per ml. Modified from Lovett (150).*

(where unencysted zoospores are removed by filtration), the beginning of this shift can be detected around 10 min, and nearly 50% of the ribosomes have entered polysomes by 20 min of germination (Fig. 45) (138). This initial rapid increase is completed by about 30 min, after which the level of polysomes continues to rise at a less rapid rate. In the presence of cycloheximide, a small percentage of ribosomes very slowly enters the polysome fraction but based on incorporation rates apparently catalyze little, if any, new polypeptide synthesis. Actinomycin D, on the other hand, fails to reduce the rate of polysome assembly or the level achieved to any significant extent until after 20 min. The level then decreases exponentially ($T_{1/2}$ = 45 min).

The simplest interpretation of the incorporation and polysome experiments is that the sudden beginning of protein synthesis results from rapid assembly of functional polysomes using the ribosomes and mRNA previously stored in the zoospore nuclear cap. The exponential decay of the first-formed polysomes in the presence of actinomycin D is presumed to represent normal turnover due to polypeptide synthesis and ribosome runoff without new mRNA production. If the initial actinomycin D-resistant polysome assembly represents all the preformed mRNA that is normally mobilized during early germination, the continued increase in polysome content after 20 min in untreated cells may indicate the contribution of newly synthesized mRNA. However, this would only be true if actinomycin D had no direct effects on protein synthesis, such as the reduction in initiation reported for HeLa cells (215).

Despite the evidence for a rapid activation of protein synthesis soon after zoospores encyst, a net increase in protein per cell cannot be detected until 80 min (Fig. 31). At least two factors contribute to the delayed onset of protein accumulation: persistence of the zoospore ribosome-associated inhibitor and protein turnover. The average elongation rates (weight-average ribosome transit times) have been compared for cells at 30 min of germination and in the early growth phase at 120 min (138) and have been found to be 66 and 32 s, respectively, indicating a twofold higher rate per ribosome in the actively growing cells. The average elongation rate and polymerization rate (5.53 amino acids per ribosome per s at 24 C) at 120 min are similar to the values reported for *Saccharomyces cerevisiae* (106, 137). When corrected for the reduced numbers of active ribosomes per cell and their 50% lower translation rate, the calculated rate of new synthesis at 30 min is one-fifth

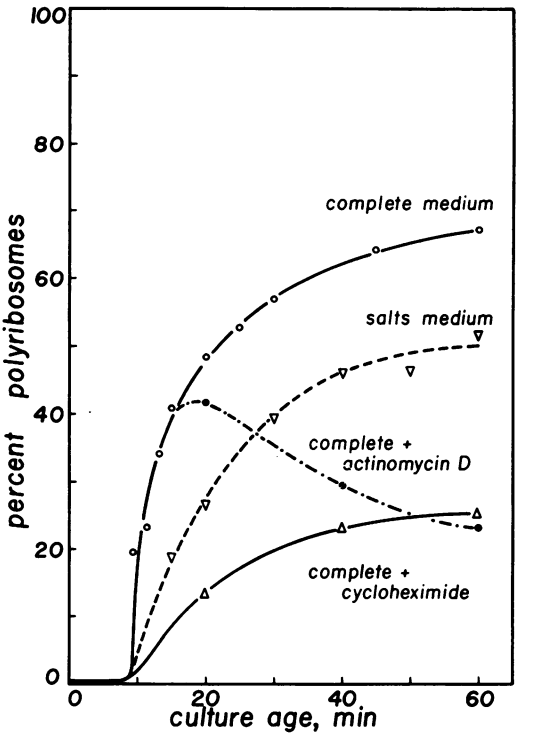

FIG. 45. *Formation of polysomes during zoospore germination. Separate cultures were grown under the conditions indicated, and the polysomes were extracted and analyzed on gradients, after the culture samples had been rapidly chilled and removed at different times. The inorganic salts medium was BSM (150) and the actinomycin D- and cycloheximide-treated cultures contained 20 and 5 μg of inhibitor per ml, respectively, from zero time. Reproduced from Leaver and Lovett (138).*

of that at 120 min or 1.5 to 2 pg/cell per h. Lodi and Sonneborn (145) have reported evidence for the turnover of preformed zoospore proteins at a rate of 5%/h during germination. Their experiments did not measure turnover of newly produced proteins, and so 5%/h may represent a minimal value, but even so this could be equal to about half of the small amount of new synthesis in the early stages. The combined effect of the reduced translation rate and turnover satisfactorily explains the failure of the cells to increase in protein content before 80 min. The reduced translation rate itself appears to be due to the persistence of the zoospore ribosome inhibitor in the high-density cultures used for our experiments (4). The presence of a similar inhibitory material in the medium following germination (C. S. Gong, unpublished data) suggests that the ribosome inhibitor is released from the cells after encystment and, further,

that it may be identical to the zoospore germination inhibitor reported by Truesdell and Cantino (251) (also mentioned as a zoospore maintenance factor by Soll and Sonneborn in reference 228).

Silverman et al. (213) have also examined protein synthesis during germination and have drawn a somewhat different conclusion from ours concerning the rates of early protein synthesis. However, their basic results are in substantive agreement with and confirm ours regarding the activation of protein synthesis after encystment. They reported that polysome formation started later and reached a higher level earlier (i.e., 80% polysomes by 30 min), but this may have been due to the use of cycloheximide, which could cause accumulation of polysomes during collection of the cells. Using [^{3}H]leucine incorporation data that was corrected for average pool specific activities, Silverman et al. (213) estimated the rate of protein synthesis to increase fourfold between pulses at 13 to 20 min and 33 to 40 min. Assuming the average protein to contain 8% leucine, the rate they calculated for cells at 33 to 40 min would be some three times as high as our estimate (138) and should yield measurable increases in protein per cell, even with a 5%/h turnover rate. Our failure to detect such an increase (150) suggests that their use of pool specific activities may have resulted in an overestimate of the amount of synthesis due to pool compartmentalization (239, 268). Our method should have been independent of variations in pool specific activity, but it will be important to resolve these differences. A considerably less certain, but independent, estimate of the relative rates of protein synthesis between the 30- and 120-min cells can be made if it is assumed that both acytinomycin D inhibition of new mRNA synthesis and a severe medium step-down result in normal polysome runoff. Polysomes were found to decay with a half-life of 45 min in the actinomycin-treated germinating cells (20 to 60 min; Fig. 45) and with a half-life of 24 min after a step-down shift at 120 min (Fig. 36). These differences in polysome half-life are in good agreement with the twofold difference we have found for the transit times measured at the same stages.

As in the case of protein synthesis, incorporation of RNA precursors can be detected well before measurable changes occur in the total RNA content per cell. Active incorporation of radioactive uracil, uridine, guanosine, or adenosine begins between 10 and 15 min, with the rates becoming linear by 20 min (Fig. 46) (138, 150, 227). Actinomycin D (5 to 25 μg/ml) inhibits this incorporation by more than 98% without blocking encystment or germ tube formation until 45 to 50 min (150). If the radioactive precursors were not transported into the cells prior to 15 min, an earlier start might elude detection; nevertheless, [^{3}H]adenosine labeling experiments have shown this not to be the case. The ATP specific activity begins to rise 30 s after zoospore inoculation, whereas measurable accumulation of [^{3}H]AMP into RNA does not begin until 10 to 15 min and becomes linear by 20 min (Lovett and Wilt, unpublished data). This means that, like protein synthesis, RNA synthesis is started up in a short period between 10 and 15 min during the maximum rise in zoospore encystment.

From the analysis of pulse-labeled RNA on polyacrylamide gels (Fig. 47), it is clear that the early transcription products include both polydisperse RNA and rRNA, with much of the latter in the form of precursors (i.e., precursors

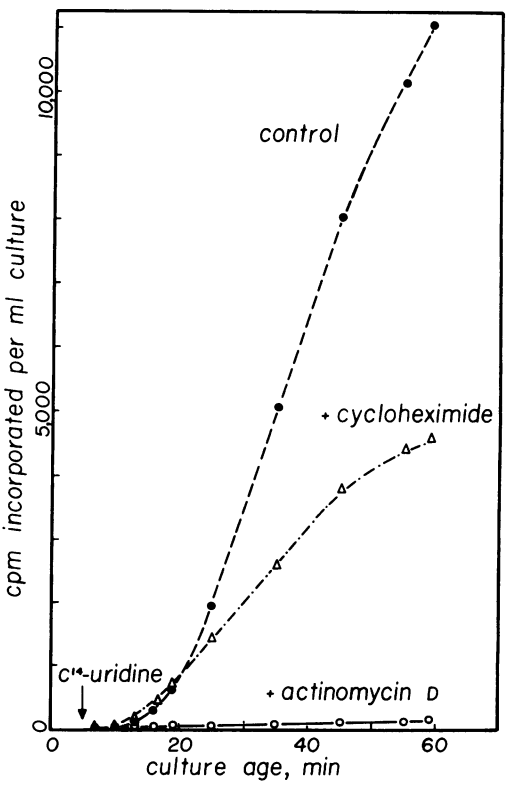

FIG. 46. *Incorporation of 2-[^{14}C]uridine during germination of Blastocladiella zoospores. Cells were germinated in complete synthetic medium to which [^{14}C]uridine was added at 5 min. Actinomycin D (20 μg/ml) or cycloheximide (5 μg/ml), when used, was present at zero time. Samples (1 ml) were removed versus time to measure incorporation. Reproduced from Leaver and Lovett (138).*

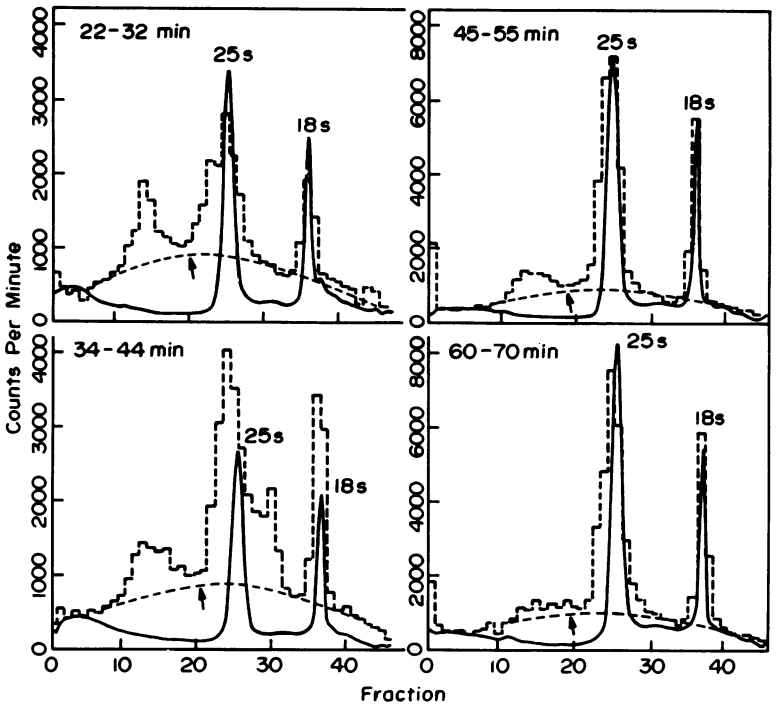

FIG. 47. *Fractionation of pulse-labeled RNA synthesized at intervals during zoospore germination. Cells germinating in complete synthetic medium were removed and pulsed for 10 min with 5-[³H]uridine. The extracted RNA was separated by polyacrylamide electrophoresis and scanned at 260 nm, and gel slices were counted. The broken line marked with an arrow represents the polydisperse fraction. ---, Absorbance at 260 nm; ————, radioactivity. Reproduced from Leaver and Lovett (138).*

accumulate during a 10-min pulse with [³H]uridine prior to 50 min of germination) (138). The evidence for sluggish processing as a potentially limiting condition in the early stages is consistent with the size of the nucleolus, which is still small and compact at this time and subsequently enlarges (from about 11% of the nuclear volume to about 40%). Actinomycin D prevents the enlargement of the nucleolus, as might be expected from its effect on RNA synthesis (Fig. 17) (14). After 50 min, precursor accumulation is insignificant, suggesting that the processing of rRNA precursors has achieved the growth-phase rate, and it is at this time that the cells begin to accumulate measurable amounts of RNA (150).

Under conditions of continuous labeling, the entry of [³H]uridine into cytoplasmic polysomes lags well behind the rapid postencystment shift of ribosomes into the polysome fraction (138). Thus, it is most unlikely that newly made RNA could contribute in any major way to early polysome formation. Furthermore, the whole-cell labeling experiments cited above demonstrate rather convincingly that virtually no new RNA is synthesized until after 15 min, at which time nearly half of the original polysome increase has already occurred. Our suggestion that new mRNA begins to enter polysomes after 20 min will require proof that the polydisperse radioactivity on the polysomes is mRNA by other criteria. Some support for this notion is provided by the large percentage of the newly synthesized RNA associated with the poly(A) fraction when RNA is extracted from zoospores germinating in medium containing [³H]adenosine (Lovett and Wilt, unpublished data). We do not yet know whether any of this putative mRNA enters polysomes at the early stages of germination. On the other hand, in the case of new rRNA it is evident that new synthesis makes no appreciable contribution to cytoplasmic ribosomes until after 26 min (138).

Cells germinated in inorganic medium share several metabolic features with growth-phase cells following a step-down to inorganic medium: they fail to increase in dry weight (Fig. 30), their RNA and protein contents change little (Fig. 31), and the polysome fraction only reaches a level of 50% by 60 min (Fig. 45). Uridine incorporation experiments, nevertheless, demonstrate that RNA synthesis does oc-

cur in these cells at a reduced and relatively constant rate through at least 70 min. The processing of the newly synthesized rRNA remains sluggish, however, and the small amount of rRNA made is apparently degraded instead of being processed into mature ribosomes (Fig. 48) (138). On the other hand, it is significant that the cells germinating in inorganic medium develop normally through and beyond the 45- to 50-min stage when actinomycin D blocks rhizoid growth. This means that the rapid accumulation of RNA normally associated with this stage is not required for rhizoid elongation. Since the cells reach this block point irrespective of when they are exposed to the antibiotic, it also suggests that actinomycin D may prevent the synthesis of an essential mRNA at this point in development (150). The cells in inorganic medium should be able to produce the necessary mRNA via turnover.

Our understanding of regulation in the zoospore stage and during zoospore germination is still far from complete, but a more coherent picture of the system is beginning to emerge. Each of the three laboratory groups working in this area has used somewhat different methods to prepare and germinate *Blastocladiella* zoospores. Since these factors can influence both the kinetics and synchrony of germination (41, 225, 228, 251), it is difficult to correlate the precise timing of events between the experiments of different groups, particularly at early stages when many changes occur within a few minutes. These uncertainties need to be kept in mind when considering the interpretation of germination events which follows.

Although there may be some disagreement about precise developmental correlations, it is generally agreed that the start-up of both RNA and protein synthesis begins after the cells encyst (RC-I stage). The incorporation curves clearly lag behind the rising encystment curve when cells are germinated in complete or inorganic medium. It is also generally agreed that most, but not all, of the events associated with encystment, i.e., events prior to outgrowth, can occur in the absence of detectable protein synthesis, and further, that the entire developmental sequence of encystment and early germ tube outgrowth can be accomplished by cells in the absence of measurable RNA synthesis. With the exceptions of flagella axoneme disassembly, disappearance of the microtubular triplets, and the deposition of the thin cyst wall, all other visible structural events of encystment (RC-I and RC-II stages) entail membrane rearrangements. In a sense, these changes, which have been described in detail (41, 150, 224, 227, 251), could be considered a type of dedifferentiation (252). On the other hand, Soll and Sonneborn, from a somewhat different perspective, have stressed these events as examples of differentiation in the absence of concurrent transcription and translation.

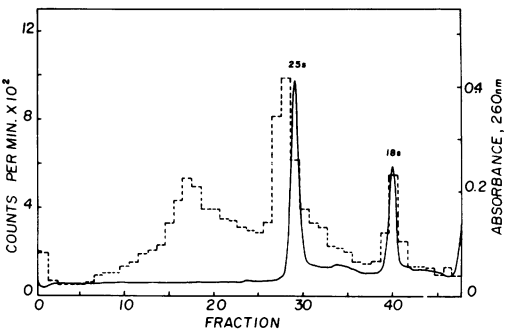

FIG. 48. *[³H]uridine incorporation into RNA during germination of cells in inorganic BSM medium. The cells were pulsed for 10 min from 51 to 61 min after inoculation. See the legend to Fig. 47 for the method used. – – –, Absorbance at 260 nm; ———, radioactivity. The amount and distribution of radioactivity incorporated in these cells after germinating 61 min in inorganic salts is similar to the earliest pulse (Fig. 47, 22 to 32 min) in cells germinated in complete medium. However, the BSM cells develop a normally branched rhizoid (Lovett, unpublished data).*

The only obviously new structures produced in the encystment stages are the cyst wall and the golgi equivalents (14). Cyst wall formation occurs without protein synthesis and is correlated with a rapid 90% depletion of the cellular glycogen pool within the first 20 min (237). An analysis of the cyst wall has not been published, but it is expected to contain chitin and/or glucan. Its rapid assembly, even in cycloheximide-containing media, means that either the polymerizing enzymes or preformed materials must be in the zoospore before encystment. There is no solid evidence for preformed wall material as is found, for example, in *Pythium* zoospores (S. N. Grove and C. E. Bracker, personal communication), but the first, second, and last enzymes for the chitin pathway are present in zoospore extracts (29, 37, 86, 152, 179). Much of the chitin synthetase is found in particulate fractions (29), and part occurs in association with isolated γ particles (37). A role for the γ particles in cyst wall formation has been put forward by Cantino and Myers (38), based on this association and the reported production of multivesiculate bodies (often found with openings through the plasmalemma) from the γ particles as they disappear during germination (250, 251).

The nine triplets of flagellum-anchoring microtubules in the zoospore disappear early during encystment (RC-I) in the absence of new protein synthesis; the degradation or disassembly of the retracted flagellar axoneme, however, does not occur as it normally would within the next several minutes (14, 138, 226). The pH 5.5 protease activity characteristic of the germination phase is not inhibited by cycloheximide in complete medium (145), so the retracted axoneme must require specific new proteins for its removal. New protein synthesis also seems a probable requirement for the organization of the functional golgi equivalents, as these are few and poorly developed in cells encysted in the presence of cycloheximide (14, 138).

The data are all consistent with the activation of both RNA and protein synthesis during the RC-I stage after the original membrane changes (138, 213), although Soll and Sonneborn interpret these as events of the RC-II stage. This may, in part, be due to the delay in the start of amino acid incorporation (i.e., at 25 to 30 min) in their DM-2 medium (227) as compared with our synthetic medium (138, 150) or that of Silverman et al. (213). On the other hand, in their inorganic germination solution, Soll and Sonneborn did find incorporation to begin earlier, which was at the time that the earliest dinsintegration of nuclear caps was also detected (Fig. 3) (227). In our view the experimental results suggest strongly that polysomes begin to increase as soon as the membrane of the nuclear cap loses it previous continuity (and connections with the nuclear membrane), even while most of the ribosomes are still clustered around the nucleus (138, 213).

The kinetics of polysome formation (Fig. 45) and those for uracil and uridine incorporation clearly indicate that the earliest essential protein synthesis does not require prior or concomitant transcription of RNA to make new ribosomes, tRNA, or mRNA. Furthermore, one can assume, a priori, that some minimum of all the required protein components must preexist in the zoospore since no new proteins could be made in their absence. The demonstration of cell-free synthesis in zoospore extracts (Table 2) clearly supports such an assumption. In terms of macromolecular constituents, the activation of protein synthesis must take place utilizing entirely preformed materials already stored in the zoospore. Conversely, the regulation of synthesis in the zoospores may be expected to involve mechanisms to prevent both the function and the degradation of these macromolecules in a readily reversible way. If nucleoside triphosphates are limiting, the early rise in respiration (39, 237) may indicate an increased rate of energy metabolism to produce these compounds before significant RNA and protein synthesis can occur. The necessity to provide substrates for this metabolism could be a partial cause for the rapid depletion of glycogen (237) and lipid (168, 219) reserves during germination. The timing of the respiratory rise is correlated with the steady increase in ATP specific activity of zoospores germinating in medium containing [^{3}H]adenosine (Lovett and Wilt, unpublished data). The zoospore protease (145) could also give rise to increased levels of free amino acids.

We cannot yet fully explain how protein and RNA synthesis are suppressed in zoospores. The ribosome inhibitor (3, 4, 208) represents one probable control at the level of translation, but we still know too little about its mechanisms of action to be sure it has no other effects such as, for example, on initiation. The sudden appearance of the inhibitor soon after inducing differentiation and its loss during germination certainly imply that it has a regulatory role. The presence of about 90% free 80S ribosomes and ribosomal subunits and free mRNA particles further suggests that initiation steps prior to elongation are suppressed. However, the effect, if any, of the ribosome inhibitor on initiation is still untested. Initiation could also be expected to be deficient if the initiation factors are excluded from the cap, if methionyl-$tRNA_F^{met}$ is lacking, or if specific blocking proteins are bound to the stored mRNA. These factors are presently being examined. The function of the nuclear cap membrane remains somewhat enigmatic. The evidence available does not strongly support a compartmentalizing function, but we cannot yet be certain whether it has a regulatory role or whether it serves a secondary, protective function. With regard to regulation of RNA synthesis, it may be significant that the nuclear envelope has no pores outside the area covered by the cap since the zoospores contain the same RNA polymerases as growth-phase cells (114). Definitive conclusions concerning regulation in the zoospores obviously must await further information.

The uncertainty concerning zoospore regulation also limits our understanding of how the biosynthetic systems are reactivated during encystment. Nevertheless, the germination process is known in sufficient detail to encourage some tentative suggestions of possible mechanisms. Zoospores have high levels of both cyclic AMP (cAMP) and cGMP, and both decrease rapidly during the first 45 min of germination (212). cAMP phosphodiesterase activity is also

at its highest level in zoospores and drops precipitiously during the first 20 min of germination (157). It is not known how these changes are related to the concomitant activation of metabolism, but a significant role(s) might be expected based on the extensive evidence for the participation of cAMP in the regulation of both glycogen (223) and protein (267) metabolism in other organisms. The structural significance of the membrane changes during germination has been discussed elsewhere (41, 224, 226, 251), but the correlation between the activation of biosynthesis and these membrane transormations during encystment also encourages a view that removal of permeability barriers may be significant. It releases the closely packed ribosomes, may permit the release of the ribosome-associated inhibitor, and reestablished cytoplasmic continuity between the ribosomes and the bulk of the elongation factors and, perhaps, of the initiation factors. Disorganization of the cap membrane also removes the barrier between the ribosomes and the mitochondrion and between the latter and the nucleoplasm through the nuclear pores. At the very least, this should facilitate transfer of mitochondrially generated ATP for biosynthetic reactions. This interpretation would suggest macromolecule synthesis as a secondary consequence of the membrane reorganizations, which seems plausible if it is recalled that encystment, followed by synthesis, seems to be an invariant and all-or-none sequence once it is triggered. To assign the cap membrane the primary regulatory role, on the other hand, seems unsatisfactory because it seems to lack any general applicability. With the exception of species in the *Blastocladiales*, the zoospores of other fungi that lack the uninterrupted cap membrane (even in cases where the ribosomes do aggregate next to the nucleus as a "nuclear cap") also fail to display macromolecule synthesis (Cooper and Fuller, personal communication; Ferguson and Lovett, unpublished data; 141). This encourages an expectation that the primary regulation of protein synthesis will be found at the level of initiation, which would be the most efficient point to block the entire process (263).

At the present time, three firm conclusions can be drawn concerning postencystment protein synthesis: (i) the net amount is very small; (ii) a large number of different polypeptides are produced from the stored mRNA (i.e., become labeled even in the presence of actinomycin D) (213); and (iii) some unknown fraction of these proteins is essential for development. Ribosomal structural proteins make up more than 20% of the new protein at 30 min (138, 151) and could contribute many of the labeled bands observed by Silverman et al. (213). The identity of other new proteins is unknown. The germination protease studied by Lodi and Sonneborn (145) seems not to be a candidate since it is made during zoospore differentiation, but these authors did suggest that it could have an important role in the intracellular events of this stage by modifying other proteins. The effects of stage-dependent cycloheximide addition have shown that one or more proteins must be made before the rhizoid can begin to form at 25 to 30 min (213), as has the lag in rhizoid initiation after removal of cycloheximide from cells blocked at encystment (227). An obvious possibility for this synthesis would be proteins required for golgi center organization or function or some material needed for cell wall secretion (e.g., secretory vesicles) (14). Since L-glutamine:D-fructose 6-phosphate aminotransferase (glucosamine phosphate isomerase [glutamine forming]; EC 5.3.1.19), D-glucosamine 6-phosphate *N*-acetyltransferase, and chitin synthetase are already present in zoopores, synthesis of their structural proteins would not seem to be necessary; other proteins required for this pathway or for glucan synthesis have not been examined.

Despite the inessentiality of new RNA before 40 to 50 min, the transcription of all types begins immediately after encystment. New mRNA apparently begins to enter the polysome fraction between 15 and 20 min (138), but its quantitative contribution still requires analysis. Silverman et al. (213) demonstrated very pronounced qualitative differences in the proteins synthesized by cells in the presence and absence of actinomycin D. The production of some proteins stopped by 45 min in actinomycin D, whereas others did not, and those apparently resistant to inhibition were in the lower-molecular-weight range. They also found the average size of the polysomes to shift to smaller sizes in the antibiotic-treated cells, although it is difficult to judge whether the size shift represented preferential synthesis of a specific class of smaller proteins or other effects of the inhibitor over the longer exposure times. Their experiments did show rather convincingly that continued mRNA synthesis is required to maintain the production of a normal spectrum of proteins and, further, that many different mRNA's are translated at early times. In has been estimated that probably no more than one-fourth to one-half of the stored mRNA [poly(A)-associated RNA] in the zoospore nuclear cap is used to assemble the polysomes formed first (151). The fate of the remainder is unknown. It might

be expected that they would enter polysomes at a slow rate throughout germination, although they do not seem to do so effectively in actinomycin D-treated cells. Alternatively, the "excess" mRNA might represent messages for proteins needed when cells begin growth in different environments, with the unused transcripts subject to destruction. Both alternatives would require a mechanism for the selective activation of the required messages from a larger population, perhaps through special proteins that are characteristically associated with eukaryotic mRNA's (207) or through the blocking nucleotides at the 5′ end of mRNA molecules (173). As indicated earlier, these mechanisms could also be involved in the regulation of zoospore protein synthesis.

The immediate start of rRNA synthesis and preferential accumulation of RNA per cell after 40 min, compared with the lag in protein accumulation until after 80 min, can be explained as a response to the growth conditions, independent of the developmental program of the cells. During the growth phase, a reduction in protein synthesis causes a stringent shutdown of rRNA production and prevents the synthesis of excess ribosomes (Lovett, manuscript in preparation). During germination, preferential rRNA synthesis may be interpreted as a response in the opposite direction to readjust the ribosome concentration to achieve maximal synthetic rates in complete medium (138). Additional evidence for this concept is provided by the demonstration of ribosomal protein synthesis before 80 min and the delay in active protein accumulation until the RNA/protein ratio has risen from 0.32 in the zoospores to the ratio of 0.41 characteristic of the growth phase (Fig. 32) (138). The lag in RNA before 40 min is probably due to sluggish precursor processing, which may in turn be caused by a requirement for the reorganization or expansion of the nucleolus. As expected, the RNA/protein ratio fails to increase significantly in cells germinating in inorganic medium (Fig. 32), and it is, thus, not a requirement for normal morphological development.

The experiments with inorganic medium have helped to verify the distinction between regulatory growth responses and the developmental program for germination, although the results obtained with cells germinated in inorganic medium need to be interpreted with some caution. It seems reasonable to assume as a first approximation that encystment and the "turn-on" of biosynthesis are entirely normal in inorganic media, but later the cells must accomplish any new synthesis of materials at the expense of endogenous substances via turnover. It is not yet clear when this begins, but it could start as early as 30 min when the stored glycogen is largely depleted (237) and the rhizoid begins to form. In any case, active turnover may severely complicate attempts to measure developmentally significant synthesis in cells germinating under starvation conditions. The situation is further complicated by the demonstration of Hennessy and Cantino (107) that shifting cells to buffer at 30 min induces microcyclic sporogenesis, i.e., an entirely different developmental program. The failure of cycloheximide to block leucine incorporation by more than 80% in inorganic germination solution compared with complete medium (227) and its only partial inhibition of the germination protease, which is not observed with complete medium (145), further indicate a very different metabolic situation in the two types of media. A necessity to complete development (including a significant amount of rhizoid elongation) under conditions of severe nutrient limitation may be a frequent problem for cells germinating in nature if they need to become established on insoluble organic substrates. For this alternative developmental program, a mechanism ensuring the minimal essential synthesis to produce a rhizoid at the expense of intracellular turnover would have important survival value. It also seems probable that the balance point between this program and a switch to the microcyclic sporulation program would depend on some critical level of nutrients in the external environment or on the ratio between these and the level of utilizable endogenous materials.

The ultrastructural changes during gametogenesis (18, 192) and zoosporogenesis (10) in *Allomyces* species resemble the process described for *Blastocladiella* zoosporogenesis in most important details. The diploid zoospores (81, 108) and haploid meiospores (184) of *Allomyces* are quite similar to *Blastocladiella* zoospores in their organization, except for the smaller side body, additional small and scattered mitochondria, and numerous lipid droplets. The processes of encystment and rhizoid outgrowth described for *Allomyces* zoospores (108) and meiospores (129, 185) also closely resemble the same stages of *Blastocladiella* zoospore germination through the first events; in *Allomyces* the subsequent hyphal growth at the pole opposite the rhizoid begins later.

RNA and protein metabolism have not been studied extensively during germination in *Allomyces* species, but experiments with both zoospores and meiospores have given results very similar to those with *Blastocladiella*. In both

Allomyces systems the incorporation of radioactive amino acids and uracil (or uridine) begins at or immediately after encystment (28, 185, 186). In leucine-lysine synchronized cultures of *A. neo-moniliformis*, DNA replication occurs between 0 and 10 min and mitosis between 40 and 65 min at 34 C (no motile period with the technique used) (186). In similar meiospore germination experiments, mitosis occurs between 25 and 70 min (185). *Allomyces arbuscula* zoospores can encyst in the presence of either cycloheximide or actinomycin D but can only produce a rhizoid in the presence of the latter (28). Burke et al. (28) have concluded from these results that protein synthesis is required for rhizoid formation and that the mRNA templates for the essential proteins are preexistent as stored mRNA in the zoospores. The kinetics of specific RNA synthesis (i.e., mRNA, rRNA, and tRNA) have not been examined during *Allomyces* spore germination, and there is no experimental basis for the conclusions of Olson and Fuller (186) and Olson (185) that the whole-cell incorporation data represent mRNA synthesis.

DISCUSSION

Experimental work on the induction and metabolism of differentiation in most fungi is either absent or very fragmentary. What is known on the subject has been discussed extensively in several comprehensive and specialized reviews (16, 90, 151, 220, 221, 222, 241, 253, 261), and it would serve no useful purpose to review the general problem of fungal morphogenesis again here. It does seem appropriate, however, to take a critical look at the evidence now available on the roles of RNA and protein synthesis in fungal development. The objectives of the discussion that follows are threefold: (i) to compare the data on RNA and protein metabolism obtained using higher fungi with those just described for *Blastocladiella emersonii*, (ii) to point out the general conclusions that can be drawn from this area of research with fungi, and (iii) to stress the interesting similarities between many fungi and other eukaryotic and prokaryotic microogranisms in the conditions that induce differentiation and in a number of the metabolic changes that subsequently occur in the differentiating cells.

Sporulation

The conditions necessary for the induction of asexual sporulation in fungi may vary depending upon the group or the specific organism. For example, light may have an improtant role in triggering the sporulation of some fungi, e.g., *Blastocladiella britannica* (110, 112, 113), *Trichoderma* (92), *Physarum polycephalum* (55), *Pilobolus* (189), etc., but the mechanism of action is not at all clear. On the other hand, there are numerous examples of species in which a limitation of growth leads to sporulation (222). Generally, this limitation has been imposed by restricting or removing either the source of nitrogen, the source of carbon, or both (or the substitution of a less readily metabolized substrate). In the case of *Neurospora crassa*, sporulation can be induced by either manipulation of the carbon and nitrogen components in liquid medium (183) or by simple starvation in phosphate buffer (233). *Aspergillus nidulans* mycelia grown in nonshaken, submerged liquid culture do not sporulate but can be induced to do so by filtration and incubation of the mycelial mat moistened with medium (7, 85). The mycelium cannot be induced under these conditions until it reaches a stage of "developmental competence." Strains of both *Penicillium chrysogenum* (194) and *Aspergillus niger* (176) will sporulate in continuous liquid culture if severe growth limitation is imposed by providing only slightly greater than the maintainence level of glucose or a relatively poor carbon source such as citrate, respectively. Although it may be hazardous to generalize, these results do suggest that starvation or near starvation may be a very common inducer of sporulation in conidiating fungi. It is also an essential, but not sufficient, condition for the induction of sporulation in *Physarum polycephalum*, which requires light as well (55). Starvation is a well-known method for inducing sporulation in a variety of aquatic fungi besides *Blastocladiella* (67, 230). In the case of *Achlya bisexualis*, the protoplasm of young, growth-phase mycelia can be almost entirely converted into zoosporangia by starvation in 0.5 mM $CaCl_2$ (94, 247). The induction of differentiation in fungi by various degrees of starvation and many of the consequent metabolic responses are strikingly similar to the induction methods and responses reported for bacteria and cellular slime molds: the process of differentiation is characteristically an aerobic one, with accompanying changes in many of the enzymes of the tricarboxylic acid cycle, glyoxalate shunt, etc.; the conditions that induce sporulation often result in the appearance of secondary metabolism and production of extracellular enzymes such as proteases; and, finally, extensive intracellular turnover of RNA and protein takes place. All of these characeristics have been well documented in both bacterial sporulation and cellular slime mold morphogenesis. One might say, tongue in cheek, that the situa-

tion in fungi also resembles those more extensively studied systems in that the interelationships of the observed changes and the causative factors are poorly understood! At least one important reason for this is that the conditions used to induce sporulation typically present the cells with a severe step-down in all available nutrients or, alternatively, with a large excess of carbon in the absence of the nitrogen compounds needed to maintain balanced biosynthesis. These changes could be expected to have large effects on intermediary metabolism (91, 246), and it seems probable that it is these effects that have been measured in many of the enzymological studies, discussed in the review by Smith and Galbraith (222). Workers in bacterial sporulation have been plagued with similar problems in trying to assess which of the many changes in starved bacteria are intrinsic to the process of sporulation and which are metabolic manifestations of the release from catabolite repression (77, 206). This problem has also received relatively little attention until recently in the work with *Dictyostelium* (102). In work with fungi, these distinctions have not often been clearly tested, but in a few instances it has been demonstrated that enzymatic changes that are normally associated with sporulation can be altered without affecting the process, e.g., trehalase (103) and succinic dehydrogenase in *Neurospora* (185, 233), and uridine 5'-diphosphoglucose (UDPG) pyrophosphorylase in *Physarum* (134). Much of this work has concentrated on intermediary metabolism; however, since energy and intermediates would be required for new synthesis, the presence of a tricarboxylic acid cycle and related enzymes could be a necessary, but not sufficient, condition for sporulation (77).

An excellent example of the kinds of problems encountered with the developmental analysis of metabolic enzymes comes from the work of Hutterman et al. (119) who have provided an unequivocal demonstration of the de novo synthesis of glutamic dehydrogenase during starvation-induced spherulation (sclerotization) of *Physarum polycephalum* by using density-labeling techniques. This enzyme increases about ninefold in salt-induced spherulation but little, if any, in mannitol-induced spherulation. However, glutaric acid, which inhbitis the enzyme, also prevents spherulation under both circumstances (118). Thus, large changes in the specific activity of an enzyme per se may be irrelevant to the success of differentiation whereas some minimal activity may be essential for the basic cellular metabolism that supports it. The same experiments have provided evidence that some enzyme changes in induced cultures may represent altered balances in the rates of their synthesis and degradation during the period of extensive decrease in the protein content of starving plasmodia (120). In this regard, it seems significant that the glutamic dehydrogenase increased little in the mannitol cultures that lost only about 30% of their protein while differentiating in synthetic medium (118) but increased ninefold in the starvation medium (120) in which about 60% of the protein was lost (91). A variety of evidence suggests that the decrease in protein content of *Physarum* is accompanied by active protein turnover. Prelabeled proteins decreased in specific activity with a half time of about 10 h (205), and both sporulation and spherulation require new protein synthesis throughout most of the respective processes (204, 205). Cycloheximide and actinomycin D block the enzyme changes characteristic of spherulation and, if added during differentiation, cause a rapid decrease in some enzyme activities (119, 120). Although such results have not yet provided quantitative data on turnover, they clearly indicate that it is a significant activity and further suggest that both RNA transcription and translation are required. Significant losses in RNA (45) and increased ribonuclease levels (46) also accompany differentiation in *Physarum*. Turnover is indicated by the increased specific activity of RNA in the presence of [^{3}H]uridine, despite the losses in total RNA (45), and by the decrease in specific activity of prelabeled RNA during differentiation (47). However, problems of precursor permeation and the failure of actinomycin D to block more than 80% of the uridine incorporation during differentiation (45, 203) make it difficult to interpret the results of the studies of RNA synthesis and the rates of turnover in *Physarum*. All classes of RNA do appear to be made at a low rate in both spherulation and sporulation (45, 47, 203, 204), and hydridization-competition experiments indicate that some of the RNA synthesized during differentiation may differ from the growth phase (45, 47, 203, 204). However, the function, if any, of the putative new or unique RNA transcripts has not been demonstrated, although some candidates have been suggested for their translation products, e.g., spore wall proteins (122) and acid nuclear proteins (143).

Sporulation in the yeast *Saccharomyces cerevisiae* has also been studied in considerable detail (for reviews, see references 100, 248), and this species has many useful characteristics for experimental analysis of the cell cycle and sporulation (104). With glucose-grown cultures, *S. cerevisiae* can only be induced to sporulate efficiently if the cells were taken from the station-

ary phase (54, 101). This result with *Saccharomyces* illustrates well the typical requirement for aerobic metabolism during sporulation. Mitochondrial development and respiratory metabolism are repressed during growth on glucose but increase rapidly when the glucose is exhausted (59, 255). Cultures shifted from midlog-phase glucose cultures to potassium acetate sporulation medium sporulate poorly and only after a long delay, whereas cells removed from the same stage of acetate-grown cultures undergo sporulation earlier and efficiently (101). Respiratory-deficient mitochondrial mutants fail to sporulate (210), except if induced to sporulate immediately after mutation while their mitochondrial enzymes still function (135). Although sporulation in yeast involves meiosis and premeiotic DNA replication (214), it shares the common feature of induction by the shift of the cells to a high carbon-low nitrogen medium (typically 1% potassium acetate with or without small amounts of yeast extract). In glucose-grown cells, such a shift during the growth phase requires the cells to respond simultaneously to a release from glucose repression, adaptation to a new carbon source, and nitrogen starvation. In the case of acetate-grown cells, only nitrogen depletion should be involved, but even with this system it is necessary to distinguish between responses to starvation and sporulation-specific events if, in fact, these are truly separable, and not dependent, events. Because both glucose and acetate-grown *S. cerevisiae* cells have been used by different laboratories, the timing of the events reported is not always comparable. In either system the dry weight, RNA, and protein content continue to increase for a few hours after shifting the cells to acetate medium, after which they decline to a varying extent depending upon the conditions used (53, 54, 69, 111).

A round of DNA replication doubles the DNA content starting at T_2 or T_4 (hours after cells are placed in sporulation medium) for cells from acetate- or glucose-grown cells, respectively (53, 69, 111), and it remains essentially constant throughout the remaining events of meiosis and spore formation.

The large differences reported for the magnitude of RNA and protein increases and decreases during sporulation clearly suggest that the magnitude of these changes are subject to the conditions used for both pregrowth and the induction of sporulation (53, 111, 156). Such differences have led to equally large variations in the estimates of the amount of new RNA and protein synthesis and turnover (42, 53, 69, 111, 156). For example, using different diploid strains, the same presporulation growth conditions, and only slightly different sporulation media, it has been reported, on the one hand, that the RNA content of the cells increased by 70% from T_0 to T_{10} (40%, T_0 to T_3) (69) and, on the other, that very little new RNA synthesis takes place before T_4 and that this includes very little rRNA (42). It seems difficult to rationalize the latter results, particularly because one would not expect such a large increase in total RNA without rRNA synthesis. Furthermore, earlier work had reported a significant accumulation of the 20*S* precursor to 18*S* rRNA (123), and others have reported active precursor incorporation during the earliest stages (111, 166). RNA synthesis (measured by adenine incorporation) reaches a peak at about T_2 in acetate-grown cells, after which it declines steadily (111). With glucose-grown cells, two incorporation peaks have been reported, one at T_{10} and the other at T_{20-25}. (It may be significant that the sporulation medium in the latter experiment contained 50 μg of adenine and 75 μg of arginine/ml.) Three temperature-sensitive mutants behaved similarly at the nonpermissive temperature (70). Continuous labeling with adenine (T_0 to T_6 or T_0 to T_{12}) showed rRNA synthesis and 20*S* precursor RNA accumulation, but the amount of label in mature 18*S* and 25*S* rRNA decreased at the later time period, an observation consistent with the turnover of newly synthesized rRNA (123). Clearly defined rRNA was not observed at any time using 10-min pulses of [^{32}P]phosphate, although some tRNA was made (42). The pattern of incorporation with the majority of radioactivity in RNA between 8*S* and 16*S* and the nonribosomal base ratios would be consistent with mRNA synthesis and turnover. Chaffin et al. (42) concluded that the new synthesis did not utilize materials released by RNA turnover, but this interpretation depended upon some unverified assumptions used to interpret a triple label experiment.

The recent reports of Hopper et al. (111) and Magee and Hopper (156) have shed some important light on the problems of sporulation-specific events versus starvation responses. They used midlog-phase cells grown in acetate to avoid the carbon source adaptation and respiratory development characteristic of the commonly used glucose-grown cultures. They also compared the responses of a heterothallic sporulation diploid (a/α) with corresponding homozygous diploids (α/α or a/a) that failed to sporulate in the standard acetate medium. The patterns of incorporation for RNA and protein precursors were found to be the same with both sporulating and nonsporulating cells, as was the char-

acteristic accumulation of glycogen that occurs during early stages in induced cells. The radioautography of proteins labeled at different points between T_0 and T_{10} and separated by gel electrophoresis showed them to differ from pulse-labeled vegetative cells but to be similar for both sporulating (α/a) and nonsproulating (α/α) diploids. That is, new bands appeared and others disappeared, but none of the changes was unique to the α/a sporulating cells (111) Both RNA and protein were also found to be degraded in both types of cultures beginning at T_0 (156), and most of the nucleic acid degradation products were recovered in the sporulation medium. The extent of RNA and protein loss was significantly lower in the nonsporulating cultures (20 to 30% versus 50 to 70% for RNA and 0 to 10% versus 25 to 30% for protein) (see also reference 70). The nonsporulating cells also differed from sporulating cultures in the absence of DNA replication and glycogen utilization (156). Magee and Hopper (156) concluded that the biosynthesis of glycogen, RNA, and protein, the decreased respiration, and the rise in medium pH were not unique to sporulation but were responses to nitrogen depletion. The quantitative differences observed in RNA and protein degradation did suggest that these changes were partially specific to sporulation, whereas DNA synthesis, meiosis, and glycogen breakdown were unique to it (111). Despite a failure to identify specific proteins associated with sporulation (which could have escaped detection [111]), the process was, nevertheless, sensitive to cycloheximide inhibition throughout (69, 156). The viability of cells exposed to cycloheximide during the first 1 to 2 h in sporulation medium was greatly reduced, suggesting a need for new protein synthesis to adapt to the new conditions. Protein degradation, DNA replication, and glycogen breakdwon were also inhibited by cycloheximide, indicating a need for continued protein synthesis during differentiation (156), although none of the proteins was identified. The evidence derived from the study of yeast sporulation, like that from *Physarum*, points to a significant turnover of protein and RNA as a normal component of differentiation. The amount of essential turnover is not known, but it is of interest that amino acid auxotrophs sporulate without addition of the required amino acids (166). This would seem to provide good evidence that turnover is normally adequate in providing all the needed amino acids for new synthesis.

The only other aquatic fungus that has been studied in any detail for RNA and protein synthesis during differentiation is the oömycete *Achlya* (94, 247). In this organism the differentiation of zoosporangia and zoospores requires about 5 h and, like *Blastocladiella* and *Allomyces*, the longest phase (T_0 to T_4) is required for conversion of the vegetative hyphal tips into septate zoosporangia. The process may be induced with good synchrony by shifting young mycelia to 0.5 mM $CaCl_2$ and represents another example of induction by starvation. The losses in total RNA and protein during *Achlya* sporulation were not considered significant by Griffin and Breuker (94) although both decreased by about 20%. With an improved induction system, Timberlake et al. (247) reported that most of the mycelial cytoplasm was transferred to the developing zoosporangia. About 21% of the total protein was again lost, and these workers assessed the rates of protein synthesis, degradation, and turnover. Using incorporation rates, pool specific activities, and loss of radioactivity from prelabeld protein, they estimated the rate of turnover to be 75%/h. This would result in nearly four complete turnovers of the cellular protein during the 5 h required for differentiation, which appears exceptionally high. The evidence for active protein turnover seems incontestable, but the calculated rate should be taken with some reservation. In light of the recent reports of physical pool compartmentalization (239, 268), it seems probable that data based on the average pool specific activities may significantly overestimate the actual rates of synthesis. Nevertheless, even if one assumes a rate one-half that reported for *Achlya*, a large percentage of the amino acids released by degradation must be re-utilized at all stages. The high level of turnover is also accompanied by increased levels of protease activity (247), and development is inhibited by cycloheximide throughout.

Sporulation is also inhibited if actinomycin D is added to *Achlya* at any stage in the process (94), suggesting a continued need for new RNA synthesis. Griffin and Breuker (94) examined the patterns of RNA synthesis by fractionation of [^{3}H]uridine-labeled RNA on gradients. They concluded that rRNA and tRNA synthesis continued during sporulation and, further, that a high-molecular-weight fraction other than rRNA precursor was specifically synthesized during differentiation. Turnover was considered to be negligible since the specific activity of prelabeled RNA did not decrease. However, this is probably not valid since they did not flood the pools with cold nucleotides to lower the pool specific activities. The 1-h labeling periods and inadequate gradient resolutions achieved in the study also do not really support

the interpretation of Griffin and Breuker. In a long, 1-h pulse of vegetative cells, the precursors would be expected to contain a negligible percentage of the total radioactivity, whereas precursor accumulation would be expected to become prominent during the severe starvation conditions imposed on the mycelia in the 0.5 mM $CaCl_2$ induction medium. In our experience with *Blastocladiella* (using short, 10-min pulses), the first precursor may represent nearly 50% of the isotope incorporated into rRNA in a starvation-induction medium. Thus, low rates of rRNA synthesis and processing seem to be indicated during sporulation of *Achlya*, as well as in *S. cerevisiae* (42, 123) and *Blastocladiella*. It is not possible to determine from the published data how much new synthesis this respresents in *Achlya* or whether nonribosomal RNA is also produced. Evidence that new mRNA synthesis may be required comes from a study by O'Day and Lorgen (181) of the changes in acid phosphatase activity during *Achlya* sporulation. Fractionation experiments indicated a probable lysosomal (vacuolar?) location of the enzyme, a two- to threefold increase in specific activity during early stages, and replacement by (or conversion to) a new isozymic species. The role of the enzyme was not established, but the effect of actinomycin D and cycloheximide clearly indicated a continuous process of breakdown and resynthesis. Both antibiotics prevented the normal rise in enzyme specific activity if added at T_0 and resulted in a rapid loss of activity if added after the rise had occurred. Thus, both transcription and translation would seem to be required for the increased phosphatase level and its maintenance during sporulation (181).

From the results just summarized, *Achlya* appears to belong on the list of fungi in which starvation induces sporulation and concomitant turnover of both RNA and protein. The list is probably short only because relatively few species have been examined for RNA and protein synthesis under these cleary defined conditions. Whether sporulation in other fungi will be found to have a similar basis cannot yet be assessed, but the depletion of required nutrients could be a contributing factor in many cultures grown on solid media. Although there is extensive literature on the effects that varied carbon and nitrogen ratios have on sporulation (222), the rates of RNA and protein synthesis and their turnover have rarely been examined. Even in a well-nourished mycelium, the aerial hyphae may be subjected to carbon and nitrogen limitation since precursors could only be provided by intrahyphal transport. Such de facto "starvation" might serve as a signal to begin sporulation. The necessity for nitrogen starvation to induce sproulation in submerged *Aspergillus niger* mycelia (5) is in concordance with such a requirement. On the other hand, the induction of microcylic sporulation by liquid cultures of *A. niger* grown at 44 C (with conidia increasing in size but not producing hyphae) and shifted to 30 C (6) may mean that simple starvation is not the only mechanism for induction unless, of course, the high temperature has the same effect by incapacitating unknown, but essential, enzymes or pathways. An interaction of both physical and nutritional cues may be involved in triggering sporulation in aerial hyphae and the resultant slowed metabolism of the spores (169). Even less well understood are the mechanisms by which light or other nonstarvation treatments lead to fungal differentiation (e.g., HCO_3 or KCl induction of resistant sporangia in *Blastocladiella* [31, 93]). In organisms such as *Trichoderma* (92) and other light-induced species, light could be the inducer for aerial branching, after which a program for differentiation that is shared in common by many fungi might control sporulation.

Spore Stage

Considerably more attention has been given to the problem of RNA and protein synthesis in fungal spores and during their germination, although the number of species studied in any detail is not impressive. Most of this work has been reviewed extensively (151, 241, 261), and only the important conclusions and new results will be discussed here. The degree and extent of dormancy found in fungal spores varies over a wide range (241), and the stringency with which RNA and protein synthesis are regulated could be expected to vary similarly, even if the basic mechanisms for their control did not. Some spores enter a truly dormant, cryptobiotic state (e.g., *Neurospora* ascospores and *Blastocladiella*-resistant sporangia), whereas others, which may be produced by the same organism (e.g., *Neurospora* macroconidia and *Blastocladiella* zoospores), may be transient cells with very limited survival potential. Others fall at various points in between, with differing degrees of dormancy and a spectrum of requirements for the events that trigger germination. To germinate, some spores may only need to be hydrated; others may require the additional presence of nutrients; and still others require the removal of self inhibitors, heat shock, etc. (see Sussman and Douthit [241] for an extensive discussion of these phenomena). Only the few spore types in which RNA and protein syn-

thesis have been studied will need to concern us here.

In virtually all of the spores examined, the RNA, DNA, and protein synthetic systems have been found to be partially or totally inactive. No differences in the rRNA or tRNA have been found between spores and vegetative cells that can be directly attributable to the nonsynthetic state (261), even though some changes in the ratios of iso-accepting tRNA species were found between the spore- and early-germination-stage cells of *Rhizopus stolonifer* (165). The aminoacyl-tRNA synthetases have also been found present (117, 165, 208, 259). The general level of tRNA charging with amino acids is high in *Blastocladiella* zoospores, but a thorough study of the charging level of each individual tRNA species in any spore is lacking. The very limited data on free amino acid pools (151, 197) do not clearly suggest that the pools are limiting for protein synthesis. Quantitative measurements of the nucleoside triphosphate pools of spores are also not yet available. In *Neurospora crassa* the conidial ATP pool was found to be one-half that in growth-phase cells and did not increase during the first 6 h of germination (217). In this case, at least the level of the pool was adequate for germination, even though the flux through the pool was not measured and could have changed.

The in vitro activities of isolated spore ribosomes, when measured in poly(U)-phenylalanine assays, have usually been found to be lower than those of growth-phase ribosomes (3, 4, 116, 191, 208, 258). The actual significance of the reduced activity is uncertain, but damage due to nuclease nicking during cell rupture and fractionation is a probable contributing factor (4, 116, 151). Spore ribosomes occur largely as free, inactive 80S ribosomes, but the mechanical procedures required to rupture the spores, the generally high nuclease levels in fungi, and prior washing steps usually have led to considerable uncertainty in deducing their actual status in vivo (151). The typical aqueous washing media used to obtain pure conidial suspensions can partially (49) or substantially (169) activate protein synthesis, leading to the presence of polysomes in ungerminated cells. On the other hand, Brambl (22) has shown that *Botryodiplodia* spores contain a low level of polysomes even if isolated in nonaqueous media. A reduced rate of protein synthesis and the presence of some polysomes may, thus, be characteristic of conidia formed in a moist, sticky matrix, such as in *Botryodiplodia* and *Fusarium* (49), but not of the air-dry conidia produced on the elevated conidiophores of *Neurospora* and probably other species. As suggested by Mirkes (169), dehydration itself may be a significant factor in limiting synthesis in such spores.

Other protein components required for protein biosynthesis, including the initiation and elongation factors, have not been studied systematically in spores. Some evidence has been provided for elongation factor activity in crude spore extracts of *Uromyces* (271), *Botryodiplodia* (260), and *Peronospora* (109). We have now purified both elongation factors I and II from the zoospores of *Blastocladiella* (Gong and Lovett, unpublished data). In any case, at least minimal amounts of these and other essential proteins, such as initiation factors, would be expected to be present even in fully dormant spores (151). It seems probable that many spores have stored mRNA (some being clearly present where polysomes are detected), although to date most of the evidence for stored, inactive spore mRNA is largely circumstantial (151, 261). The poly(A)-associated RNA of *Blastocladiella* zoospores (Lovett and Wilt, 154) and *Schizosaccharomyces* ascospores (188), however, provides direct evidence for an mRNA fraction in these spores.

The specific mechanisms by which RNA and protein synthesis are suppressed in the spore stage are unknown and will remain so until one or more spore types have been examined for the presence and activity of all the numerous components. A ribosome-associated inhibitor analogous to that of *Blastocladiella* zoospores has not been reported for any other fungi, although the germination inhibitor of *Glomerella cingulata* does inhibit amino acid incorporation by conidia (144). A self-inhibitor of *Dictyostelium discoideum* spores has also been reported to inhibit amino acid incorporation (8), but its identification as *N'N'*-dimethylguanosine has been questioned (124, 245). Other spore germination inhibitors have not been implicated as regulators of protein synthesis, and in any case the chemical identification and mode of action of those that have been so interpreted will need to be clarified before a primary regulatory role to prevent spore protein synthesis can be fully accepted.

Not enough is known to propose probable mechanisms for the control of mRNA synthesis in spores. No differences could be found between the RNA polymerases of zoospores and growth-phase cells of *Blastocladiella* when tested in vitro (114). The absence of RNA polymerase II in preparations from *Rhizopus stolonifer* conidia, which was originally thought to indicate a control function (88), is now of uncer-

tain significance since all classes of RNA are synthesized in early germination before RNA polymerase II "reappears" in the extracts (195). This does not, however, eliminate possible changes in the stability or activity of the polymerases in vivo nor the potential presence of factors that regulate their activity. It is possible to suggest the most obvious targets for the regulation of protein biosynthesis and, further, that the suppression of protein synthesis itself may be adequate to prevent the transcription of rRNA and tRNA cistrons through the action of normal, growth-phase controls.

The experimental work on spores just summarized and discussed in more detail elsewhere (151) suggests that the ribosomes, tRNA, aminoacyl-tRNA synthetases, and the initiation and elongation factors are present in all spores in at least minimal amounts. It seems probable that mRNA is also present in many, if not all, fungal spores. If these assumptions are correct, then an efficient way to prevent synthesis would be to block the primary event of initiation complex formation. Such a block could result from: (i) a lack of available initiation-specific methionyl-$tRNA_F^{met}$, nucleoside triphosphates (ATP and guanosine 5'-triphosphate [GTP]), or free amino acids; (ii) physical compartmentalization of one or more components; or (iii) the presence of specific, reversible inhibitors of initiation factors or of mRNA function. Data on pools are lacking for any of the spores that have been examined for in vitro protein synthesis, and the methionyl-$tRNA_F^{met}$ level remains to be measured in any spore. Obvious physical compartmentalization seems rare, aside from the ribosomal nuclear caps of *Blastocladiella* and *Allomyces* zoospores and the ribosomal aggregations of some other chytridiomycetes (43, 48, 82, 130); whether other types of physical isolation exist in the form of membrane or vesicle associations remains to be seen. The physical compartmentalization of amino acids recently reported for growth-phase cells of *Neurospora* (239) and *Candida* (268) is certainly suggestive in this respect.

Growth-rate-associated changes in the level of initiation factors (21, 139) and protein factors that alter the specificity of initiation (96) have been reported for *Escherichia coli*. Elongation factor activity has also been found to increase exceedingly rapidly after fertilization of sea urchin eggs (74). It seems possible that analogous factors or mechanisms may be found that modulate the level or activity of the initiation and elongation factors in the spores of fungi. For example, a lack of initiation factor IF-3 could prevent dissociation of the inactive 80S ribosomes and thereby limit the availability of the active 40S ribosomal subunits needed to form the first mRNA–40S–methionyl-$tRNA_F^{met}$ initiation complex. Alternatively, an inhibitor could prevent 80S ribosome dissociation or block an active group on the ribosome required for some essential function. The association of specific proteins with eukaryotic mRNA (27) and the differences between the proteins associated with polysomal and free cytoplasmic mRNA (201) also suggest specific mRNA-protein complexes as a potential mechanism for the control of initiation (207). A requirement for 7-methylguanosine at the 5' termini of mRNA to permit translation (173) presents another potential mechanism. The presence of 80% of the poly(A)-RNA in the nuclear cap of *Blastocladiella* zoospores, but free from the ribosomes, may provide an example of such controls (151).

Spore Germination

The reactivation of the protein and RNA synthetic systems during spore germination has been a subject of considerable interest. Aside from the primary necessity to hydrate air-dry spores, the triggering mechanisms are not understood (241). With *Blastocladiella* the potassium ion concentration is critical (228); *Uromyces* uredospores (271) and *Glomerella* conidia (144) require the removal of the self-inhibitor; some spores require no nutrients whereas others, such as *Fusarium solani* (49), may require a specific carbon source or, as exemplified by *Neurospora crassa*, both nitrogen and carbon sources (169) for successful germination.

Little change has usually been noted in the total RNA, DNA, or protein content of most spores before at least 1 h after inoculation into the germination medium and often not until even later (151, 261). Although spores may begin to increase in size soon after exposure to the aqueous medium, the emergence of the germ tube is typically delayed until between 1 and 6 h. An increase in dry weight, RNA, and protein content may or may not precede this visible manifestation of germination. However, the results of isotope experiments show that synthesis begins very early and that, although it is quantitatively negligible, it represents essential functions for the completion of germination. Virtually all spores begin both RNA and protein synthesis within a few minutes after inoculation into a germination-sufficient medium, typically 10 to 15 min (151, 261). In a review of RNA synthesis, during spore germination Van Etten et al. (261) classified the fungi into the following three types: (I) spores in which RNA and protein synthesis begin simul-

taneously, (II) spores that start protein synthesis before RNA synthesis, and (III) spores in which RNA synthesis precedes protein synthesis. However, if precursor incorporation is used as a criterion, it seems probable that most fungi will be found to fall in category I, as does *Blastocladiella*, which they placed in group III. In some earlier studies, $^{14}CO_2$ (187) was used as an isotope precursor, and kinetics of RNA labeling would have been dependent not only on uptake but also on CO_2 fixation and prior steps of precursor biosynthesis. Also, in our own experience with *Blastocladiella*, the label of $^{32}PO_4$ does not equilibrate rapidly with all phosphates in the nucleoside triphosphates and enters RNA less rapidly than do [^{3}H]adenosine and [^{3}H]uridine. The point is stressed because, although this could be unique to *Blastocladiella*, in the few cases in which high-specific-activity nucleosides have been used as precursors and the RNA has been analyzed by gel electrophoresis, all species of RNA (tRNA, rRNA, and presumptive mRNA) have been labeled at very early time points (138, 169, 188, 195). Thus, most spores probably start up synthesis of all RNA species nearly simultaneously at an early stage in germination, even though the rates for different RNAs may vary. That some exceptions may, nevertheless, occur is suggested by work with *Botryodiplodia* (23) in which the start of RNA synthesis is delayed until just prior to germ tube formation at about 1.5 to 2 h.

It is not at all certain in many cases whether new RNA synthesis is actually required for early germination events (as contrasted to growth) and, if it is, what kind of RNA is needed. Many of the inhibitor experiments reported have been inconclusive, but in some species the failure of actinomycin D or ethidium bromide to prevent germination at concentrations that effectively inhibit precursor incorporation into RNA (23, 151, 198) suggests that new RNA synthesis is not necessary at the earliest stages in some fungi (261). In *Neurospora crassa* and *Blastocladiella emersonii*, the evidence for stored mRNA (17, 154), the extremely rapid appearance of significant levels of polysomes (138, 169), and the early entry of new polydisperse RNA into the polysome fraction all lead to the conclusion that both stored and new mRNA's are normally translated at or soon after the start of germination. In *Blastocladiella* and *Allomyces*, the stored mRNA is sufficient for the essential events of early development, but this mRNA must be replaced by and/or supplemented by new mRNA for growth to continue (138). A similar utilization of both stored and new mRNA seems probable for other fungi as well. Ascospores of *Schizosaccharomyces pombe* contain poly(A)-associated RNA before germination, and the amount increases about threefold during the first 30 min of germination (188). An early preferential synthesis of poly(A)-RNA has also been reported to occur during *Rhizopus stolonifer* germination by Roheim et al. (195), although the presence or absence of stored mRNA is not yet established for this species.

rRNA precursors are a very prominent fraction of pulse-labeled RNA of *R. Stolonifer* at early stages (T_0 to T_{30} min) but not when the cells have begun growth (T_{225} min) (195). Thus, in both *Blastocladiella* and *Rhizopus*, the synthesis and processing of rRNA does not reach maximal rates until growth begins, even though it is detectable very early. This acceleration of synthesis and processing is a normal accompaniment of germination in *Blastocladiella* but is not essential for it, as shown by cells germinated in inorganic media (138). In *Neurospora*, on the other hand, Mirkes (169) has shown that the absence of both a carbon and nitrogen source inhibits germination and the increase in the rate of RNA synthesis. Different fungi may, therefore, have developmental programs for RNA synthesis that respond in different ways to the presence or absence of external nutrients.

In contrast to the variable requirement for new RNA synthesis to complete germination, no spores have been found able to germinate in the absence of protein synthesis (151). Unfortunately, this universal requirement is matched by an equally general ignorance of the essential functions the new proteins perform. Numerous changes in enzyme activities and/or the amount of specific enzymes have been reported to occur during fungal spore germination (182, 249, 256), none of which has been shown to be required. The few known specific enzymes with logical roles in germination (e.g., trehalase in *Neurospora* [240] and *Phycomyces* [257] and alkaline protease in *Microsporum* [190]) are present in the spores before germination. The actual rates of protein synthesis and turnover have not been measured during the early critical stages of swelling and germ tube formation of conidia or other fungal spores. It would be of considerable interest to know whether the slow increase in protein content in such spores results from the combined effects of a low synthetic rate and protein turnover, as it does in *Blastocladiella* (138, 213, 145). *B. emersonii* zoospores can encyst and produce a sizable rhizoid entirely at the expense of endogenous materials, and uredospores of the obligate parasite *Uromyces*

phaseoli (231) can produce a germ tube, appressorium, and infection hypha without added nutrients. In contrast, the spores of *Neurospora crassa* (169), *Aspergillus niger* (270), *Fusarium solani* (50), and others require carbon and nitrogen sources in the medium for germination to occur. This suggests that spore germination in fungi may be of three types. The first, represented by *Uromyces*, supports new synthesis during germination entirely by turnover (231, 269). This may be a common, developmental program for obligate parasites or spores that normally need to penetrate insoluble substrates. The second type, represented by *Neurospora*, *Aspergillus*, and *Fusarium*, may require nutrients as a signal to trigger germination, after which a variable ratio of external and internal materials are used (i.e., synthesis perhaps being partially or completely dependent upon the medium). The third type, represented by *Blastocladiella*, apparently does not require external nutrients as a cue to start germination and may utilize either external-plus-internal or entirely internal substrates to support germination, as determined by availability. From the work with *Neurospora*, it is evident that different spores of the same fungus may have both different triggers for germination and different developmental programs for the metabolism of germination (169, 240).

Because DNA synthesis does not usually begin until the time of germ tube emergence, Van Etten et al. (261) concluded that it was not necessary for germination. Two apparent exceptions to this pattern have been reported for *Microsporum gypseum* macroconidia (9) and *Allomyces neo-moniliformis* zoospores (186). In *M. gypseum* the early DNA synthesis was reportedly detected by thymidine incorporation and preceded a net increase in DNA content by 1 h, which would not be expected if the incorporation represented nuclear DNA replication. The general inability of "true" fungi to utilize thymidine directly for DNA synthesis (95, 151, 170) and the potential to convert it to other pyrimidines (209) suggest that these incorporation data need verification. Olson and Fuller (186) found the nuclear DNA content of *A. neo-moniliformis* to double in the first 10 min after inoculation. In addition to the apparent difference in timing relative to other spore germination systems, the result seemed in direct conflict with our earlier conclusion that DNA replication closely followed mitosis in *Blastocladiella* (150). Olson and Fuller suggested that the short or nonexistent G_1 phase implied by our data was quite different from other known biological systems. However, there is good evidence for the absence of a G_1 phase in *Schizosaccharomyces pombe* (20), *Physarum polycephalum* (24), and a number of other eukaryotic microorganisms (170). J. S. Bittner and D. Therrien (personal communication) have also measured the level of DNA in *Blastocladiella* nuclei by microspectrophotometry and concluded that the DNA was replicated after the last zoosporangial mitotic divisions. Thus, the zoospores should have their DNA in the replicated form, and the radiation sensitivity of the cells through the period of mitosis is consistent with postmitotic replication (61). Olson and Fuller (186) did not measure the nuclear DNA of *Allomyces* through, or after, the first mitotic division, which could have resolved the issue with *A. neo-moniliformis*. They indicated the pregermination zoospore stage as the G_1 phase (Fig. 23 of reference 186), but this is a specialized, motile, nongrowing cell and probably has little relation to a normal cell cycle. If *Allomyces* did normally replicate its DNA with little or no G_1 phase after mitosis, as suggested for *Blastocladiella*, it seems possible that in the nongrowing zoosporangia mitosis might not necessarily be followed by the normal synthetic phase until the cells encyst in a complete medium. This would be analogous to the arrest of *Saccharomyces cerevisiae* cells in the G_1 phase under a variety of conditions, including the cessation of growth (104). The experiments with *Allomyces* do not show whether a true G_1 phase occurs in the normal, growth-phase, nuclear cycle, and it will be important to determine whether these two related organisms really do differ or whether the discrepancy is only related to the zoosporangial phase.

On the basis of the preceding, admittedly selective, discussion of fungal differentiation, it is hard to escape one important conclusion: a significant proportion of the synthetic activities examined during development of fungi probably represents cellular adjustments to the growth-supporting capacity of the medium. Such observations are of themselves important and interesting because they help to elucidate the metabolic control mechanisms of the fungi. On the other hand, however, these easily measured activities involving bulk synthesis (i.e., rRNA, tRNA, ribosomal proteins, metabolic enzymes, etc.) provide an exceedingly high background "noise" within which the developmentally significant events are still hidden. It seems apparent that to identify the latter it will be necessary to devise more selective methods and, in their present state of development, general labeling and competitive hybridization techniques seem unlikely to provide the discrim-

ination needed. Such experiments can show whether different DNA base sequences are transcribed during periods of differentiation but do not identify specific transcriptional or translational products (except in highly differentiated cells producing one or a few proteins where the translation product is already known). A large number of proteins is produced very early in fungal spore germination (213; 262; Abe and Lovett, unpublished data), and hybridization lacks the selectivity to identify a minor, but essential, transcript among the many cistrons being transcribed. A more profitable approach, in my view, would be to identify proteins whose functions are critical to the success of differentiation (sporulation or germination) and then to identify those that must be newly synthesized. This is easier to say than to do, but the biosynthesis of cell wall precursors, apical secretory vesicles, and cell wall polysaccharide-polymerizing enzymes seem a logical place to begin for the analysis of germination. Temperature-sensitive conditional mutants should also provide a means for detecting unknown, but essential, protein functions in sporulation and germination (104, 121). If the critical proteins can be identified, it should be possible to trace them to establish the time of their synthesis and the transcription of their mRNA's (including cistrons precoded in stored mRNA).

General Features of Microbial Differentiation

Perhaps the microbial developmental systems to receive the greatest attention biochemically have been the formation and germination of bacterial spores (57, 62, 77, 127, 158, 159, 199, 206, 244), differentiation in myxobacteria (64) and caulobacters (136), and the aggregation and sporulation of cellular slime molds (19, 84, 98, 128, 242, 243). This material has been extensively reviewed and need not be repeated here. Instead, a few illustrative examples will be mentioned to permit a comparison of some of the important general features of these well-studied organisms with what we know about fungi.

Despite their very different growth forms, life cycles, and taxonomic affinities, sporulation in bacteria, many fungi, and the cellular slime molds share a common characteristic in their inducibility by starvation for carbon, nitrogen, or all organic nutrients. Many show a further similarity in that the differentiation so induced is reversible up to some point of commitment (159) and that they become essentially closed systems with regard to a dependence upon, or even utilization of, external substrates. These closed metabolic systems are characterized in most cases by extensive degradation and turnover of intracellular substances (62, 98, 102, 159) and release of various materials, such as proteases, enzymes, antibiotics, etc., to the medium (62, 145, 159). A requirement for aerobic metabolism during sporulation is another common feature (77, 98). The metabolic similarities of microbial cells induced to differentiate by starvation are probably related to two other features they share in common: all are free-living and exist in competitive and rapidly changeable environments over which they have no control and, unlike the nonreproductive cells of higher organisms, each microbial nucleus not only must be totipotent but also in most cases must be able to give rise to a differentiated state that is completely reversible. This process of differentiation often involves remodeling what was previously an undifferentiated cell and, as part of becoming differentiated into a spore, it must set up the conditions for a rapid reversal or dedifferentiation to resume the vegetative phase at germination. This, too may require remodeling of the existing cell, as in fungal and bacterial spores, or abandonment via excystment by amoeboid cells. Most eukaryotic microorganisms are more versatile than bacteria in their ability to respond to various environmental changes with more than one pattern of development. *Achyla* mycelia may produce zoosporangia or thick-walled gemmae, and the secondary zoospores may encyst reversibly (230). *Physarum* and other myxomycetes produce sporangia and sclerotia (of which microspherulation is a special case) in the plasmodial stage, and the haploid amoebae can encyst and reemerge or produce flagella reversibly to become motile swarmer cells (99, 125, 126). This amoeboflagellate transformation is also characteristic of *Naegleria gruberi* (83, 132). *Blastocladiella* is equally versatile in its ability to sporulate in response to starvation at any stage of growth and to form resistant sporangia under other adverse circumstances (31). Cellular slime molds, in addition to asexual sporulation and microcyst formation, have recently been found to have a complex sexual phase that involves the formation and germination of macrocysts (177, 178). The developmental versatility of these and many other eukaryotic microorganisms suggests that such cells may carry a set of different, genetically specified developmental programs (159, 243), each of which is triggered by a specific category of environmental cues. This is a very tidy and intellectually attractive concept, but it is, unfortunately, still

largely just a concept and not a proven fact.

Relatively few eukaryotic microorganisms have been examined in detail, and even in this small sample the interpretations have often been equivocal, to say nothing of controversial. Perhaps the most actively studied system can serve as an example of the problems encountered and illustrate some of the basic similarities just summarized. In *Dictyostelium discoideum*, the enzymes leading to spore cell wall synthesis and storage sugars, both significant end products of the biochemical events of differentiation (e.g., cellulose, mucopolysaccharide, and trehalose), have been examined in detail by several laboratories (84, 128, 242). There is little disagreement that particular enzymes (e.g., trehalose-6-phosphate synthetase, UDPG pyrophosphorylase, glycogen synthetase, glycogen phosphorylase, and others) increase in specific activity during aggregation and fruiting and that some of these changes fail to occur at normal times in developmentally incompetent mutants (243). Furthermore, since actinomycin D blocked the synthesis of certain enzymes only if added several hours before their normal appearance (or change in activity), it was proposed that the mRNA transcription and translation required for their synthesis were both developmentally regulated and temporally separate events (242, 243). Firtel et al. (76), on the other hand, found that the delay effects were due to slow penetration and incomplete inhibition of the more resistant mRNA synthesis by actinomycin D (a problem noted earlier with *Physarum*) and concluded that UDPG pyrophosphorylase, at least, was transcriptionally controlled at the time its activity appeared. Hybridization experiments (75) also indicated that RNA extracted from each developmental stage of *D. discoideum* contained a small fraction of transcripts representative of differing mRNA populations. Nevertheless, at least 56% of the total genome was estimated to be expressed during the 20-h developmental sequence. Although the hybridization data are consistent with the transcription of specific mRNA's in a developmental program, the protein products they specify are unknown.

In contrast to the experiments and interpretations emphasizing transcription, Killick and Wright (128) have presented an opposing view. They propose that many of the changes in enzyme specific activity may not be the result of selective transcription at the time they appear but, rather, that unmasking via the release of inhibitors or reduced rates of enzyme degradation (turnover) could yield the same results. These authors, in turn, put forward their own view that metabolite availability and various mechanisms to modulate enzyme function can provide a more realistic explanation of the metabolic shifts in the differentiating cells. Hames and Ashworth (102), using an axenic strain of *D. discoideum*, have shown that the levels of glycogen in amoebae can be manipulated over a 100-fold range without affecting their subsequent development. Myxamoebae with high starting glycogen levels did produce significantly more end products (25 to 200% more) but, in the case of trehalose-6-phosphate synthetase at least, neither the amount nor the timing of enzyme synthesis was significantly different when compared with cells with low starting glycogen. Thus, despite the very considerable effort concentrated in a single metabolic area, there is still no generally accepted interpretation of the underlying regulatory mechanisms and their integration in a developmental program.

An important observation by Hames and Ashworth (102) was that the rates and extent of RNA and protein degradation were not altered during differentiation of the myxamoebae, despite large differences in stored glycogen. They considered this as evidence against a common view that the degradation of these materials is required primarily to provide oxidizable substrates for energy production in the closed differentiating system. They further stressed that the degradative activities might be as important in differentiation as is the new synthesis (102). I would readily concur with their suggestion that degradative activities have received less attention than they deserve. It has often been proposed that an experimental virtue of *D. discoideum* and other microorganisms is that the metabolism of growth and differentiation can be clearly separated because the latter occurs in nongrowing, starved cells (243). The clear implication is, of course, that the analysis will thereby be simpler. In practice, except for the ease of manipulation, this seems hardly to be the case in the systems so far examined, including bacteria in which the problem of metabolic responses to media changes versus spore differentiation has received considerable attention (77, 158, 206). In essence, one problem may have been substituted for another, and the relative lack of attention to the degradative component in the differentiation of eukaryotic microorganisms may represent a degree of myopia. The favored experimental organisms perform so nicely in the laboratory that we tend to forget that they must "do their thing" in nature under very different circumstances. Cells growing in the wild probably rarely have a chance to

gorge themselves with the large amounts of free sugars, amino acids, bacteria, etc., normally provided in laboratory media. In their chancy and competitive natural environment, it might well be suicidal if the success of sporulation, for example, were dependent upon storage materials they may rarely be able to accumulate. If so, the extensive degradation and turnover commonly observed could represent a preprogrammed and obligatory part of differentiation capable of supporting the entire process, irrespective of the level of endogenous reserves. From this perspective, the concept of degradation to supply energy and precursors for essential new synthesis remains viable; the experiments of Hames and Ashworth could certainly be interpreted to show how inflexible (and thus in nature, essential?) this part of the developmental program is. It will be interesting to see whether this apparent invariance extends to other eukaryotic microorganisms. Certainly the data now available suggest that it is a characteristic of *Blastocladiella* and probably of other less thoroughly studied fungi.

By stressing degradation, it is not meant to suggest that the support of new synthesis is the only important activity related to turnover in differentiation. Turnover may also function to remove existing and expendable proteins and RNA (57, 102, 158). In bacteria, the known extracellular proteases have no certain function outside the cell; the serine proteases, whose production is an obligatory component of sporulation, do not seem to be responsible for the major intracellular degradation (62). These enzymes may, however, have important regulatory functions in modifying specific proteins. For example, the size of the β subunits of *Bacillus subtilis* RNA polymerase (146) and the molecular weight of aldolase (200) are both reduced in sporulating cells, and both changes have been demonstrated using the serine protease in vitro (62, 146). These changes are considered to be functional ones, i.e., an altered template specificity of the polymerase and increased heat resistance in the aldolase. The failure of both the polymerase modification and sporulation to occur in a temperature-sensitive serine protease mutant (229), at the restrictive temperature, provides support for a regulatory role via protein modification in bacteria (62). The functional significance of the breakdown and resynthesis of preexisting vegetative cell enzymes and release of many factors to the external medium in bacteria, slime molds, and fungi remains speculative. The extensive turnover that accompanies differentiation has made it difficult to determine what fraction of the original growth-phase proteins are actually conserved in a modified or unmodified form and exactly what new and essential proteins are produced (62, 77, 199).

In conclusion, this limited and selective summary should be sufficient to illustrate the impressive number of ways in which certain true fungi (including *Blastocladiella*) and the plasmodial slime molds resemble other microorganisms in the conditions that trigger differentiation and in the general patterns of metabolism in the sporulating and germinating cells. These similarities seem likely to be a direct reflection of the shared need of such diverse organisms to ensure survival and dispersal through their ability to complete differentiation in the competitive, unstable, and rapidly changing microenvironments of their natural habitats.

ACKNOWLEDGMENTS

I wish to thank M. Murphy, P. E. Lessie, I. R. Schmoyer, T. G. Ademan, W. E. Barstow, C. S. Gong, C. J. Leaver, F. Wilt, and S. Abe for their important contributions to my work with *Blastocladiella*.

This research was made possible by Public Health Service grant AI-04783 from the National Institute for Allergy and Infectious Diseases and by a Brown-Hazen grant from the Research Corporation.

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