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STUDIES OF MITOCHONDRIAL BIOGENESIS AND SOME DEVELOPMENTAL PROCESSES OF THE CELLULAR SLIME MOLD *DICTYOSTELIUM DISCOIDEUM*

BY

LAWRENCE KOBILINSKY

A dissertation submitted to the Graduate Faculty in Biology
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy, The City University of New York

1977

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

May 3, 1977
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Chairman of Examining Committee

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of New York

ABSTRACT

STUDIES OF MITOCHONDRIAL BIOGENESIS AND SOME DEVELOPMENTAL PROCESSES OF THE CELLULAR SLIME MOLD *DICTYOSTELIUM DISCOIDEUM*

by

Lawrence Kobilinsky

Advisor: Professor Diana S. Beattie

Addition of ethidium bromide (EB) to cultures of the slime mold, *Dictyostelium discoideum*, blocks cell division after 1-2 generations. The cyanide-sensitive respiration was decreased 75% after 5 days in EB. Purified mitochondria from EB-treated cultures contained 50% the content of DNA as mitochondria from control cells. Low temperature spectroscopy studies indicated that the amount of cytochrome $\underline{c}_1$ was decreased by 80% and that of cytochrome *c* increased 2-fold. Two cytochromes *b* absorbing at 557 and 561 nm were observed; however, the content of cytochrome b_{561} alone appeared to decline in cultures treated with EB. After removal of EB, the cells resumed a normal growth rate without any discernible lag. The activity of oligomycin-sensitive ATPase, cytochrome *c* oxidase, and succinate- cytochrome *c* reductase as well as the cytochrome content began to increase after one day reaching control levels within five days.

Electron micrographs of cells treated with EB revealed that mitochondrial membranes were greatly reduced. Membranous whorls were apparent as was a profound loss of rough endoplasmic reticulum. Three days after removal of EB, mitochondria were again ovoid in shape and contained well-developed cristae.

Amoebae developing in the presence of EB form abnormally small aggregates in large numbers. The kinetics of the accumulation and disappearance of five developmentally regulated enzymes are characterized. EB alters the kinetics of two enzymes, α-mannosidase and alanine transaminase which normally increase in activity from the onset of starvation. The kinetics of accumulation and disappearance of tyrosine transaminase and alkaline phosphatase, which start to accumulate only after the completion of aggregation, are identical for amoebae developing in the presence or absence of EB.

Vegetative amoebae growing axenically in the early logarithmic phase can be induced to extend microprojections, filopodia, from their cell surfaces in response to 1.0 mM cyclic 3',5'-adenosine monophosphate. Cyclic GMP, AMP or ADP at the same concentration have no effect. 2-4 dinitrophenol (0.1 mM) or sodium azide (1 mM) do

not prevent the induction by cAMP. Induced cells can be more extensively agglutinated with Concanavalin A at 0.5 mg/ml than non-induced cells. Fluorescent labeling studies indicate that cell surface receptor sites for this lectin are capable of moving within the plane of the membrane. Scanning electron microscope studies confirm the presence of numerous filopodia on the surface of cells from cultures at the mid-logarithmic phase of growth as well as in the pre-aggregative stage of development. Furthermore, treatment of the cells with colchicine or griseofulvin or cooling to 3°C did not prevent the induction of filopodia suggesting that microtubules are not involved. Addition of cytochalasin B or procaine caused the immediate and reversible collapse of filopodia suggesting that the microfilaments within the core of the filopodia may play a role in the formation and maintenance of these structures.

Analysis of the respiratory chain of spores of _D. discoideum_ which lack a cyanide-sensitive respiration indicates that cytochromes a-a_3, b and c-c_1 are present at levels identical to those found in the vegetative amoebae. Enzymes of both the respiratory chain and the citric acid cycle are present in spores in quantities at least as high as are found in amoebae. The activities of glutamic dehydrogenase and oligomycin-sensitive ATPase are reduced in the spores 30% and 56% respectively.

Intact spores appear to lack a cyanide-sensitive respiration as a result of inadequate quantities of respiratory substrate and more importantly as a result of a lack of the cofactor NAD^+. The emergence phase of spore germination is sensitive to the antibiotic chloramphenicol which is a specific inhibitor of mitochondrial protein synthesis. It is concluded that germination requires the early synthesis of NAD^+ and generation of respiratory substrates and one or more mitochondrially synthesized proteins.

ACKNOWLEDGEMENTS

Throughout the early years of my graduate education at The City College of N.Y. and then at Brooklyn College, I became especially interested in two specialized areas of the biological sciences, namely, mitochondrial biogenesis and the biochemistry of developmental processes. Having read several scientific articles written by Dr. Diana S. Beattie, who had been working in these two fields, I decided to come to the Mt. Sinai School of Medicine where she became my senior advisor. The years spent at Mt. Sinai were both educationally stimulating and scientifically productive. I would like to thank Dr. Beattie for her guidance and helpful suggestions throughout this investigation. Her enthusiasm and insights made these studies both enjoyable and successful.

I would especially like to thank my wife, Estelle, for her constructive discussions of every aspect of this work and for her patience, love and understanding which were so vital to the completion of this thesis.

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CHAPTER 1

LITERATURE REVIEW AND GENERAL INTRODUCTION

LITERATURE REVIEW AND GENERAL INTRODUCTION

A. LIFE CYCLE

The cellular slime mold Dictyostelium discoideum, is a member of the Class Acrasieae; Family Dictyosteliaceae. It was first discovered by K.B. Raper in a North Carolina forest (1). D. discoideum is a eukaryotic soil microbe which normally feeds on the bacteria of forest detritus. As long as nutrients are available, the amoebae grow and divide by binary fission and are physiologically, biochemically and ultrastructurally identical (2). However, following the depletion of its food source, it undergoes a series of developmental and morphological changes as shown in Figure 1. The cells become mutually attractive and collect in large streaming patterns in groups consisting of up to 10^5 amoebae forming aggregates whose surfaces become covered with a slime sheath, hence the name slime molds. The aggregate rises and then falls to one side and now morphologically resembles a small worm or slug. Unlike the myxomycetes, the cells never become fused and can be mechanically or chemically separated at any time throughout development. The multicellular slug may migrate depending on environmental conditions; however, a migratory period is not essential for further development. When migration ceases, the anterior of the slug becomes elevated and a series of morphogenetic events ensues termed culmination, which ultimately gives rise to the fruiting body or sorocarp. This structure stands several millimeters high

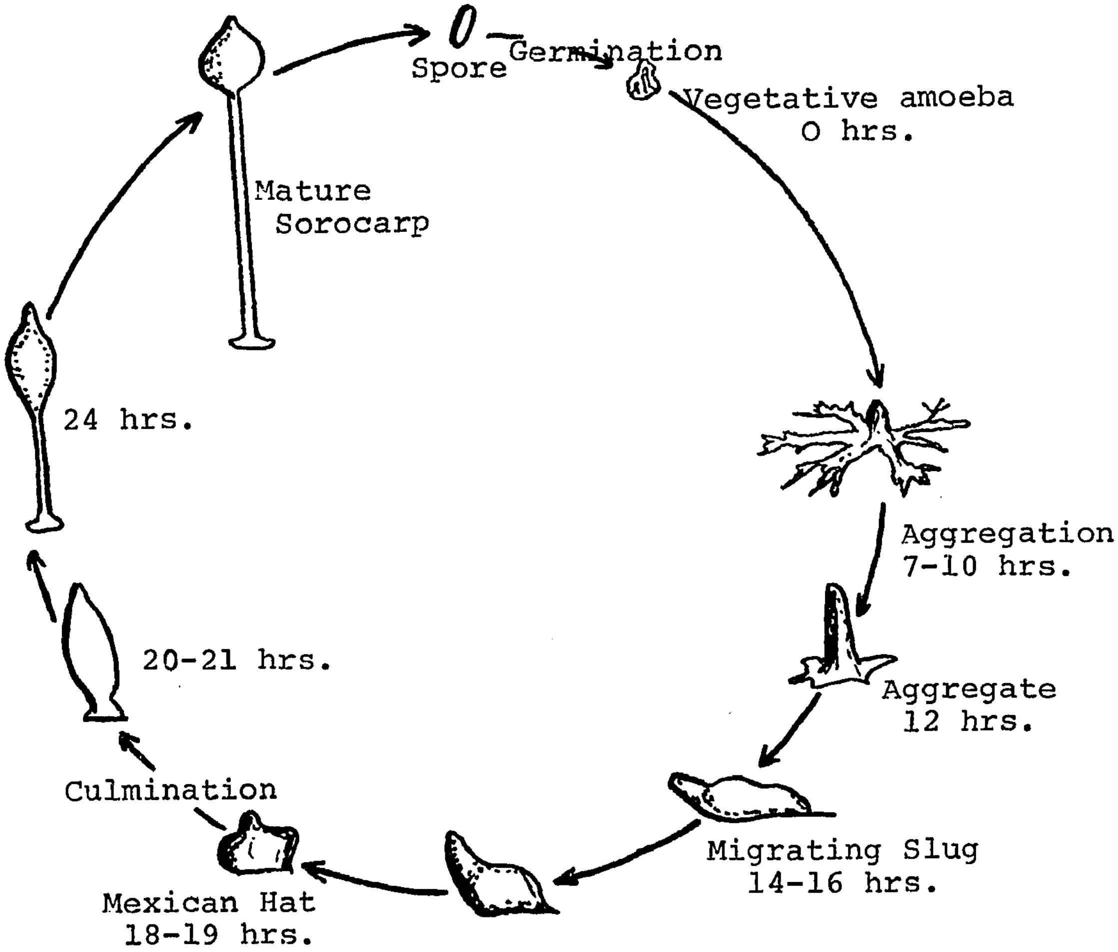

Figure 1.
The Life Cycle of Dictyostelium discoideum

and consists of vacuolated stalk cells and dormant spores in the ratio of 1:2. Unlike the stalk cells which die after vacuolization, the spores can remain viable after prolonged periods of starvation and dehydration. A single spore placed in the proper environment e.g. high humidity, temperature between 22°C and 27°C, and in the presence of oxygen, will germinate and give rise to a small vegetative amoebae thus completing the life cycle. The entire process is completed within 24-26 hrs and can be made almost 100% synchronous (For reviews see references 3 and 4).

B. DICTYOSTELIUM DISCOIDEUM VEGETATIVE AMOEBAE

D. discoideum became of great interest to microbiologists and developmental biologists when techniques were developed which allowed growth of *D. discoideum* in the laboratory. Although many different types of bacteria could serve as the food source, *Escherichia coli* B/r and *Klebsiella aerogenes*, both of which are non-mucoid bacteria, became the most commonly chosen organism. Amoebae of the wild type strain, NC-4, can be grown in either liquid culture or on solid media with a doubling time of about 4-5 hrs. Sussman and Sussman (16) isolated the first axenic strain, Ax-1, which was able to grow on a medium without bacteria containing Proteose-Peptone, dextrose, yeast extract, liver extract and fetal calf serum. Since that time, two additional axenic strains (Ax-2, Ax-3) have been isolated (7,8) which could grow on a semi-defined medium consisting of Proteose-Peptone, yeast extract and dextrose (8). All axenic strains grow with a doubling time of approximately 12-15 hrs. The Ax-3 strain grows optimally and at an exponential rate when dextrose is used as the sugar source (Figure 2). Stationary phase is reached when the cell density approaches 2×10^7 cells/ml. The isolation of these axenic strains made it possible to grow large numbers of cells without the problems associated with bacterial contamination.

D. discoideum amoebae growing on a solid surface in the presence of bacteria, will move directly toward this food source. Although it is not known precisely how the amoebae are able to move along a substrate, it has been suggested that actin and myosin which are known to be present in these amoebae (9-11) may form a contractile apparatus similar to that found in voluntary muscle (12). As yet, however, no histological evidence in support of this hypothesis has been presented. The attraction toward bacteria has been shown to be chemotactic. Bacteria secrete folic acid, which results in a chemical gradient upon which the amoebae can orient and move toward areas of higher concentration.

C. DEVELOPMENT OF D. DISCOIDEUM

1. Aggregation

When amoebae have consumed all of the food source, starvation triggers the beginning of their developmental program and multicellular aggregates form within 6-10 hrs.

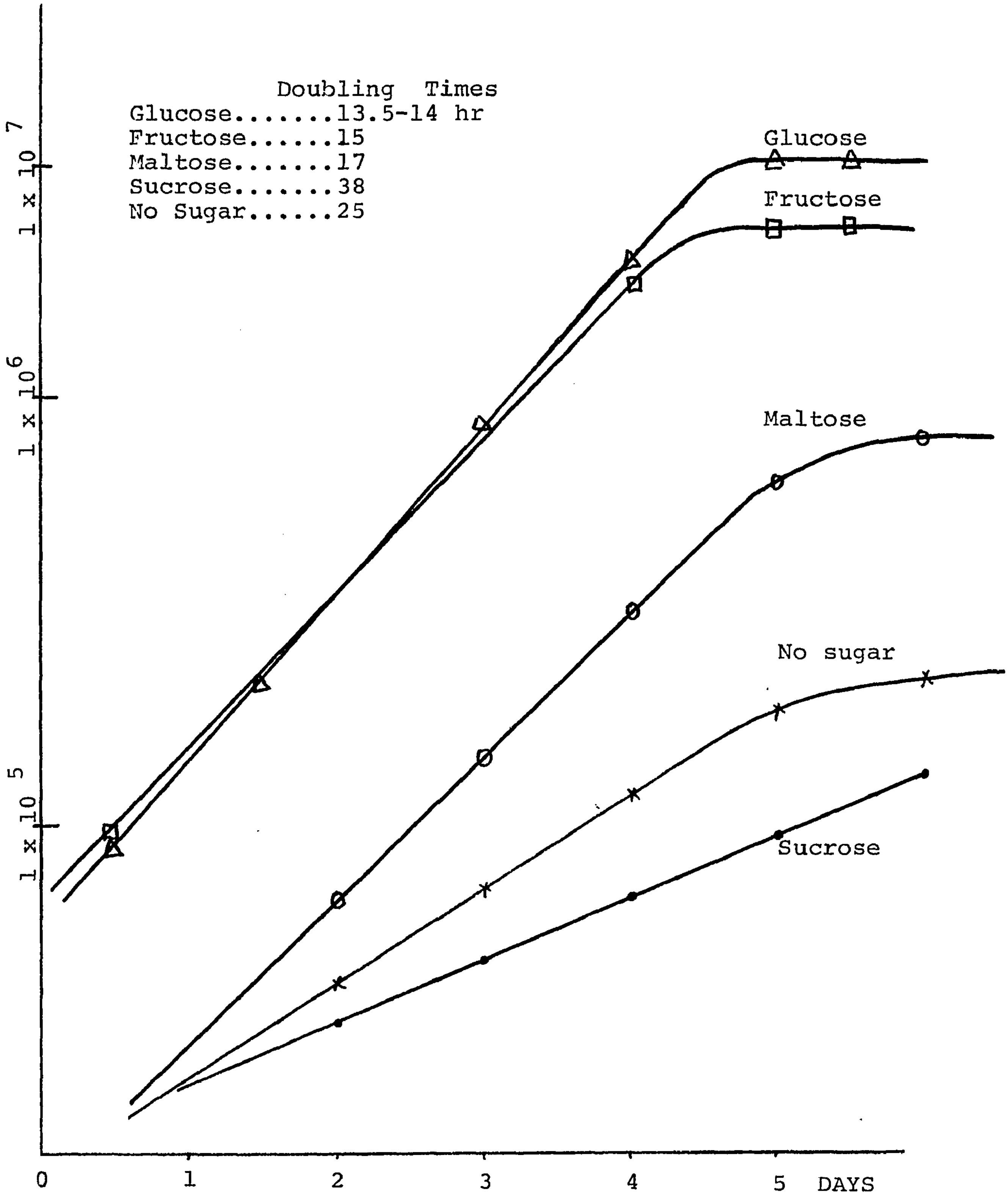

Figure 2. Growth Curves for D. discoideum amoebae, strain A-3 grown in the presence of various sugars.
Log number of cells vs. time

The gathering of up to 10^5 amoebae toward a central cell or group of cells is another example of a chemotactic response, this time to a substance secreted by the center, initially called acrasin (14, 15). Subsequently, Bonner et al. identified the active agent in acrasin to be cyclic adenosine monophosphate (cAMP) (16). Since then it has been shown that aggregation requires the synthesis and secretion of cAMP as well as the recognition and response to this agent. Three factors are known to regulate the level of cAMP in the environment. The membrane bound adenylate cyclase of slime molds converts ATP to cAMP. The specific activity of this enzyme remains constant throughout the developmental sequence (17, 18). Another enzyme, cAMP phosphodiesterase degrades cAMP into 5'-AMP and is active before aggregation (19, 20). Upon starvation cAMP builds up in the environment primarily as a result of the secretion of a heat stable factor which specifically inhibits the phosphodiesterase (19). This regulatory mechanism produces the chemical gradient upon which amoebae can orient and locomote (21). In addition to a gradient of cAMP, a minimum concentration of 10^{-5} M Ca^{2+} is required for normal aggregation (22). Furthermore, amoebae do not seem to secrete cAMP into the medium at a constant rate. A cell stimulated by a cAMP signal will respond by itself secreting a pulse of cAMP about 15 sec after the signal was recieved. Only after a short refractory period can a second cAMP secretion be made. This kind of signalling could explain the observation that during aggregation amoebae migrate toward a center in concentric waves (23, 24).

Approximately 7-10 hrs after starvation begins the amoebae increase dramatically in their adhesiveness. In vitro experiments have suggested that cells first form loose associations with neighboring cells which can be easily dissociated with EDTA. Within a short period, tighter bonds develop which are no longer sensitive to EDTA (25). It is not clear how the EDTA sensitive bonds are formed; however, the finding that Ca^{2+} increases the cohesiveness of amoebae (26) suggests that this divalent cation forms intracellular bridges providing some stability to the associate. This would explain the sensitivity of these associations to the Ca^{2+} chelator EDTA.

Aggregating cells possess antigenic determinants on their cell surfaces not present on vegetative cells. Furthermore, these determinants are not found on amoebae of an aggregateless mutant strain (23, 27, 28). Beug et al have found that specific antigenic determinants accumulate at the same period of time (9 hrs) when amoebae become cohesive in the presence of EDTA (29, 30). Rosen et al have isolated from the surfaces of aggregating cells a protein, discoiden, which consists of 4 subunits each 25,000 daltons (31). Discoiden accumulates simultaneously with the increased cohesion of cells and may be a significant factor in linking the aggregating cells. In addition to these modifications, there appear to be ultrastructural changes of the cell membrane as well. Electron micrographs of freeze fractured membranes indicate the presence of spherical projections, perhaps large protein complexes, within the membranes of cells that had been developing for 8 hrs. (32). These spheres were larger but fewer in number than those found within the plasma membranes of vegetative amoebae suggesting that smaller spheres coalesce at the time cells become adhesive. Most interesting is the finding that treatment of vegetative cells with cAMP induces identical alterations (33). Thus the experimental evidence to date suggests that as cells aggregate in response to a cAMP gradient, specific cell surface proteins are synthesized. These antigenic components cause increased cell adhesiveness ultimately resulting in tight cell binding.

2. The Slug

The slug (pseudoplasmodium) which is formed as the aggregate topples is surrounded by a slimy sheath which is secreted by peripheral cells. The slime is hydrophobic and consists of polysaccharides containing mostly glucose, N-acetylglucosamine, galactose, mannose,and a protein which is high in asparagine content (4). Under certain conditions the slug may migrate for minutes, hours or days. This migration period can be eliminated with no effect on subsequent development. High humidity (34), low ionic strength (35), and unidirectional blue light at 430 nm or green light between 550-590 nm (36, 37) all induce migration. In addition the slug is thermotropic and will orient and migrate in a temperature gradient as low as 0.05°C/cm (34, 36). The anterior of the slug consists of cells which had entered the center of the aggregate earliest, while amoebae that entered the aggregate last make up the posterior portion of the slug. Cells in the anterior 1/3 of the slug ultimately become vacuolated stalk cells while those in the posterior 2/3 of the slug become spores (38). During the aggregation stage, prior to the formation of strong intracellular bonds, mixing of cells can still take place so the fate of a particular cell is not yet determined. However, once the cohesive system is fully developed, the position of particular cells within the slug is fixed (39, 40). At this time pre-stalk and prespore cells can be distinguished histologically, morphologically and biochemically. Ultrastructurally the presence of specialized vessicles can be detected only in cells destined to become spores e.g. cells in the posterior of the slug (2, 41). These vacuoles are important for the formation of the outer spore wall (42). They develop during late aggregation and mark the first ultrastructural differentiation during development. It should be noted, however, that under certain conditions these cells can de-differentiate and are then capable of re-differentiating into the alternate cell type. Amoebae from a slug which has been experimentally disaggregated will become vegetative once again if provided with a food source. Furthermore, if the prespore and prestalk cells of the slug are separated by bisecting the slug, each part can re-differentiate the appropriate missing cell type within a few hours. The part containing only prestalk cells synthesize prespore vessicles in a proportion of the cells, while the part containing only prespore cells, lose the prespore vessicles. Thus two new slugs are formed with each containing the two cell types in the proper proportion (46). Apparently the regulatory controls which determine which cell type an undifferentiated cell will become reside in the cell population rather than in the individual cells themselves.

3. Biochemistry of Development

The biochemical events during development have been studied in great detail as a result of the ability to synchronize the developmental stages. Synchrony is attained when cells are washed free of nutrients and pipetted onto millipore filters supported on absorbant pads which are saturated with a buffered salt solution (47). Development proceeds in the absence of cell division (48). The dry weight decreases 50% (49, 50) with simple first order kinetics (51). The decrease in dry weight is presumably due to the loss in total protein content during development (52). The rate of oxygen consumption decreases from about 0.32 μl oxygen/min/mg dry weight of vegetative cells to 0.08 μl oxygen/min/mg dry weight during culmination (53).

During this period there is also a 60% loss of RNA, the bulk of which is ribosomal RNA. The polysaccharide content remains constant throughout development as does the nuclear DNA content (of axenically grown cells). Despite all of these observations, the developmental process is a very active period of macromolecular synthesis. Cocucci and Sussman have found that rRNA synthesis continues during development (54). Glycogen is converted to other polysaccharides i.e. cellulose, trehalose (55). Although there is not very significant amounts of gluconeogenesis from amino acids during development, the ribose residues resulting from degradation of RNA are converted to hexoses and ultimately become polysaccharides (4). A mucopolysaccharide also accumulates during culmination (56).

Perhaps the most important events that occur following starvation of amoebae are embodied in new protein synthesis. Actin, which is present in vegetative amoebae, increases dramatically with starvation of amoebae and then decreases over the next 6 hrs (57). Presumably this structural protein is important to the ameboid movement of aggregating cells. Some enzymes which are present in vegetative cells can also be found during all stages of development in approximately the same specific activity i.e. adenyl cyclase (17), or pyrophosphatase (58), the enzyme which cleaves pyrophosphate thus regenerating inorganic phosphate to be used for synthetic reactions. Lactic dehydrogenase, aspartate transaminase, glutamate dehydrogenase and homoserine dehydrogenase are a few more enzymes whose specific activity remains essentially unchanged (59). (For a more complete discussion see reference 4).

On the other hand some enzymes which are found in vegetative cells decrease in specific activity as cells become starved and start to develop. Examples of these are trehalase (60,61), threonine deaminase -1 (62) and β-glucosidase-1 (63).

Approximately 13 enzymes are now known to be developmentally regulated in Dictyostelium discoideum (4, 64). These enzymes,each of which seems to be a single gene product, increase in their specific activity at various stages of the developmental process. Based on experiments utilizing transcriptional and translational inhibitors each seems to require both a prior transcription of RNA and a period of de novo protein synthesis which occurs simultaneously with the observed increase in specific activities. Each of these enzymes has its own characteristic kinetics of accumulation and disappearance during development (4). The enzymes of amino acid metabolism include: leucine aminopeptidase, alanine transaminase, threonine deaminase-2 and tyrosine transaminase. The hydrolytic enzymes are: N-acetylglucosaminidase, α mannosidase, alkaline phosphatase and β glucosidase-2. The remaining enzymes are related to carbohydrate metabolism and are: trehalose-6 phosphate synthetase, UDPG pyrophosphorylase, UDPgalactose polysaccharide transferase, UDPgalactose epimerase and glycogen phosphorylase (4).

The analysis of the kinetics of accumulation of these enzymes in different mutant strains known to be temporally deranged or blocked in a particular morphological stage indicates that there is a genetic mechanism of temporal control in the synthesis of these enzymes. In a fast developing mutant strain, FR-17, analysis of five developmentally regulated enzymes indicates that although they are made at a faster rate they still accumulate in proper sequence (65, 66). The same result has been shown for the early enzymes of a slow developing strain, GN-3. Although these enzymes do not seem to accumulate in a cascade reaction, e.g. each enzyme accumulates only after the previous one has been synthesized, it appears as if there are eight sequential stages of development which insure the proper order of accumulation of these 13 enzymes (64).

It should be noted however that analysis of many mutant strains by Loomis et al indicates that the synthesis and accumulation of a developmentally regulated enzyme is not strictly linked to the synthesis and accumulation of the next normally accumulating enzyme. In mutant strains which have altered acetylglucosaminidase and α mannosidase, the remaining enzymes still appear when expected. (See Loomis, reference #4 for a more thorough discussion)

D. MITOCHONDRIA OF D. DISCOIDEUM

Dictyostelium discoideum is an excellent organism for studying developmental processes at the sub-cellular, cellular and multicellular levels. Vegetative amoebae have a relatively rapid generation time and can be harvested in quantities large enough to be used for biochemical analysis. The ability to synchronize the amoebae in both the feeding phase (67) as well as the developmental phase is an added bonus for the biochemist and molecular biologist. Despite the obvious advantages of studying sub-cellular biochemical processes in this simple eukaryote, little is known concerning mitochondrial functions such as respiratory driven ATP synthesis and even less is known about the biogenesis of these organelles.

Dictyostelium discoideum is an obligate aerobe (53) despite reports to the contrary (68,69). Amoebae cannot survive in the presence of respiratory poisons or uncouplers of oxidative phosphorylation. Furthermore, vegetative amoebae cannot grow in an atmosphere of purified nitrogen. Even though the spores have a very low rate of oxygen consumption (70), the process of spore germination is strictly aerobic and is blocked by 2-4 dinitrophenol (5×10^{-4}M), an uncoupler of mitochondrial oxidative phosphorylation and by 7.5×10^{-4} M sodium azide, a respiratory poison (71). Clearly mitochondria play a vital role in the process of germination. Ultrastructurally, mitochondria of spores are spherical, crenated and studded with cytoplasmic ribosomes (4). Following activation of spores however, these ribosomes are no longer present. Mitochondria are now elongate and less dense and a marked cyanide-sensitive oxygen consumption commences almost immediately. In an early report (1960), Takeuchi (72) attempted to correlate changes in the activities of succinic dehydrogenase and cytochrome c oxidase with the development of D. discoideum. He in fact had measured the activity of the enzyme complex succinate-cytochrome c reductase rather than succinic dehydrogenase. Nevertheless this enzymatic activity is also restricted to the inner mitochondrial membrane. His studies indicate that after development begins the preaggregative cells have 2.5 times the activity of succinate-cytochrome c reductase while the activity of cytochrome c oxidase declines over 50% during the same period.

Little has appeared in the literature since that time concerning the respiratory chain or the oligomycin-sensitive ATPase complex of D. discoideum mitochondria.

Over 90% of the total proteins of mitochondria are translated on cytoplasmic ribosomes and transported into the mitochondria in a subsequent step (73). The remaining proteins are very insoluble and are translated on the chloramphenicol sensitive mitochondrial ribosomes. These proteins numbering between eight and ten are exclusively localized on the inner mitochondrial membrane. They include the three large subunits of the cytochrome oxidase complex, four subunits of the oligomycin-sensitive ATPase complex and at least one subunit of the b-c_1 complex (Complex III) of the respiratory chain (73).

The cell contains two distinct genetic systems as well as two distinct translational systems. D. discoideum mitochondria contain their own DNA (probably of the closed circular form), ribosomes and translational machinery. Synthesis of a functional inner mitochondrial membrane requires coordination of the two systems, the controlling elements of which are not yet fully understood. Control of mitochondrial membrane biogenesis is exerted at several levels. Since the outer and inner membranes have different turnover rates control must be exerted to maintain a functional mitochondrion. Secondly, there must be some control governing the synthesis and integration of enzyme complexes into the mitochondrial membrane e.g. succinic dehydrogenase, NADH dehydrogenase, oligomycin-sensitive ATPase. Thirdly, controls must exist to regulate the synthesis and assembly of the individual enzyme complexes i.e. the cytochrome oxidase complex consists of 7 subunits, 3 of which are translated on mitochondrial ribosomes and the remaining 4 are translated on cytoplasmic ribosomes. In addition, a mechanism must exist which controls the insertion of the heme groups into the various cytochromes. The terminal step in the synthesis of these prosthetic heme groups takes place in the mitochondrial matrix (73).

One method of determining what controls exist to coordinate membrane assembly is to disrupt the two systems artificially and to determine how this affects the overall process. Stuchell, Weinstein and Beattie (74,75) using this approach treated D. discoideum vegetative amoebae with ethidium bromide and then examined the activities of the various components of the respiratory chain and the oligomycin-sensitive ATPase complex. Ethidium bromide is a cationic phenanthridinium dye which has been shown to bind preferentially to closed circular DNA by intercalation between the bases (76). This results in the inhibition of the replication and transcription of mitochondrial DNA (77-79). The effects on the respiratory chain caused by ethidium bromide treatment of D. discoideum are: 1. 80% decrease in the activity of cytochrome oxidase 2. 50% decrease in succinate-cytochrome c reductase activity. In addition the activity of the oligomycin sensitive ATPase complex which is responsible for respiratory driven ATP synthesis is reduced 80%. The results of this study indicated that for these enzymatic activities, mitochondrial transcription and presumably translation is essential.

Treatment of Saccharomyces cerevisiae with this drug results in the irreversible formation of cytoplasmic petites characterized by a complete loss of all mitochondrial cytochromes except cytochrome c (80). However, the effects of ethidium bromide treatment of D. discoideum are completely reversible following removal of the drug. The kinetics by which the activity of the various respiratory chain components returns to control levels can therefore be determined resulting in a better understanding of the process of mitochondrial membrane assembly. This investigation is described in the 2nd chapter of this thesis. In Chapter 3, I have explored the effects of ethidium bromide on various stages of the developmental process. There appear to be several effects of this drug which cannot be explained simply by interaction with mitochondrial DNA. The effects on aggregation may have resulted from the cationic nature of this molecule. The fourth chapter is a study of the induction of filopodia on early logarithmically growing vegetative amoebae (strain A-3) by cyclic 3',5'-adenosine monophosphate. A possible mechanism of aggregation is suggested as a result of these studies. The fifth and last chapter describes the respiratory competence of the spores of D. discoideum and suggests that spores lack a cyanide-sensitive respiration as a result of inadequate amounts of respiratory substrate and cofactor NAD^+.

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CHAPTER 2

The Reversibility of the Ethidium Bromide-Induced Alterations of Mitochondrial Structure and Function in the Cellular Slime Mold, *Dictyostelium discoideum*

ABSTRACT

Addition of ethidium bromide to cultures of the slime mold, Dictyostelium discoideum, caused a cessation of cell division after 1-2 generations. The cyanide-sensitive respiration in whole cells was decreased 75% after 5 days in ethidium bromide. Purified mitochondria from ethidium bromide-treated cultures contained 50% the content of DNA as mitochondria from control cells. Low temperature spectroscopy studies indicated that the amount of cytochrome $\underline{c}_1$ was decreased by 80% and that of cytochrome $\underline{c}$ increased 2-fold in mitochondria from treated cells. Two cytochromes $\underline{b}$ absorbing at 557 and 561 nm in slime mold mitochondria were observed; however, the content of cytochrome b_{561} alone appeared to decline in cultures treated with ethidium bromide. The effects of ethidium bromide were fully reversible. When the drug was removed, the cells resumed a normal growth rate without any discernible lag. The activity of oligomycin-sensitive ATPase, cytochrome $\underline{c}$ oxidase, and succinate-cytochrome $\underline{c}$ reductase as well as the cytochrome content began to increase after one day returning to control levels within five days.

Electron micrographs of whole cells treated with ethidium bromide revealed that mitochondrial profiles were elongated and had greatly reduced cristae. Numerous membrane whorls were apparent as was a profound loss of rough endoplasmic reticulum. Three days after

removal of ethidium bromide, mitochondria were again ovoid in shape and contained well-developed cristae. In all of the cells during recovery, there was a single large vacuole which appeared to enclose a large portion of the cell volume forming a new cellular compartment which may simplify the breakdown of previously damaged organelles.

INTRODUCTION

Ethidium bromide and the acridine dyes which preferentially intercalate between the bases of covalently closed circular DNA (35) have been shown to be specific inhibitors of the replication and transcription of mitochondrial DNA (6,9). Exposure of facultatively aerobic yeast to ethidium bromide results in the irreversible formation of cytoplasmic petites characterized by a complete loss of all mitochondrial cytochromes except cytochrome _c_ (29); however, ethidium bromide treatment of various mammalian cell types, such as human fibroblasts (23), mouse L cells (12,30), or SV-40 transformed cells (27) results in alterations in cytochrome levels which are completely reversible following removal of the drug. Likewise, addition of ethidium bromide to cultures of the cellular slime mold _Dictyostelium discoideum_ results in drastic changes in mitochondrial activities. The cellular slime mold grows logarithmically as a unicellular amoeba with a generation time of 12-15 hours when supplied with adequate nutrients. Previously, it was reported that after addition of ethidium bromide to cultures of slime mold amoebae, cell division ceased after 1-2 generations (32). Mitochondria purified by sucrose gradient centrifugation from cultures treated with ethidium bromide for 5 days, a time equivalent to 8-10 generations, had an approximately 80% decrease in activity of both cytochrome *c* oxidase and oligomycin- sensitive ATPase and a 50% decrease in succinate-cytochrome *c* reductase activity (33). By contrast, the specific activities of succinate and NADH dehydrogenases were identical in mitochondria from both control and ethidium bromide treated cells suggesting that continued synthesis of new mitochondrial membranes must occur when mitochondrial transcription has been blocked.

In the present study the characterization of mitochondria obtained from the slime mold after treatment with ethidium bromide has been extended both biochemically and morphologically. The rate of cyanide-sensitive respiration in whole cells was decreased 75%, as anticipated by the decreases in activity of various components of the respiratory chain. Low temperature difference spectroscopy permitted the resolution of cytochromes *c* and $\underline{c}_1$ and revealed the presence of two *b*-type cytochromes in slime mold mitochondria. After treatment with ethidium bromide, the content of cytochrome $\underline{c}_1$ was decreased while that of cytochrome *c* was increased. In addition, the content of only one of the *b*-type cytochromes was partially lowered.

Electron micrographs of cells grown in the presence of ethidium bromide reveal alterations in mitochondrial morphology which are completely reversible if cells are washed free of the drug and allowed to grow in fresh nutrient medium. The time course in which the activity of various respiratory chain components returns to control levels suggests that the inner mitochondrial membrane may be assembled in an asynchronous manner.

MATERIALS AND METHODS

Cell Culture and Preparation of Mitochondria - Axenic cultures of *Dictyostelium discoideum* strain A-3, were grown at 22-23°C as described previously (32). When the cells had grown to a density of 0.5 - 2.0 x 10^6 cells/ml, ethidium bromide was added to a final concentration of 10 μg/ml. The flasks were wrapped with aluminum foil and incubated on a New Brunswick gyratory shaker at 200 rpm. Cells used for controls were grown to a density of 1-3 x 10^6 cells/ml. Stationary phase is reached at a density of 1-1.5 x 10^7 cells per ml. Mitochondria were prepared from control and ethidium bromide-treated cells by sucrose gradient centrifugation as described previously (33).

Enzyme Assays - Cytochrome *c*oxidase, succinate-cytochrome *c* reductase, succinate dehydrogenase, NADH dehydrogenase, and NADH-cytochrome *c* reductase were each assayed at 23°C in a Gilford spectrophotometer as described by Kim and Beattie (11). ATPase activity was measured at 22°C in a medium containing 3.0 mM $MgCl_2$, 10 mM ATP, 50 mM Tris HCl, pH 9.0, and approximately 1.0 mg of mitochondrial protein in a final volume of 1.0 ml (33). Aliquots were removed at 1 min intervals over a 5 min period. The reaction was terminated by addition of 0.5 ml of 6.6% perchloric acid, and following centrifugation 1.0 ml of the clear supernatant was removed for phosphate analysis.

Respiratory Studies - Oxygen uptake was determined at 20°C with a Clark Oxygen electrode. Approximately 1.0-1.5 mg mitochondrial protein or 3-4 mg whole cell protein was added to 3 ml of a buffer containing in 200 ml, 10.93 gm mannitol and 20 ml each of a 0.1 M solution of Tris, pH 7.5, KCl and KH_2PO_4. Succinate and ADP were added to a final concentration of 3.3 and 0.5 mM respectively.

Chemical Determinations - Mitochondrial DNA was determined by the method of Morse and Carter (21). Gradient purified mitochondria were precipitated in 10% cold trichloroacetic acid. Following centrifugation at 755 x g for 20 min, the supernatant was discarded and the pellet was hydrolyzed in 2.0 ml of a 5% trichloroacetic acid solution by boiling for 30 min. The suspension was brought to 2.0 ml with 5% trichloroacetic acid, centrifuged at 755 x g for 15 min and the supernatant assayed for deoxyribose content according to the method of Sevag *et al*. (28) using calf thymus DNA Type 1 (sodium salt) as standard. The assay consisted of adding two volumes of diphenylamine reagent to 1 volume of unknown, heating in a boiling water bath for 10 min and recording the absorbance at 595 nm on a Gilford spectrophotometer. Diphenylamine was recrystallized from 70% ethanol and made 1% w/v in glacial acetic acid. Concentrated H_2SO_4 (2.75 ml) was added to 100 ml of this acid solution. Protein was determined by the method of Lowry *et al*. (18) using bovine serum albumin as standard.

Spectral Studies - The room temperature difference spectrum of whole cells or purified mitochondria was determined in a Cary model 15 spectrophotometer. Whole cells were sonicated and mitochondria were solubilized with 0.4% Triton X-100. The reference sample was oxidized with a few grains of ferricyanide and the experimental sample was reduced by the addition of a few crystals of sodium dithionite. The concentration of cytochromes in nanomoles was calculated using the extinction coefficients of Rieske (24). The low temperature ($77^{\circ}K$) spectrum of purified mitochondria was determined using an Aminco DW-2 UV-Vis spectrophotometer fitted with a Dewar flask in which the cuvettes were bathed in liquid nitrogen. The spectrophotometer was operated in the split beam mode using a 1.0 nm slit width. All samples were mixed with glycerol (1:1) prior to freezing. Reduction and oxidation of the sample was achieved as described above. In addition, where indicated, samples were reduced with 10 mM ascorbate and 8 μM tetramethylphenylenediamine.

Electron Microscopy - Cells were prefixed by adding a 6.5 per cent gluteraldehyde fixative to the culture medium in a 1:10 ratio. Cells were then harvested by centrifugation at 750 x g for less than one min at $4^{\circ}C$ and fixed in 6.5 % gluteraldehyde buffered with Veronal acetate, pH 7.3, at room temperature. Post fixation was in Veronal acetate buffered osmium tetroxide (Palade's fluid). Cells were then washed and stained with 1% uranyl acetate and dehydrated by gradual transition through alcohols of increasing concentration. The cells were infiltrated and embedded in Maraglas. Thin sections were examined with a Zeiss EM 9S-2 or a Jeol 100B Electron Microscope.

Materials - Ethidium bromide (3,8-diamino-5-ethyl-6-phenyl-phenanthridinium bromide) and streptomycin sulfate, both grade B, were purchased from Calbiochem; Triton X-100 was obtained from Rohm and Haas. Dichlorophenolindophenol, grade 1, phenazine methosulfate, bovine serum albumin (fraction five), cytochrome c (horse heart type III or VI), were obtained from Sigma; proteose peptone and yeast extract were obtained from Difco and sucrose (density gradient grade) from Schwarz-Mann. Oligomycin was obtained from ICN Pharmaceuticals Inc. and dissolved in 95% ethanol prior to use. Diphenylamine was obtained from Pfaltz and Bauer Inc.

RESULTS

Effect of Ethidium Bromide on Mitochondrial Function-

Previously, it was reported (32) that after addition of ethidium bromide to cultures of slime mold amoebae, growth continued for 1 to 2 generations at which time, cell division ceased. The amount of protein in these cultures, however, continued to increase such that the protein per cell in the cultures treated with ethidium bromide for 5 days was double that of control cells (Table I). Similar changes in cellular protein content were also observed in stationary phase cells which contain almost three times the amount of protein per cell as cells in the logarithmic phase of growth. In addition, the activity of certain mitochondrial enzymes such as succinate dehydrogenase, also varies with the growth phase. Hence, control cells used for all studies were obtained from the early or mid-logarithmic phase of growth.

The data of Table I also confirm published reports (7, 10) that ethidium bromide blocks the replication of mitochondrial DNA in the slime mold. The amount of DNA present in purified mitochondria from ethidium bromide-treated cultures was decreased 50% as compared to mitochondria from control cells. Ethidium bromide also acts to block the transcription of mitochondrial DNA and consequently several enzymes of the respiratory chain were decreased after cultures were treated with ethidium bromide for 5 days. The activities of oligomycin-sensi-

TABLE I

Effects of Ethidium Bromide on Slime Molds

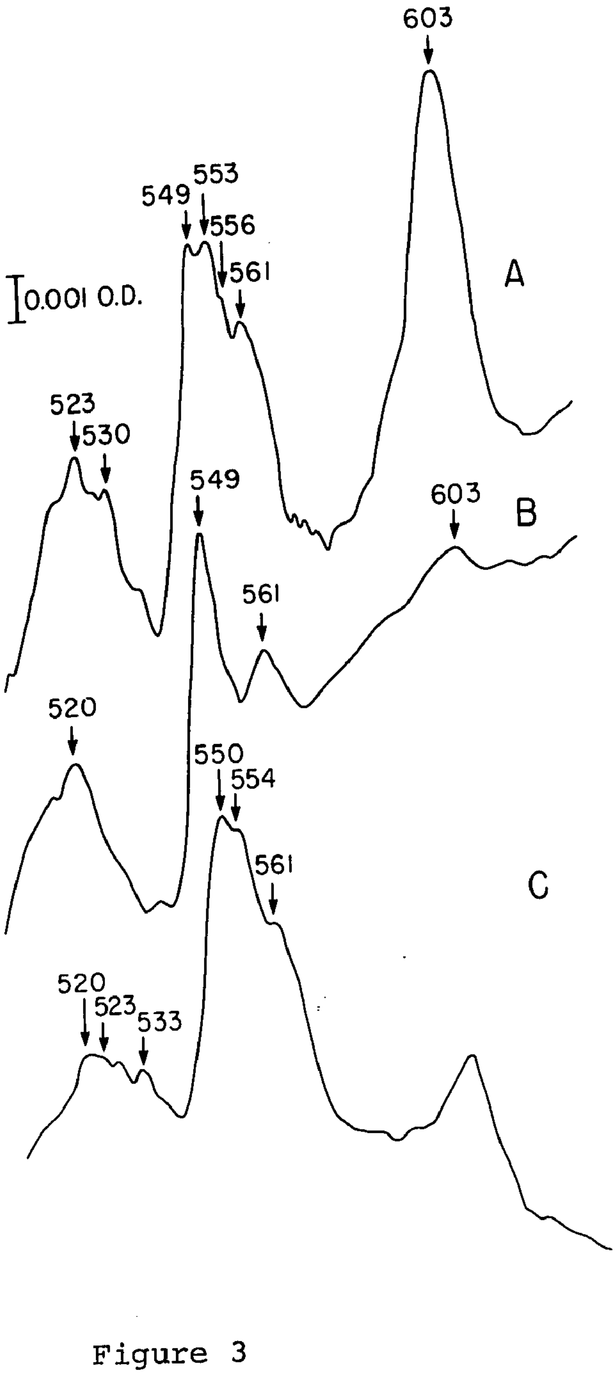
		Control	Ethidium Bromide	Change %
Protein	mg/10^8 cells	6.4	13.4	+109
DNA	μg/mg mito. protein	6.36	3.38	-49
Oligomycin-sens. ATPase	μM P_i/min/mg	0.495	0.124	-75
Cytochrome Oxidase	k/mg	1.36	0.58	-58
Succinate-cytochrome *c* reductase	nmoles/min/mg	186	125	-33
NADH-cytochrome *c* reductase	nmoles/min/mg	729	427	-41
Succinate Dehydrogenase	nmoles/min/mg mito. protein	192	168	Insig.
Succinate Dehydrogenase	nmoles/min/μg mito. DNA	30.2	49.7	+65
Cytochrome *a*-a_3	nmoles/mg mito. protein	.266	.072	-73
Cytochrome *b*	nmoles/mg mito. protein	.296	.185	-38
Cytochrome *c*-c_1	nmiles/mg mito. protein	.421	.715	+69
Whole cell respiration	natoms O/min/mg	34.1	12.9	
+ Cyanide	natoms O/min/mg	8.5	7.7	Insig.
Cyanide-Sens. respiration	natoms O/min/mg	25.6	5.2	-75

tive ATPase and cytochrome c oxidase were decreased 75% and 58% respectively in purified mitochondria from ethidium bromide-treated cultures as compared to controls (Table I). In some experiments (33), larger decreases, up to 85%, in cytochrome oxidase activity were observed. In addition, both NADH and succinate cytochrome c reductase activities were significantly decreased in these mitochondria although to a lesser extent. By contrast, the activity of succinate dehydrogenase remained unchanged in the mitochondria after treatment with ethidium bromide. The significant effect of these decreases in respiratory chain activity on cell metabolism is indicated by the 75% decrease in cyanide-sensitive respiration in whole cells (Table I). This inhibition of respiration coupled with the decrease in oligomycin-sensitive ATPase activity (a measure of the ability to synthesize ATP) may explain the cessation of cell growth. The lowered level of energy production may be sufficient to maintain cell viability and some protein synthesis, but insufficient for the cells to undergo cell division.

Spectral analysis at room temperature of the purified mitochondria from control and ethidium bromide-treated cells mirrored the decreases in enzymatic activity of cytochrome oxidase and succinate or NADH cytochrome c reductases (Table I). The total amounts of cytochromes a-a_3 and b were decreased 73% and 38% respectively while that of cytochromes c-c_1 was increased 69% above control levels after treatment with ethidium bromide. Difference spectroscopy at low temperature permitted the resolution of both c-type cytochromes and revealed the presence of two b-type cytochromes (Figure 3). In the trace of control mitochondria reduced with dithionite (Figure 3 trace A), the absorption maxima at 550 and 554 nm are presumably due to cytochromes c and c_1 while those at 556 and 561 nm represent b-type cytochromes. Further evidence for these assignments is apparent in Figure 4 , in which control mitochondria were first reduced with ascorbate-tetramethylphenylenediamine (trace A) and then reduced with dithionite using the ascorbate reduced mitochondria in the reference cuvette (trace B). In trace A, the absorption band at 603 nm due to cytochromes a-a_3 is apparent as well as the two bands at 550 and 554 due to cytochromes c and c_1. The presence of two bands at 520 and 525 nm is also apparent in the beta region of the spectrum. Since ascorbate- tetramethylphenylenediamine acts to reduce the respiratory chain at the level of cytochromes c and c_1, further addition of dithionite using the ascorbate reduced mitochondria as the reference, will show the reduction of only b type cytochromes. Figure 4 , trace B reveals two distinct peaks at 556 and 562 nm which most likely represent two different forms of cytochrome b.

Mitochondria purified from slime mold cultures treated with ethidium bromide for 5 days were also examined by low temperature difference spectroscopy. As seen in

Figure 3. Low temperature ($77^{o}K$) difference spectra of purified mitochondria from: trace A, control cells; trace B, cells treated with ethidium bromide for five days; trace C, cells which had been washed free of ethidium bromide and allowed to recover for three days. Samples were reduced with dithionite and oxidized with ferricyanide. Protein concentration was 2 mg/ml.


603
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533

Figure 3

Figure 4. Low temperature (77°K) difference spectra of purified mitochondria from control cells. Trace A: sample was reduced with ascorbate-tetramethylphenylenediamine. Trace B: dithionite reduced with the reference cuvette containing mitochondria reduced with ascorbate-tetramethylphenylenediamine. Protein concentration was 2 mg/ml.

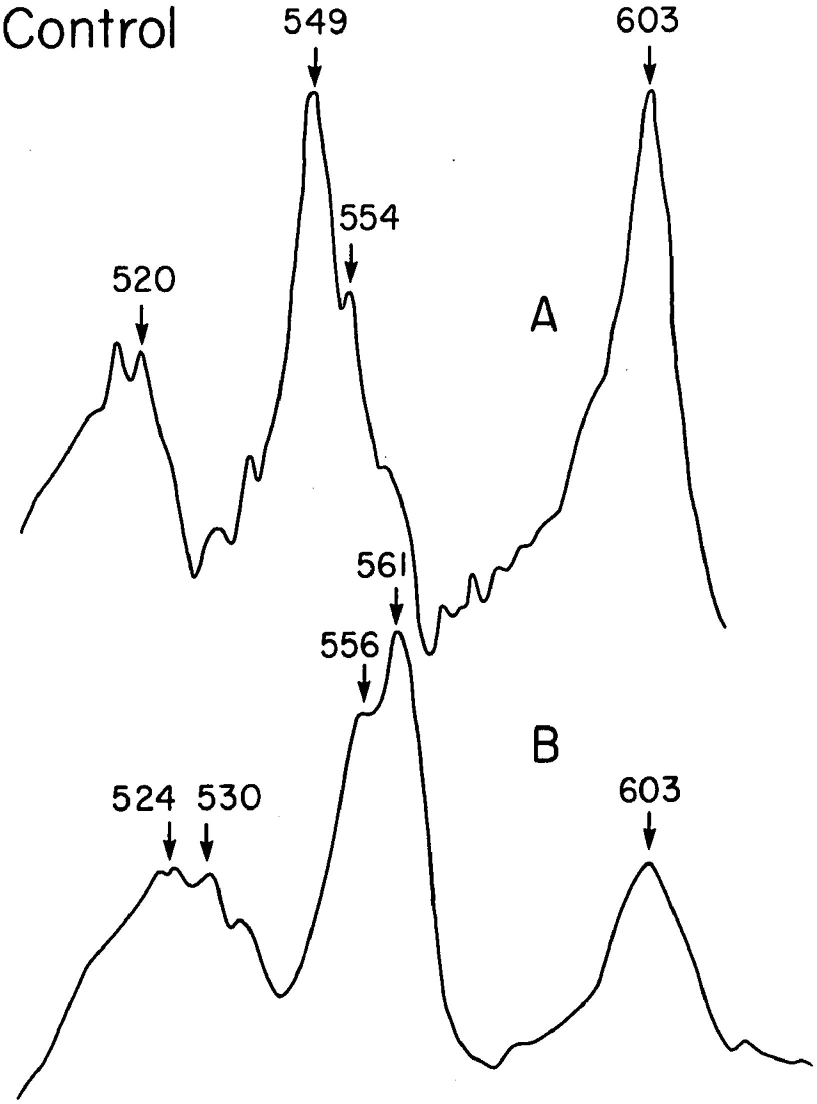


Figure 4

Figure 3 , trace B, the bands at 603 and 561 nm due to cytochromes $\underline{a}$-$\underline{a}_3$ and presumably one of the $\underline{b}$-type cytochromes were greatly reduced as compared to the control mitochondria. The cytochrome $\underline{c}$ region of the spectrum was also altered significantly in the mitochondria after ethidium bromide treatment revealing an almost symmetrical peak at 549 nm due to cytochrome $\underline{c}$ with only a slight shoulder at 554 nm due to cytochrome $\underline{c}_1$. Reduction of these mitochondria with ascorbate further emphasized the prominence of the band due to cytochrome $\underline{c}$ and the pronounced loss of cytochrome $\underline{c}_1$ (Figure 5 , trace A). The beta region of the spectrum also reveals a single symmetrical band at 519 nm due to cytochrome $\underline{c}$. The presence of two $\underline{b}$-type cytochromes in the mitochondria from ethidium bromide-treated cultures was apparent when the dithionite reduced mitochondria were analyzed using ascorbate reduced mitochondria as the reference. Although it is difficult to quantitate the absolute decrease in amounts of the two $\underline{b}$-type cytochromes after ethidium bromide treatment, these spectra suggest that the cytochrome $\underline{b}$ absorbing at 561 nm is lowered considerably more than that absorbing at 555-556 nm. The trace B spectrum obtained after dithionite reduction (Figure 3 , trace B) also indicates a pronounced decrease in absorbance at 561 nm.

Figure 5. Low temperature (77^{o}K) difference spectra of purified mitochondria from cells treated with ethidium bromide for 5 days. Trace A: sample was reduced with ascorbate-tetramethylphenylenediamine and oxidized with ferricyanide. Trace B: dithionite reduced with the reference cuvette containing mitochondria reduced with ascorbate-tetramethylphenylenediamine. Protein concentration was 2 mg/ml.

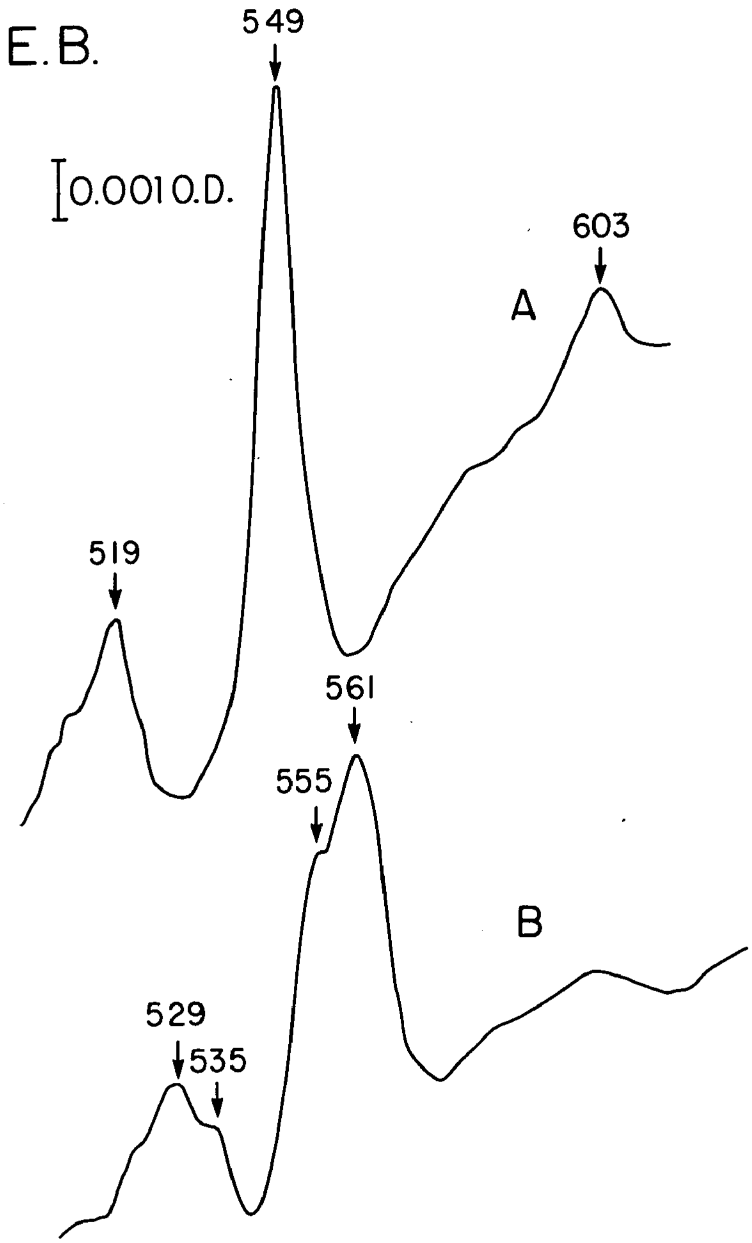


Figure 5

Recovery of Slime Molds From Ethidium Bromide Treatment - The effects of ethidium bromide on slime mold amoebae appear to be completely reversible. When cells were removed from the ethidium bromide medium by low-speed centrifugation, thoroughly washed to remove the drug, and resuspended in fresh nutrient medium, growth and cell division began immediately without a detectable lag. Examination of the cells by difference spectroscopy at room temperature (Figure 6) indicated that the cytochrome spectrum did not return to that of the control until 5 days after removal of the ethidium bromide despite the immediate return to the normal growth pattern. The whole cell spectrum 2 days after removal of the ethidium bromide indicates that levels of cytochrome $\underline{a}$-$\underline{a}_3$ (605 nm) are still greatly depressed. In these cells, the band at 550 nm due to cytochrome $\underline{c}$ remains elevated especially when compared to the band at 562 nm due to the $\underline{b}$-type cytochromes.

Spectral analysis of purified mitochondria from cells after removal of ethidium bromide confirmed the lag in recovery of the mitochondrial cytochromes (Figure 7). The amount of both cytochromes $\underline{a}$-$\underline{a}_3$ and the $\underline{b}$-type cytochromes remained nearly constant for the first two days after removal of ethidium bromide and then rapidly increased reaching control levels at different times. Cytochromes $\underline{a}$-$\underline{a}_3$, the most severely reduced by ethidium bromide treatment, did not recover completely until 5

Figure 6. Room temperature difference spectra of whole cells solubilized in 0.4% Triton X-100 from: Upper trace, control cells; second trace, cells which had been treated with ethidium bromide for 5 days; third trace, cells which had been allowed to recover for 5 days. Cuvettes contained between 4-6 mg protein.

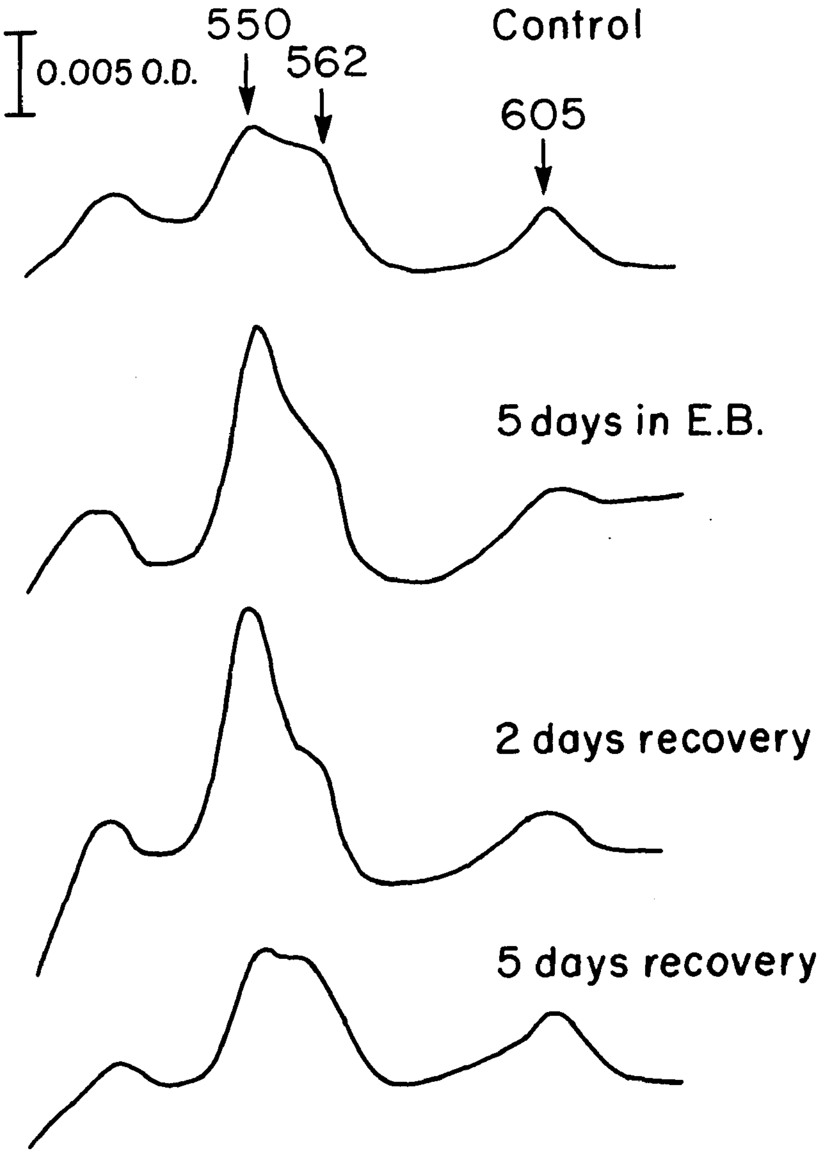
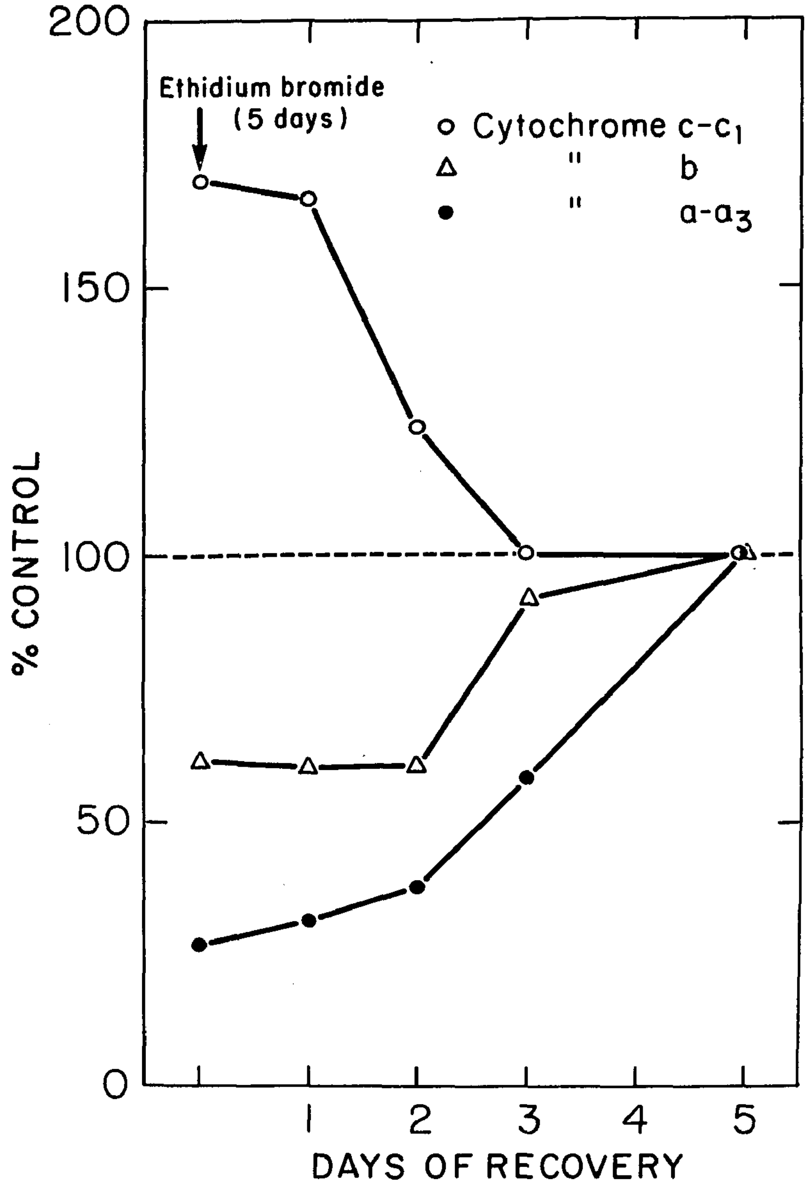

0.005 O.D.
550
562
Control
605
5 days in E.B.
2 days recovery
5 days recovery

Figure 6

Figure 7. Time course of the recovery of cytochromes based on room temperature difference spectroscopy of purified mitochondria. Control values for cytochromes $\underline{c}$-$\underline{c}_1$, cytochrome $\underline{b}$ and cytochromes $\underline{a}$-$\underline{a}_3$ were 0.421, 0.296 and 0.266 nmoles/mg respectively.



200
150
100
50
0
% CONTROL
1
2
3
4
5
DAYS OF RECOVERY
Ethidium bromide
(5 days)
○ Cytochrome c-c1
△ " b
● " a-a3

Figure 7

days after removal, while cytochrome b reached the control level after 3 days. Likewise, the amount of cytochrome c rapidly decreased once the ethidium bromide was removed reaching control levels within 3 days. Low temperature spectroscopy of the purified mitochondria three days after ethidium bromide removal confirmed the partial recovery of the band at 603 nm due to cytochromes $\underline{a}$-$\underline{a}_3$, and the complete recoveries of the bands at 562 and 550 nm of cytochromes b and c respectively. The prominent peak at 554 nm due to cytochrome $\underline{c}_1$ has also reappeared and returned to the control level. (Figure 3, trace C)

In agreement with the spectral data, the enzymatic activities of cytochrome oxidase and succinate-cytochrome c reductase returned to the control levels with similar kinetics as the cytochromes (Figure 8). The activity of both enzymes increased very little during the first day and then increased rapidly reaching the control level after 5 days. The activity of oligomycin-sensitive ATPase also remained unchanged the first day after removal of ethidium bromide, then gradually increased reaching control levels within 5 days. Figure 8 also shows the activity of succinate dehydrogenase assayed in whole cells. Previously, it was reported that the specific activity of succinate and NADH dehydrogenase had nearly doubled in whole cells maintained in ethidium bromide for 5 days despite the identical activity of these enzymes in purified mitochondria from control and ethidium bromide-

Figure 8. Time course of the recovery of mitochondrial enzymes expressed in terms of % control. Succinate dehydrogenase activity was assayed in whole cells solubilized in 0.4% Triton X-100. All other enzyme activities were monitored in purified mitochondria. Control values were for succinate dehydrogenase (34.3 nmoles of substrate oxidized/min/mg whole cell protein), cytochrome oxidase (1.36k/mg mitochondrial protein), oligomycin-sensitive ATPase (0.495 μM P_i/min/mg mitochondrial protein), and succinate-cytochrome c reductase (186 nmoles/min/mg mitochondrial protein).

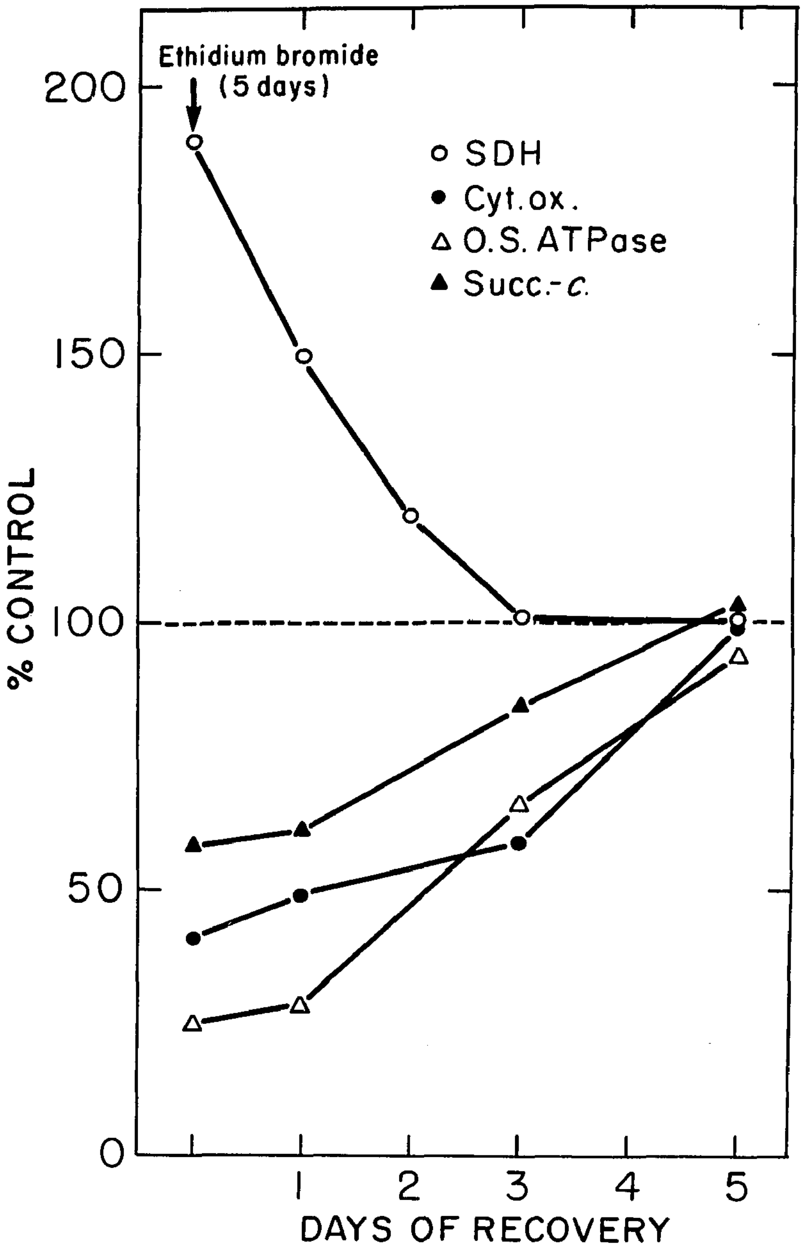

Ethidium bromide
(5 days)
○ SDH
● Cyt.ox.
△ O.S. ATPase
▲ Succ.-*c*.
% CONTROL
200
150
100
50
0
1
2
3
4
5
DAYS OF RECOVERY

Figure 8

treated cells. The specific activity of succinate dehydrogenase in whole cells decreased rapidly reaching control levels within 3 days after removal of the drug.

Electron Microscopy - Ultrastructural studies of control and ethidium bromide-treated cells were performed to investigate the effects of the drug on mitochondrial morphology. Figure 9 shows a control cell in the vegetative stage of growth. Higher magnification indicates the presence of spherical to ovoid-shaped mitochondria containing numerous cristae (Figure 10). In addition, rough endoplasmic reticulum is abundant and often seen closely apposing each mitochondrion. The well-developed infoldings of the inner mitochondrial membrane can be seen in Figure 11 . Control cells contain numerous mitochondria, lysosomes and a nucleus containing 1-3 nucleoli (Figure 12). After two days in ethidium bromide many mitochondria have become elongated and twisted(Figure 13), while the number of cristae appear reduced (Figure 14). Structures consisting of numerous membranes are present in most cells (Figure 15). These electron micrographs strongly suggest that after two days in ethidium bromide, the mitochondria become twisted eventually forming these whorled membranous profiles (Figure 16 and 17). Despite these alterations in mitochondrial morphology, rough endoplasmic reticulum is still apparent in these cells.

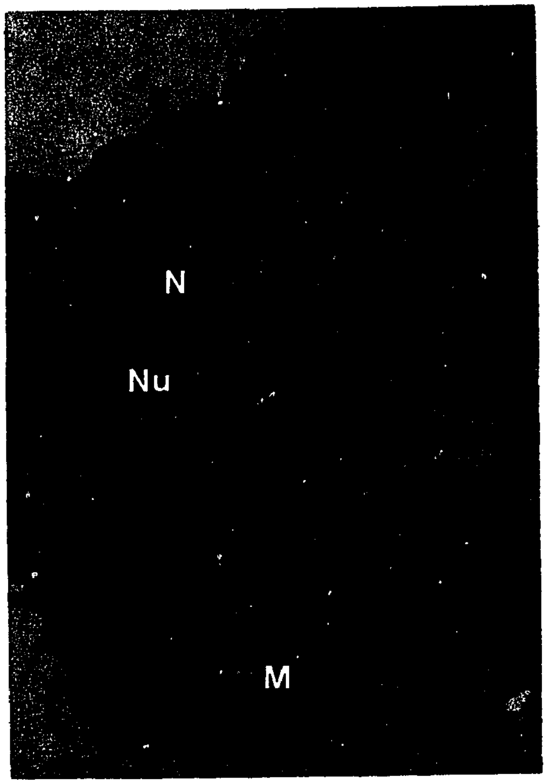
After 5 days of exposure to ethidium bromide, even

Figure 9. Thin section through a vegetative amoeba. Spherical to ovoid mitochondria (M) can be seen as well as a Nucleus (N) containing a single nucleolus (Nu). X 7500.

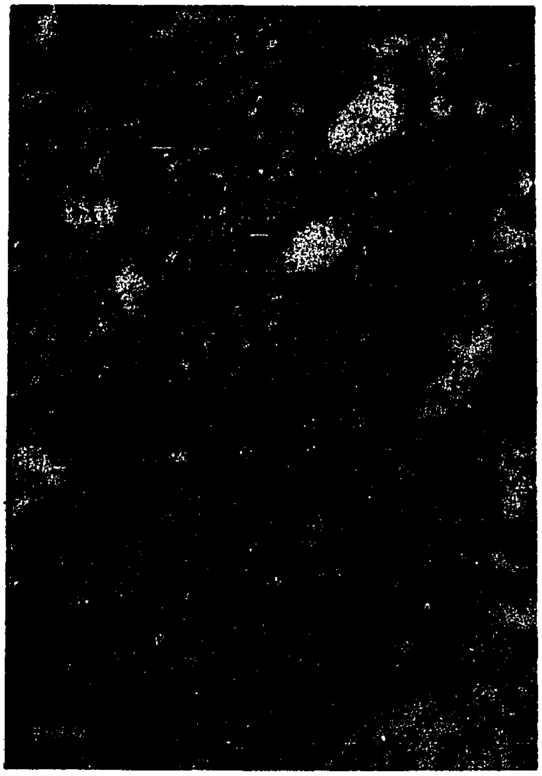
Figure 10. Higher magnification reveals the presence of rough endoplasmic reticulum (RER) adjacent to each mitochondrion. Mitochondria contain numerous cristae. X25,000.

Figure 11. Mitochondria from control amoebae have well-developed tubular cristae with random orientation. X50,000.

Figure 12. Control cell containing numerous mitochondria, a nucleus (N) containing 2 nucleoli and lysosomes. X25,000.


N
Nu
M


RER
10



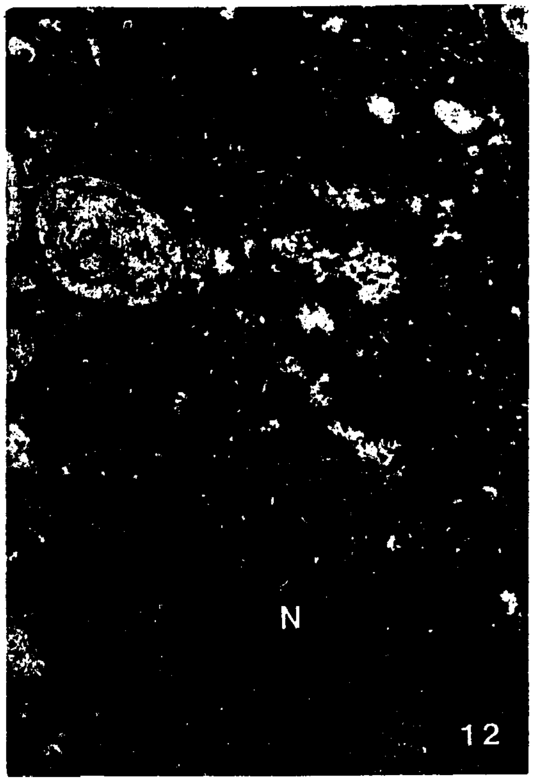

N
12

Figure 13. Thin section through a cell which had been treated with ethidium bromide for 2 days. Some mitochondria appear elongated and twisted (arrow). X 20,000.

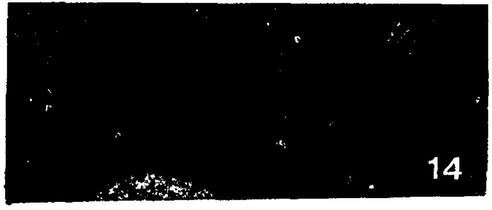
Figure 14. Elongated mitochondria after treatment with ethidium bromide contain fewer infoldings of the inner membrane X 35,000.

Figure 15. Cells treated with ethidium bromide for 2 days contain membranous structures. X 70,000.

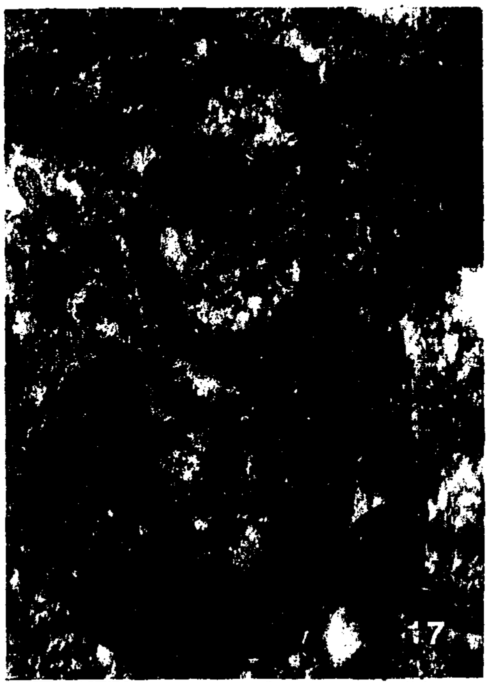
Figure 16. Rearrangement of mitochondrial membranes with an electron translucent area (et) appearing within the matrix after two days in ethidium bromide. X 70,000.

Figure 17. Mitochondria appear greatly twisted suggesting that some whorled membrane structures present in ethidium bromide-treated cells result from mitochondrial membranes which have become twisted upon themselves. X 70,000.


c
c
c
13


14


16


17

more striking morphological effects, are observed. In general, cells appear to lack the organized appearance of control cells and many whorled membrane figures are observed in all cells (Figures 18 and 22). Rough endoplasmic reticulum is no longer prominent, while structures still identifiable as mitochondria appear greatly deranged (Figure 19). Elongated mitochondria contain no apparent infoldings and cristae are totally absent from some mitochondria (Figures 20 and 21). Structures other than mitochondria are also evidently affected by drug treatment for this period of time (Figure 22).

Biochemical evidence described above has demonstrated that when the cells are washed free of ethidium bromide and allowed to grow in nutrient media for three days, the levels of mitochondrial enzymes have almost completely recovered from the effects of the drug. Electron micrographs of such cells show that most mitochondria are ultrastructurally indistinguishable from those of control cells (Figure 24); however, the general appearance of the cells is still morphologically abnormal (Figure 23). All cells at this stage of recovery have a large proportion, approximately 30-75% of their volume, compartmentalized in a single large vacuole within which is an amorphous material resembling that found in lysosomes (Figure 23). A single membrane within the vacuole encloses many abnormal membrane structures. Most mitochondria appear to be normal with well-organized cristae (Figure 24).

Figure 18. A cell after ethidium bromide treatment for 5 days containing whorled membranes (W) and twisted mitochondria (TM) X 10,500.

Figure 19. Altered mitochondria (M) lacking inner membrane infoldings after ethidium bromide treatment for 5 days. A whorl (W) appears to be forming within a mitochondrion. X 28,000.

Figure 20. Elongated and twisted mitochondria without noticeable cristae after 5 days of ethidium bromide treatment. X 28,000.

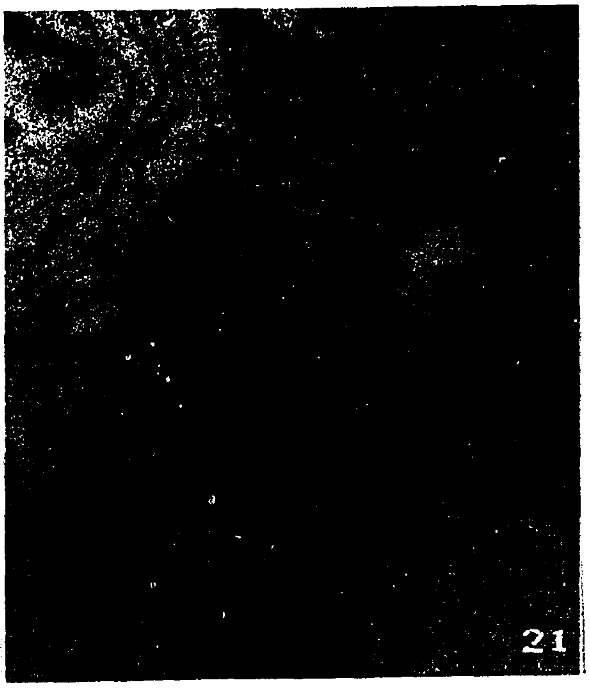
Figure 21. Mitochondrion showing rearrangement of the membranes after 5 days of ethidium bromide treatment. X 54,000.

Figure 22. Lysosomes, membranous whorls and mitochondria after five days of ethidium bromide treatment. X 36,000.

18

19

20



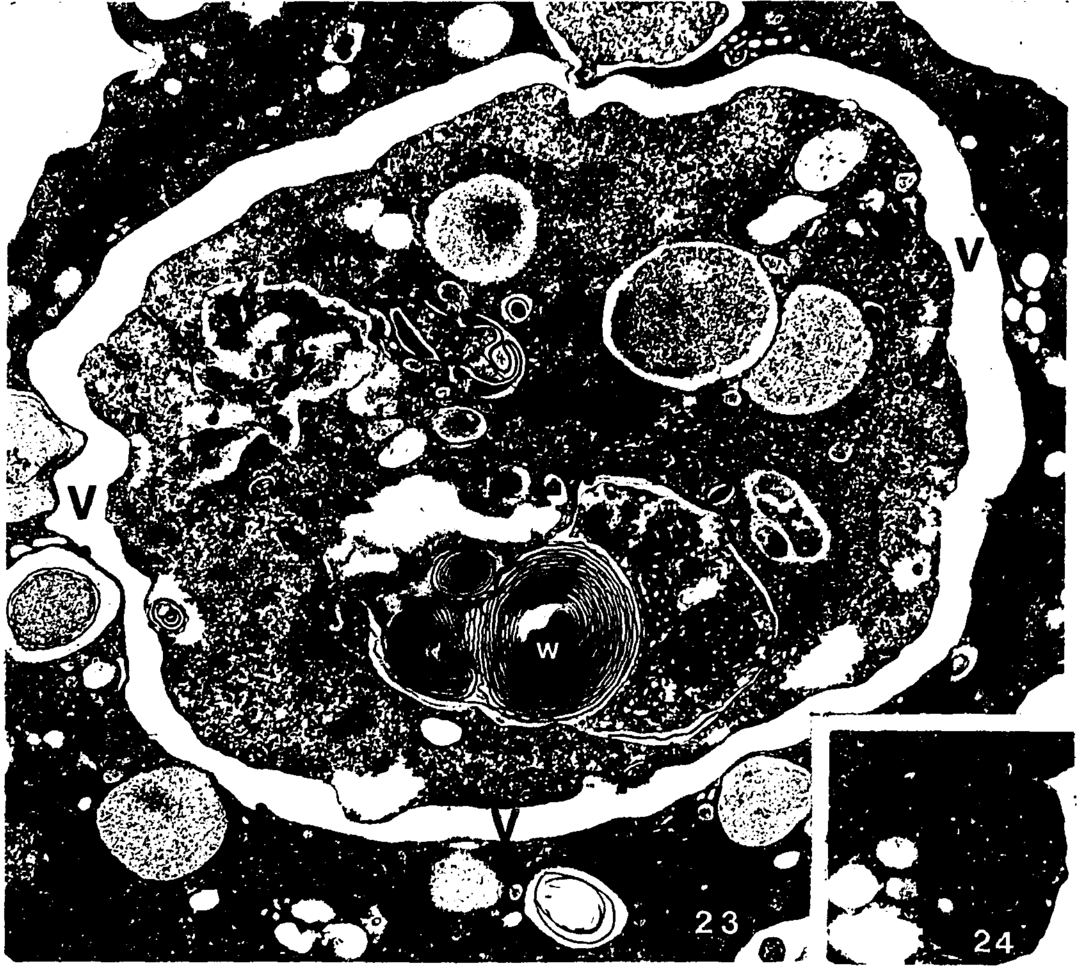
21

Figure 23. Intact cell 3 days after removal of ethidium bromide. Large vacuole (V) observed in all cells. Membranous whorls present (W). X 20,000.

Figure 24. Mitochondria showing properly oriented cristae. X 25,000.

Figure 25. Higher magnification of membranous whorl (w) o bserved in Figure 21. X 40,000

Figure 26. Rough endoplasmic reticulum (RER) in close juxtaposition to mitochondria containing numerous cristae. X 40,000


V
V
V
W
23
24


W
25


26

Whorled membrane figures are very obvious at this time and are almost always found within the large inner compartment (Figure 25). Rough endoplasmic reticulum is again present in abundance in close juxtaposition to mitochondria (Figure 26).

DISCUSSION

The present report describes the effects of ethidium bromide treatment on mitochondria of the cellular slime mold, *D. discoideum.* Previously it was reported that when cultures of vegetative amoebae were grown in the presence of ethidium bromide for 5 days, the activities of several segments of the respiratory chain and oligomycin-sensitive ATPase were decreased significantly suggesting that the mRNA for some proteins of these enzyme complexes was transcribed from mitochondrial DNA. In this study, it was determined that the amount of DNA present in sucrose gradient-purified mitochondria from cells after 5 days in ethidium bromide was 50% that present in mitochondria from control cells. These results indicate that the drug blocks the replication of mitochondrial DNA in agreement with previous studies (9, 10, 14, 20, 22). Furthermore, these results suggest that new synthesis of mitochondrial DNA must stop almost immediately, despite the continued synthesis of cellular proteins including mitochondria. As a result, each new mitochondrion formed under these conditions has a lowered amount of DNA.

In a previous study (33), it was reported that mitochondria purified from cells treated with ethidium bromide for 5 days had reduced levels of cytochromes *a*-a_3 and *b* and increased levels of cytochromes *c*-c_1 as determined by difference spectroscopy at room temperature. In the present report, I have been able to resolve the *c*-type cytochromes with absorption maxima at 549 and 554 nm by low temperature difference spectroscopy using either dithionite or ascorbate-tetramethylphenylenediamene reduction (Figure 3 A, 4A). In addition, two *b*-type cytochromes with absorption maxima at 556 and 561 nm can be identified (Figure 3 , trace A). The presence of two forms of cytochrome *b* is even more apparent when the dithionite-reduced mitochondria were analyzed using ascorbate-tetramethylphenylenediamine reduced mitochondria in the reference cuvette (Figure 4 , trace B). Similar resolution of *c* and *b* type cytochromes by low temperature spectroscopy has been achieved in mitochondria from many cell types (2-5, 17, 26, 34). Of great significance was the observation that the amount of cytochrome $\underline{c}_1$ was decreased approximately 80% in mitochondria purified from cultures treated with ethidium bromide for 5 days while the amount of cytochrome *c* increased nearly two fold (Figure 5 , trace A). Previously, Ross *et al.* (25) had reported that the apoprotein of cytochrome $\underline{c}_1$ is synthesized on extramitochondrial ribosomes; however, formation of the holo-cytochrome $\underline{c}_1$ in growing yeast cells requires mitochondrial protein synthesis. The data obtained with slime molds treated with ethidium bromide for 5 days further suggests that the elaboration of complete cytochrome $\underline{c}_1$ requires mitochondrial transcription.

It is also of some interest that of the two *b*-type cytochromes present in slime mold mitochondria, only that with an absorption maximum at 561 nm is apparently reduced by treatment with ethidium bromide (Figure 3 , trace B; Figure 5 , trace B). Little change in the amount of the cytochrome *b* with the absorption maximum at 556 nm is observed suggesting that synthesis of only one of the two *b* cytochromes depends on mitochondrial transcription. Previous studies had indicated that the biosynthesis of the b-c_1 region of the respiratory chain requires mitochondrial protein synthesis (16). Specifically, Weiss has shown that cytochrome *b* is synthesized on mitochondrial ribosomes in *Neurospora crassa* (36). Although the latter study did not indicate which of the two cytochrome *b*'s known to be present in *Neurospora* mitochondria (34) had been isolated, it is of some interest that Von Jagow and Klingenberg (34) showed that chloramphenicol had a more profound inhibitory effect on the biosynthesis of cytochrome b_T (absorbing at 562 nm) than on cytochrome b_K (absorbing at 556 nm).

All of the effects of ethidium bromide described above are completely reversible. When the amoebae were washed free of the drug and resuspended in fresh medium, cell division commenced immediately without any apparent lag. Despite the immediate resumption of a normal growth rate, there was a pronounced lag in the reappearance in the mitochondrial membrane of various respiratory chain components. For example, after removal of ethidium bromide, the content of cytochromes $\underline{a}$-$\underline{a}_3$ began to increase immediately at a slow rate while that of cytochrome $\underline{b}$ did not increase for two days. After this time, the amount of both cytochromes increased rapidly reaching control levels after 5 days (Figure 7). By contrast, the elevated levels of cytochrome $\underline{c}$ returned rapidly to control levels within 3 days with only a short one-day lag period. Similar kinetics of recovery were observed when the activities of either cytochrome oxidase or succinate-cytochrome $\underline{c}$ reductase were examined. Furthermore, the activity of oligomycin-sensitive ATPase returned to the control level by 5 days after a short lag.

Figure 8 also shows the decline in the specific activity of succinate dehydrogenase in whole cells after the removal of ethidium bromide. In previous studies (33), it was shown that the activities of succinate and NADH dehydrogenases were identical in purified mitochondria from control and ethidium bromide-treated cells despite the presence of twice the activity of these enzymes in whole cells treated with ethidium bromide for 5 days. These results had suggested that continued formation of new mitochondrial membranes with identical amounts of succinate and NADH dehydrogenases must occur after cell division has ceased due to the effects of ethidium bromide. Hence, the amount of mitochondria or mitochondrial protein per cell must have increased nearly 50%. After removal of ethidium bromide, the specific activity of succinate dehydrogenase in whole cells rapidly declines reaching control levels within 3 days. The kinetics of recovery of this enzyme and the other respiratory chain enzymes during recovery from ethidium bromide suggests that the mitochondrion is assembled in an asynchronous manner as previous studies with yeast in our laboratory had also suggested (11,16).

After treatment with ethidium bromide, gross alterations in cellular morphology were observed. The earliest observations, 2 days after addition of the drug, indicated that mitochondrial profiles had become elongated and twisted lacking a well-organized cristal membrane system (Figures 13 and 14) in contrast to the spherical-shaped mitochondria of control cells. These changes became progressively more severe such that after 5 days, the mitochondrial cristae were almost completely absent, although the outer membrane remained intact. This observation correlates well with the biochemical data indicating large decreases in the activity of inner membrane components of the respiratory chain. Similar effects of ethidium bromide on mitochondrial morphology have been described in HeLa cells (12,13,15) chinese hamster fibroblasts (19) and mouse L cells (12,30). Likewise, treatment with chloramphenicol, a specific inhibitor of mitochondrial protein synthesis (1) also results in a damaged appearance of mitochondria (8,12,31) suggesting that the presence of proteins synthesized on mitochondrial ribosomes is required for the maintenance of mitochondrial structure.

Exposure of slime mold amoebae to ethidium bromide also results in the appearance of many whorled membrane bodies in the cells (Figures 23 and 25). The presence of these myelin-like figures has been reported in other cell types following treatment with ethidium bromide or chloramphenicol (12,13,15,19,30,31). The suggestion that these whorled membranes arise from detached cristal membranes is supported by electron micrographs of cells treated with ethidium bromide for 2 days (Figures 15 and 17). Mitochondrial membranes appear to be in the process of becoming twisted upon themselves.

Ethidium bromide treatment also appears to affect other cellular structures as well as the mitochondria. Control cells contain well-developed rough endoplasmic reticulum some of which can be observed in close apposition to the mitochondria (Figure 10). After exposure to the drug for 5 days, the amount of rough endoplasmic reticulum is reduced to insignificant levels and none is seen adjacent to any of the mitochondrial profiles.

Three days after removal of ethidium bromide, the cells appear to have recovered morphologically. Most mitochondria once again appear spheroid with well-organized cristal membranes (Figure 24) and are close to rough endoplasmic reticulum. At this stage of recovery all cells appear to have compartmentalized damaged membranes and organelles within a single large vacuole, sometimes occupying 75% of the total cell volume. This structure appears to consist of a vacuole enclosed by a single membrane and surrounded by an amorphous material which in turn is enclosed in a unit membrane. The material between the two unit membranes appears similar to that found in lysosomes. Preliminary observations with the Gomori stain procedure indicate the presence of acid phosphatase activity within the vacuole. These recovery vacuoles vary in size but are quite distinct from structures found in control cells, and should not be confused with the osmoregulating vacuole present in vegetative amoebae. The compartmentalization of damaged membranes in these vacuoles suggests that this may be a more efficient way of removing or degrading them and I have not ruled out the possiblity that this vacuole fuses with the cell plasma membrane "secreting" damaged cell material. Alternately this vacuole may simply represent a phagocytized cell as these amoebae are known to cannibalize other cells when starved.

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CHAPTER 3

THE EFFECTS OF ETHIDIUM BROMIDE ON THE DEVELOPMENT OF THE SLIME MOLD *DICTYOSTELIUM DISCOIDEUM*

ABSTRACT

Amoebae developing in the presence of 10 μg/ml of ethidium bromide form abnormally small aggregates in large numbers. The kinetics of the accumulation and disappearance of five developmentally regulated enzymes are characterized. Ethidium bromide appears to have little effect on the kinetics of the appearance of three enzymes, acetylglucosaminidase, α-mannosidase and alanine transaminase which normally increase in activity from the onset of starvation. The kinetics of accumulation and disappearance of tyrosine transaminase and alkaline phosphatase, which start to accumulate only after the completion of aggregation, are identical for amoebae developing in the presence or absence of ethidium bromide.

INTRODUCTION

Following the removal of a food source, the vegetative amoebae of *Dictyostelium discoideum* begin a series of developmental stages which include aggregation, slug formation, culmination, and ultimately construction of a fruiting body, or sorocarp. During this developmental sequence, many physical and biochemical changes occur i.e. a 50% decrease in dry weight (1), a decrease in the rate of oxygen consumption (2) and a decrease in RNA content (3). The protein content has also been shown to decrease in dry weight (4). Despite this dramatic decrease in protein content, protein synthesis continues throughout development. A major portion of this synthesis represents enzymes which accumulate during the various stages. Certain enzymes are present only in vegetative cells i.e. trehalase (5) while others are present during both feeding and the developmental stages i.e. adenyl cyclase (6) or pyrophosphatase (7). Approximately thirteen enzymes, however, accumulate during discrete periods of development. These enzymes represent single gene products and all require a period of RNA synthesis followed by cycloheximide sensitive protein synthesis for the accumulation to occur (4). The pattern of accumulation appears to be regulated by the overall developmental pattern (8,9).

Ethidium bromide treatment of *Dictyostelium discoideum* vegetative amoebae has been found to cause significant inhibitory effects on the mitochondrial respiratory chain (10). Cytochromes $a-a_3$ and b are decreased over 80% and 35% respectively and cytochrome c oxidase and oligomycin-sensitive ATPase, the enzyme complex which is responsible for respiratory driven ATP synthesis are both decreased 80%.

This report examines the effects of ethidium bromide on the kinetics of accumulation of five developmentally regulated enzymes.

MATERIALS AND METHODS

Cultures of *Dictyostelium discoideum*, strain A-3 were grown at 22°C in axenic medium (11) containing 0.2 mg/ml streptomycin sulfate on a New Brunswick gyratory shaker at 200 rpm. When the amoebae had grown to a density of $0.5-2.0 \times 10^6$ mg/ml, ethidium bromide was added to a final concentration of 10 μg/ml. Cells used for controls were grown to a density of $1-3 \times 10^6$ cells/ml. For developmental studies, untreated or ethidium bromide treated cells were washed free of nutrient medium by centrifugation at 600 X g and repeated washings with a pad diluting fluid (PDF) consisting of 1.5 g KCl, 1.07 g $MgCl_2 \cdot 6H_2O$, 1.6 g K_2HPO_4, 1.8 g KH_2PO_4 and 0.5 g streptomycin sulfate per liter (pH 6.4). These cells were then resuspended in PDF and approximately 7.5×10^7 cells were placed onto blue millipore filters saturated with either PDF alone or PDF containing ethidium bromide (10 μg/ml). Developmental studies using amoebae which had been grown in axenic medium with or without ethidium bromide gave identical results . As a result most experiments were performed using untreated vegetative amoebae. All petri dishes were wrapped in aluminum foil to prevent breakdown of light sensitive ethidium bromide and more importantly, to prevent the induction of prolonged slug migration by light (12). During the developmental process, the temperature was maintained at 22 ± 0.5°C. Every two hours, cells were washed off of millipore filters with 2 ml PDF and frozen at -10°C until ready for analysis.

Enzyme Assays.

Samples were thawed out and solubilized with 0.4% Triton X-100. The following enzymes were assayed using the methods of Loomis: alkaline phosphatase (13), acetylglucosaminidase (14), and α mannosidase (15). Tyrosine transaminase was assayed according to the method of Pong and Loomis (16) and alanine transaminase according to the method of Firtel and Brackenbury (17). Protein was estimated by the method of Lowry et. al. (18) using boving serum albumin as a standard.

Materials

p-nitrophenyl phosphate, p-nitrophenyl N acetylglucosamine, p-nitrophenyl α mannoside, p-hydroxybenzaldehyde, L-alanine, L-tyrosine, pyridoxal phosphate, α ketoglutarate, lactic acid dehydrogenase (type III), NADH, and bovine serum albumin were purchased from Sigma, St. Louis, Mo. Streptomycin sulfate, grade B, was purchased from Calbiochem, San Diego, Calif.

RESULTS

Every two hours following the initiation of development, cells were washed off of their filter pads, solubilized with 0.4% Triton X-100 and their total protein determined. The kinetics of the decrease in total protein which occurs with development is shown in Fig. 27 . Twenty hours after starvation has begun the protein concentration has been reduced by more than 50% presumably due to protein degradation and subsequent oxidative metabolism of the amino acids. The kinetics are identical for cells developing in the presence or absence of ethidium bromide. For this protein determination as well as for all enzymes assayed, no difference is found between cells which (prior to starvation) had been grown in the presence of 10 μg/ml of ethidium bromide for five days or cells which had not been so treated.

Amoebae that start their developmental sequence in the presence of ethidium bromide are unable to form aggregates as large as those developing on filters saturated with PDF alone. Although smaller aggregates are formed, they are more numerous. These aggregates continue their developmental sequence in a normal manner giving rise to mature sorocarps within 26-30 hrs. Three enzymes which normally increase in activity immediately after starvation begins were assayed to determine if the accumulation of any of these developmentally regulated enzymes is also effected by ethidium bromide. At the

Figure 27. The loss of total protein as development proceeds. Millipore pads are washed completely free of cells with 2 ml of PDF solution and the protein concentration is determined by the method of Lowry. Ordinate: protein (mg/ml). Abscissa: time in hours after initiation of starva[illegible]

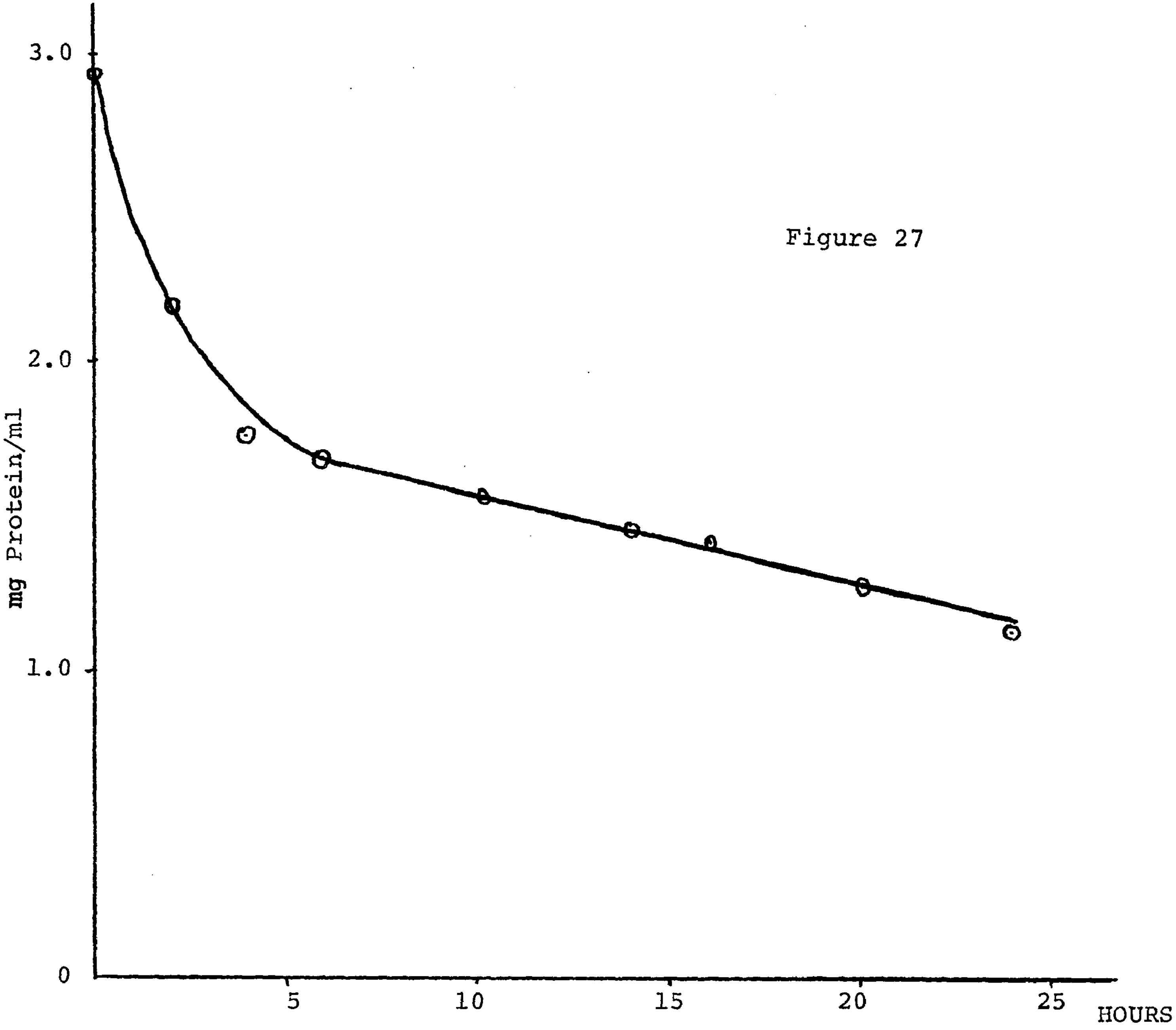


Figure 27

onset of starvation, N-acetylglucosaminidase activity accumulates with identical kinetics in the presence or absence of ethidium bromide (Fig. 28). No significance is attributed to the lower specific activity found for the ethidium bromide treated amoebae as there is a high degree of variability in the determination of absolute specific activities for this enzyme assay.

The kinetics of accumulation of α-mannosidase (Fig. 29) and alanine transaminase (Fig. 30) for cells developing the the presence or absence of ethidium bromide appears to be similar. Like N-acetylglucosaminidase, these enzymes normally start to accumulate immediately after starvation begins reaching peak activities at 22 and 16 hrs of development respectively. Cells developing in the presence of ethidium bromide appear to accumulate these enzymes at approximately the same rate with maximum accumulation occurring at the same time. Unlike α- mannosidase however, after the maximum accumulation of alanine transaminase, there is a much more rapid disappearance of the enzyme in cells treated with ethidium bromide compared to untreated cells.

The accumulation of tyrosine transaminase and alkaline phosphatase activities throughout development were also characterized. These enzymes normally begin to accumulate only after the aggregation phase is completed. For both enzymes, no significant difference was found between ethidium bromide treated and control amoebae in the

Figure 28. Change in the specific activity of N-acetylglucosaminidase. EB: cells developing in the presence of PDF containing 10 μg/ml ethidium bromide. Control: cells developing in PDF alone.

Figure 28

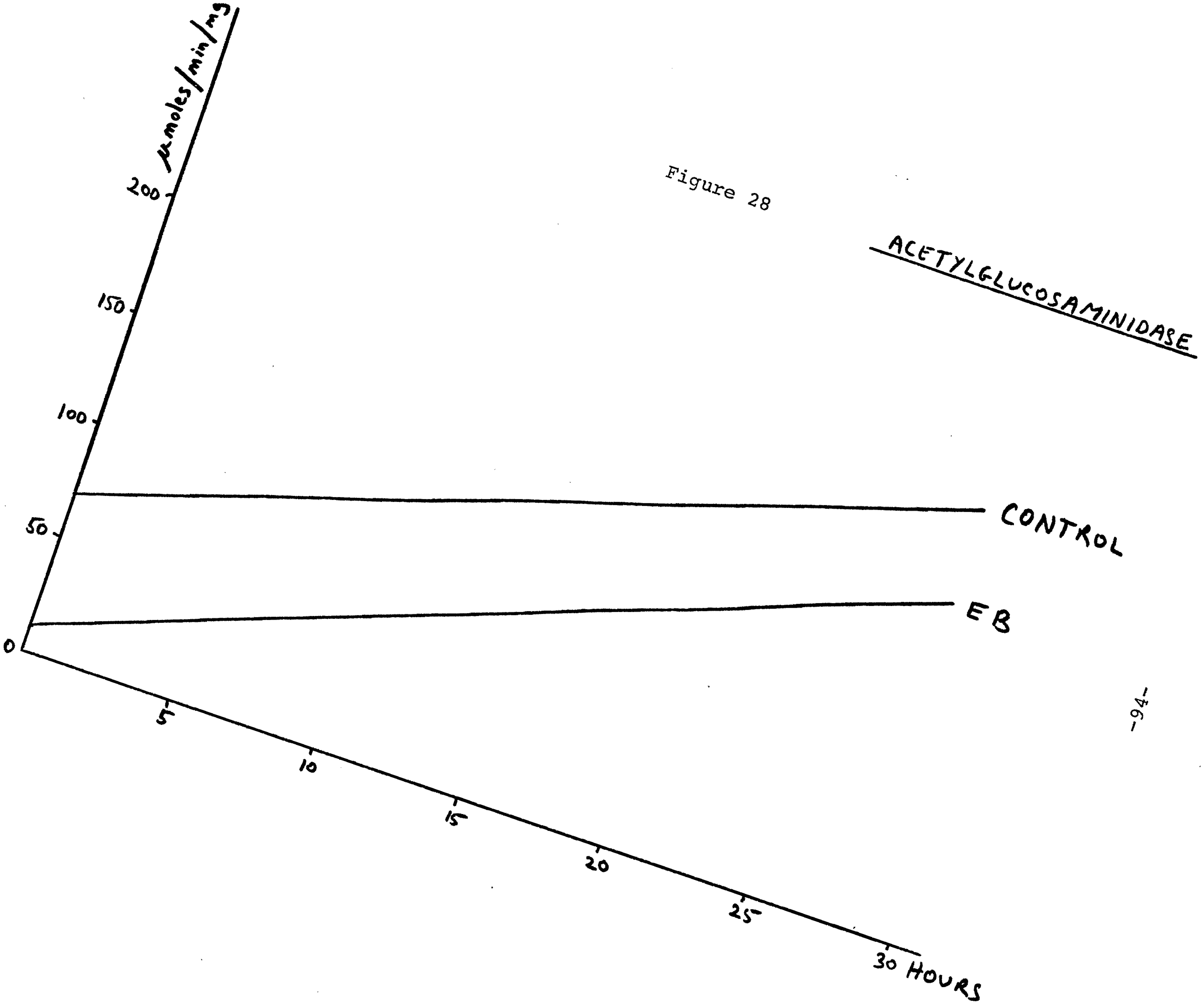

ACETYLGLUCOSAMINIDASE
μmoles/min/mg
0
50
100
150
200
5
10
15
20
25
30 HOURS
CONTROL
EB

Figure 29. Kinetics of accumulation of α mannosidase during development. EB: cells developing in the presence of PDF containing 10 μg/ml ethidium bromide. Control: cells developing in PDF alone.

Figure 29 α-MANNOSIDASE

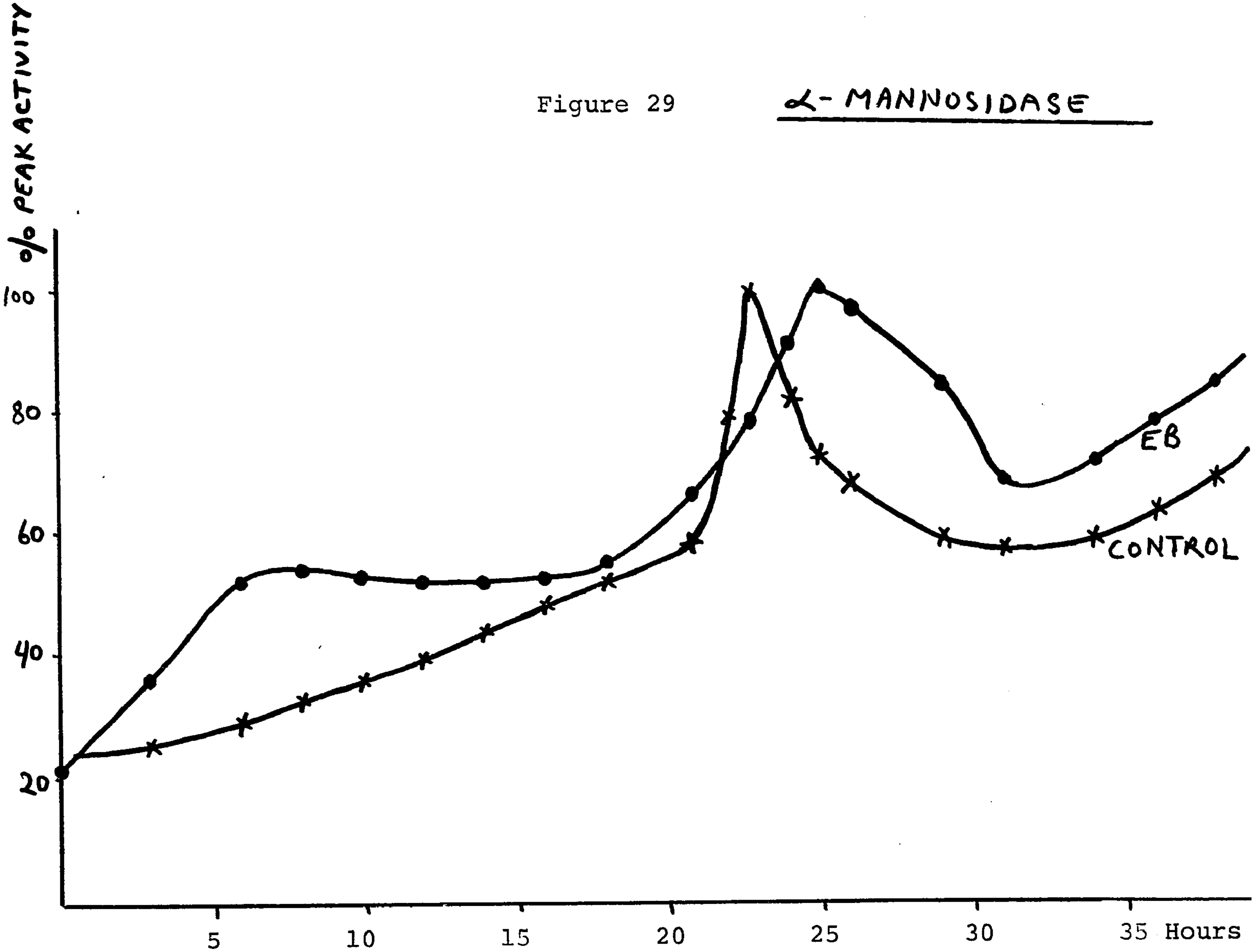
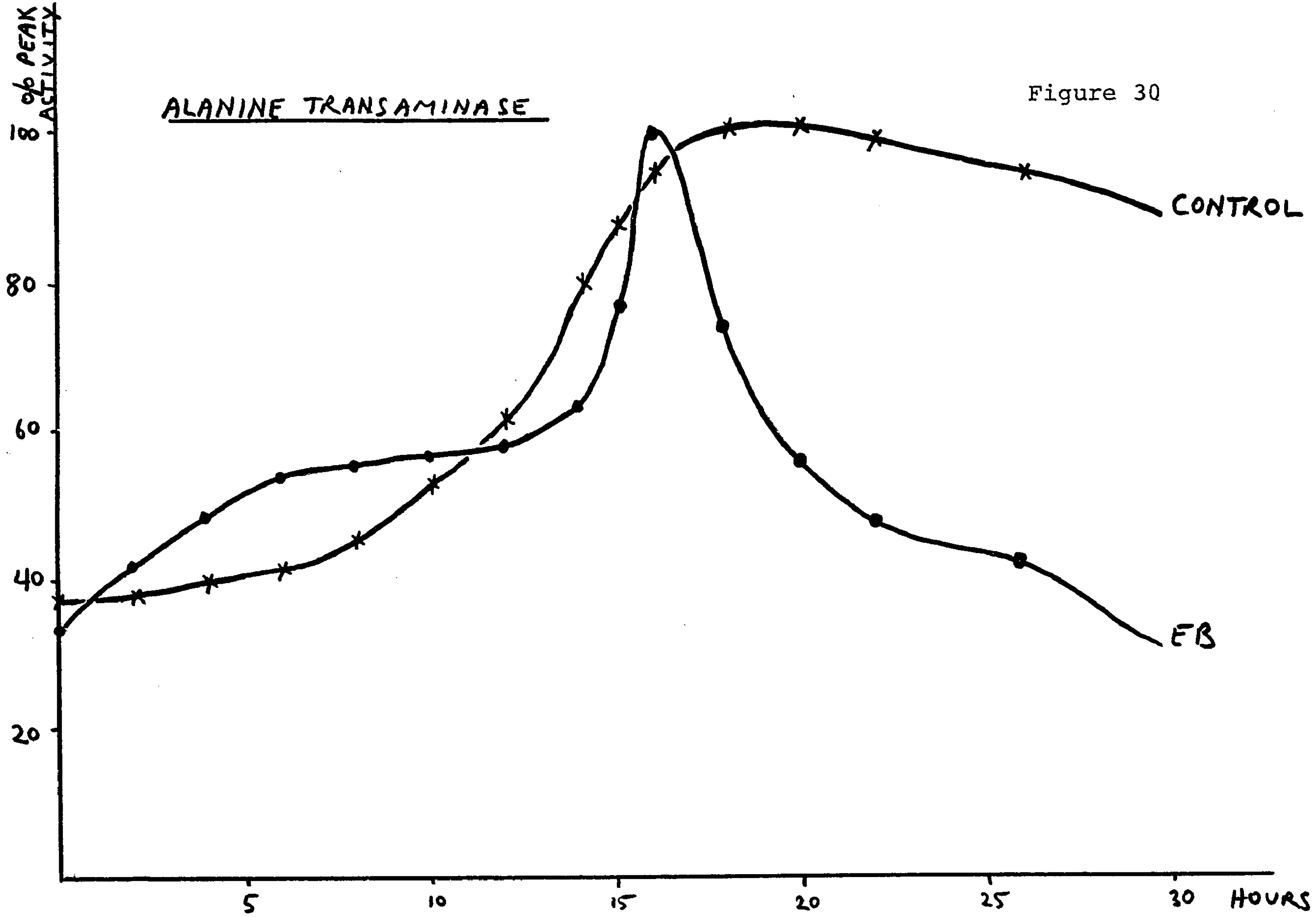


Figure 30. Kinetics of accumulation of alanine transaminase during development. EB: cells developing in the presence of PDF solution containing 10 μg/ml ethidium bromide. Control: cells developing in the presence of PDF solution alone.

Figure 30


ALANINE TRANSAMINASE
% PEAK ACTIVITY
100
80
60
40
20
5
10
15
20
25
30
HOURS
CONTROL
EB

kinetics of the accumulation or disappearance of enzyme activity (Figures 31 and 32).

Figure 31. Kinetics of accumulation of tyrosine transaminase during development. EB: cells developing in the presence of PDF solution containing 10 μg/ml ethidium bromide Control: cells developing in PDF solution alone.

Figure 31 TYROSINE TRANSAMINASE

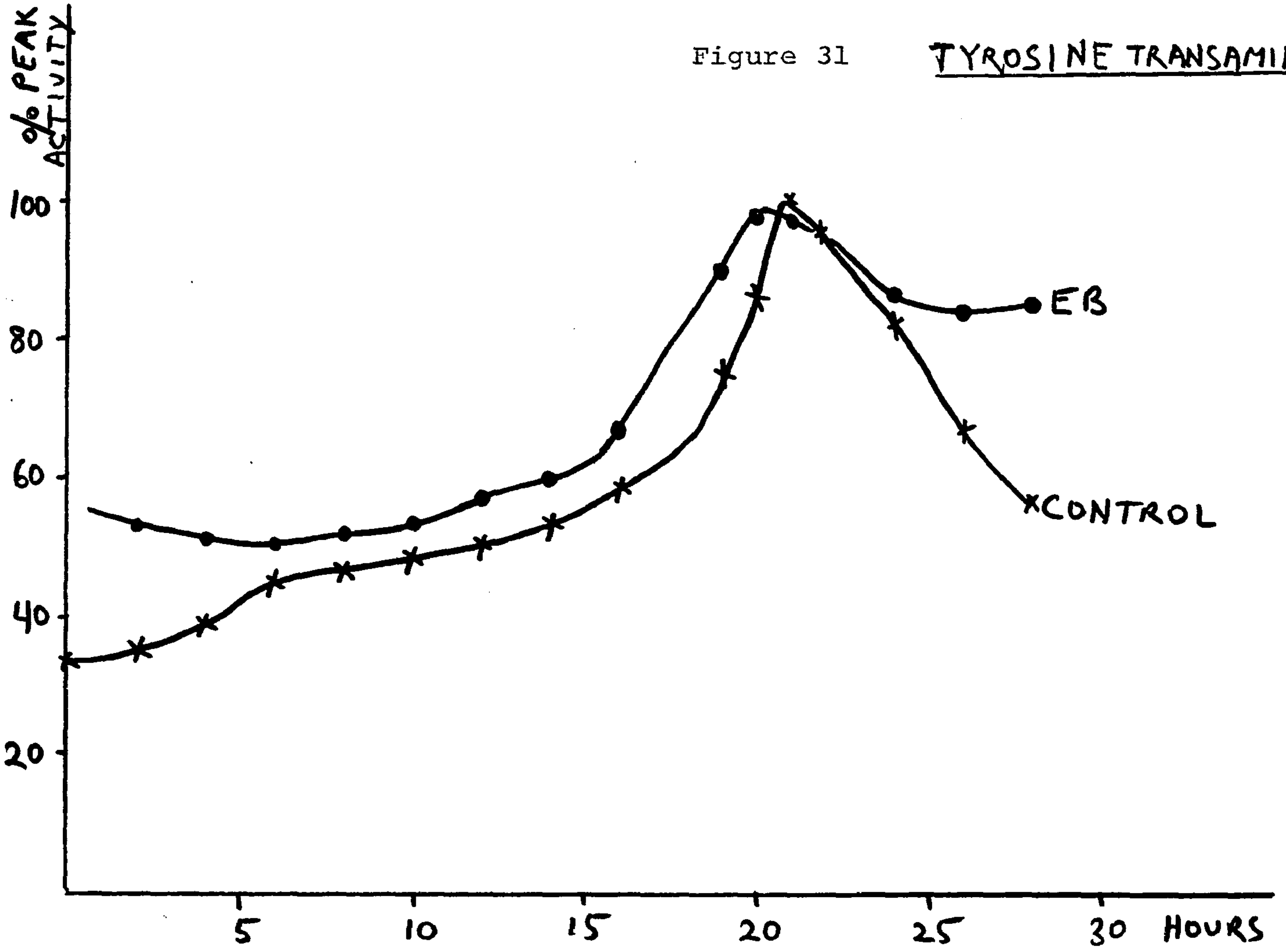
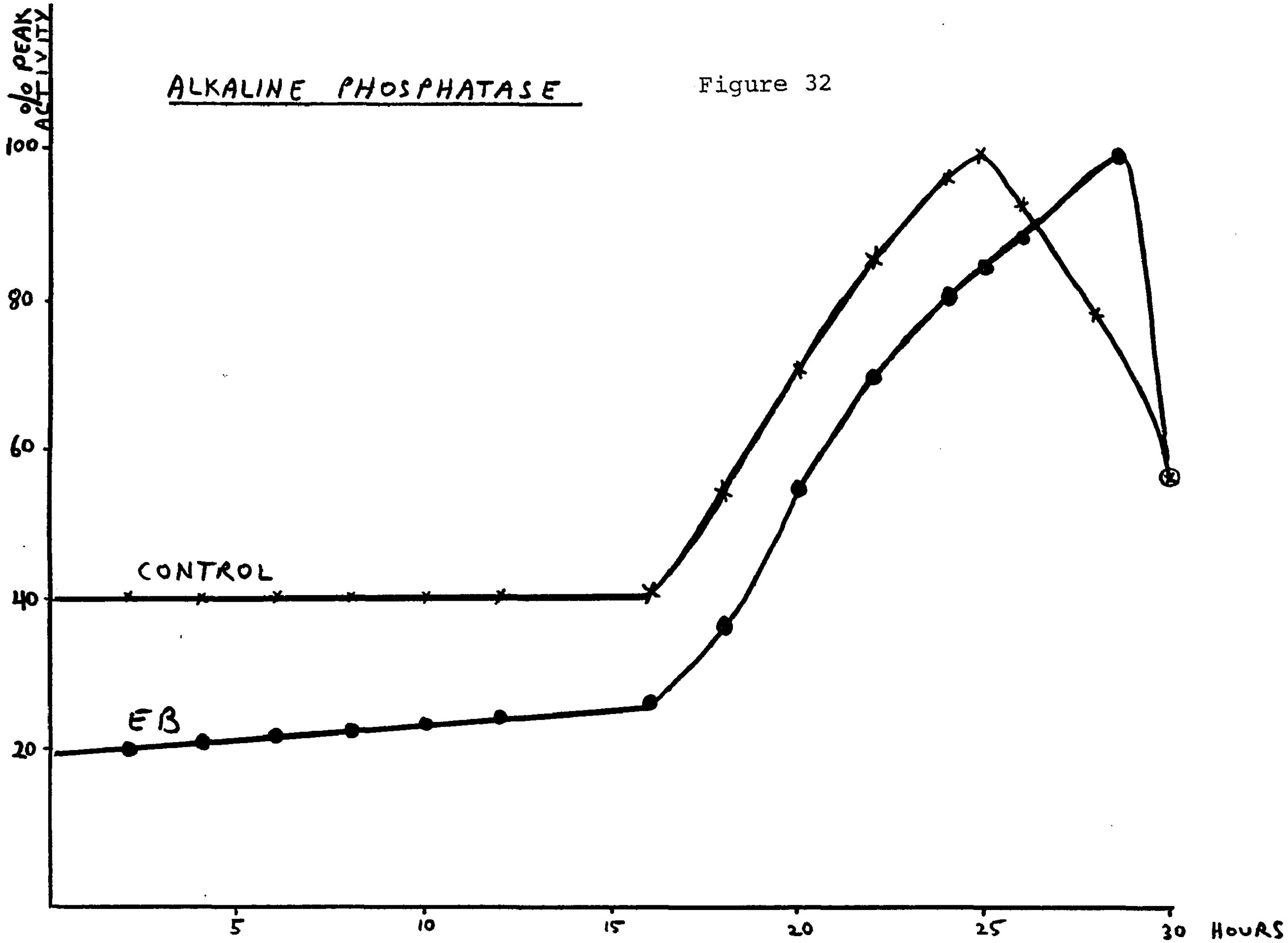


Figure 32. Kinetics of accumulation of alkaline phosphatase during development. EB: cells developing in the presence of PDF solution containing 10 μg/ml ethidium bromide. Control: cells developing in PDF solution alone.

Figure 32


ALKALINE PHOSPHATASE
% PEAK ACTIVITY
100
80
60
40
20
CONTROL
EB
5
10
15
20
25
30
HOURS

DISCUSSION

The cellular slime molds are excellent organisms with which to examine the biochemical events which occur during the transition from a unicellular to a multicellular organism. Amoebae washed free of nutrients and placed on filter supports develop in a synchronous manner. Thus biochemical analyses can be made during various stages of the developmental sequence. It appears that the major source of energy during development is derived from amino acid catabolism rather than carbohydrate utilization since the level of total carbohydrate remains constant throughout development (4). It is therefore not surprising to find decreased levels of protein in developing amoebae. A decline in the total protein content of the developing slime mold has been reported for the NC-4 strain (3). The results of this study confirm and extend this observation to the axenic A-3 strain as well.

Little is known about the regulatory mechanisms which control the size of the aggregates that form approximately 8-10 hrs post starvation. Although aggregation can take place in the presence of ethidium bromide, the number of cells entering the aggregate is restricted. This indicates that these amoebae are capable of secreting and responding to the chemotactic agent, cyclic 3',5'-adenosine monophosphate. It is not known how ethidium bromide causes this effect. Perhaps amoebae treated with ethidium bromide become deficient in ATP and cannot synthesize or secrete large quantities of cAMP. This would result in a steeper gradient of cAMP, thereby limiting the size of the aggregate. Alternately, fewer cells may be able to respond to the cAMP gradient in which case the aggregate would again be smaller than an aggregate forming from untreated amoebae.

The results of studies on five developmentally regulated enzymes suggest that ethidium bromide treatment has little effect on the accumulation of these enzymes. Although the kinetic studies of α-mannosidase and alanine transaminase accumulation suggest that ethidium bromide treatment may cause a premature appearance of these two "early" enzymes, the variability in the activities of these enzymes when assayed from experiment to experiment precludes any statement concerning the significance of their early accumulation. Ethidium bromide is known to interact with closed circular DNA (20) and as a result is a specific inhibitor of the replication and transcription of mitochondrial DNA (21, 22). Treatment of slime mold vegetative amoebae with ethidium bromide causes cessation of cell division and inhibition of the respiratory chain (10). In addition, the activity of oligomycin sensitive ATPase, the enzyme complex responsible for respiratory driven ATP synthesis, decreases by 80% as a result of treatment with ethidium bromide. It is therefore not surprising that treatment of slime mold amoebae with this drug results in some developmental alterations. Despite this, mature sorocarps can still be formed suggesting that normal development can still take place.

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CHAPTER 4

THE INDUCTION OF FILOPODIA BY CYCLIC 3',5'-ADENOSINE MONOPHOSPHATE IN THE VEGETATIVE AMOEBAE OF THE CELLULAR SLIME MOLD *DICTYOSTELIUM DISCOIDEUM*

ABSTRACT

Dictyostelium discoideum vegetative amoebae growing axenically in the early logarithmic phase can be induced to extend microprojections, filopodia, from their cell surfaces in response to cyclic 3',5'-adenosine monophosphate. Cyclic 3',5'-guanosine monophosphate, adenosine monophosphate or adenosine diphosphate at concentrations of 1.0 mM have no effect. Treatment with 0.1 mM 2-4 dinitrophenol or 1.0 mM sodium azide does not prevent the induction by cyclic adenosine monophosphate. The induced cells can be more extensively agglutinated with Concanavalin A at 0.5 mg/ml than non-induced cells. Fluorescent labeling studies indicate that cell surface receptor sites for this lectin are capable of moving within the plane of the membrane.

Scanning electron microscopic studies confirm the presence of numerous filopodia on the surface of cells from cultures at the mid-logarithmic phase of growth as well as in the pre-aggregative stage of development. Furthermore, treatment of the cells with colchicine or griseofulvin or cooling to 3°C did not prevent the induction of filopodia suggesting that microtubules are not involved. Addition of cytochalasin B or procaine caused the immediate and reversible collapse of filopodia suggesting that the microfilaments within the core of the filopodia may play a role in the formation and maintenance of these structures. A model is presented which describes a possible mechanism

whereby cells may aggregate via the cyclic adenosine monophosphate induced filopodia.

INTRODUCTION

The life cycle of the eukaryotic cellular slime mold, Dictyostelium discoideum is better understood than that of any other member of the Acrasiales. D. discoideum is an excellent organism for studying intercellular interactions. During the vegetative phase of its life cycle this organism exists as a free-living phagocytic amoeba. While nutrients are available, the amoebae grow in size and undergo binary fission to form daughter cells morphologically identical to the parent cell. When vegetative amoebae are washed free of nutrients and placed on a solid substrate, they enter a developmental sequence in which up to 10^5 cells aggregate into masses called pseudoplasmodia. This mass of cells forms a slug-like multicellular creature which may or may not migrate depending on environmental conditions (1-5). When migration ceases, the slug enters a culmination phase wherein a series of morphogenetic movements results in the formation of a fruiting body or sorocarp. Thus the life cycle is divided into two distinct phases.

The biological changes which occur in logarithmically growing cells in the ameboid phase of the life cycle when nutrients are no longer available have been extensively studied. These non-dividing amoebae are attracted to a focal cell or group of cells by a chemotactic agent, initially termed Acrasin (6,7), which contains cyclic 3',5'-adenosine monophosphate (cAMP) as the active agent (8-12). The receptor for the chemotactic response appears to be localized on the cell surface which has been shown to be the site of cAMP phosphodiesterase as well as a cAMP binding protein (13). In addition to its chemotactic role, the cell surface undergoes antigenic and biochemical modifications in the preaggregative stage (11, 12, 14-19) at which time the cells become mutually adhesive (20). Scanning electron microscope analysis has indicated that the membrane undergoes a series of morphological changes which are associated with the transition from growth to development (21). In this report, evidence is presented that filopodia which are normally found only on mid-log or preaggregative phase cells can be induced on early log phase cells which normally lack them, by the chemotactic agent cAMP. These results suggest that the initial contacts between individual cells undergoing aggregation may occur as a direct result of these filopodia. The role of microtubules and microfilaments in the maintenance of filopodial structure has been investigated.

MATERIALS AND METHODS

D. discoideum (strain A-3 from William F. Loomis, University of California, San Diego) was grown axenically (22) in a medium containing 0.2 mg/ml of streptomycin sulfate as described previously (23). Early logarithmic cells were taken from a culture with a cell density less than 10^6 cells/ml whereas mid-log cells were from a culture with a cell density of 1-4 X 10^6 cells/ml . To obtain preaggregative cells, mid-log cells were washed free of nutrient media and then 10^8 cells were placed on black Millipore filters supported on pads saturated with a buffer containing KCl (1.5 g/l), $MgCl_2 \cdot 6H_2O$ (1.07 g/l), potassium phosphate buffer (1.0 mM), pH 6.4, and streptomycin sulfate (0.5g/l). Cells were allowed to differentiate for 5.5 h at 22°C before use. All experiments unless specified otherwise were performed at this temperature.

Cells for dark field microscopy were taken directly from axenic medium without washing and examined immediately under a Zeiss microscope fitted with a dark field condenser.

Cells for electron microscopy were prefixed by adding a 6.5% gluteraldehyde fixative to the culture media in a 1:10 ratio. Cells were harvested by centrifugation and fixed in 6.5% gluteraldehyde buffered with veronal acetate, pH 7.3, at room temperature. Postfixation was in veronal acetate buffered OsO_4 (Palade's fluid). The cells were then washed and stained with 1% uranyl acetate and dehydrated by gradual transition through alcohols of increasing concentration. The cells were infiltrated and embedded in Maraglas. Silver sections were examined under a Zeiss EM 9S-2 Electron Microscope.

Cells for scanning electron microscopy were prefixed by the addition of pre-cooled (4°C) charcoal filtered gluteraldehyde, pH 6.4, to the axenic media giving a final concentration of 2.5%. After 2 h at 4°C cells were centrifuged and suspended in gluteraldehyde made 2.5% with phosphate buffered saline (PBS) consisting of 1.5 g KCl, 0.5 g $MgCl_2 \cdot 6H_2O$, and 0.5 g streptomycin sulfate in one liter of 0.04 M phosphate buffer, pH 6.4. Cells were again centrifuged, washed with PBS and transferred to 2% OsO_4, pH 6.4 at 4°C for 1 h with occasional stirring. Cells were then washed twice in PBS and dehydrated through a graded alcohol series from 50-100% ethanol. Cells were then transferred to coverslips, air dried and inspected by dark field microscopy. Previous studies with D. discoideum had indicated that air drying produced results identical to those obtained by critical point drying (21). The coverslips were mounted on stubs and coated with gold and palladium (60:40) in a Denton Vacuum evaporator supplied with a rotary coating unit. The specimen mount was tilted at 45° during the analysis performed on a JEOL JSM-35 scanning electron microscope operated at 20-25 KV. Photomicrographs were made with Polaroid Type 55 P/N film.

Fluorescence Microscopy

Fluoresceinisothiocyanate (FITC) labeled Concanavalin A (fl-Con A) was prepared by a modification of the method of Clark and Shephard (24). Concanavalin A (Con A) was made 20 mg/ml in chilled carbonate buffered saline (0.9% NaCl), pH 9.5. To this was added FITC at a concentration of 0.1 mg/ml. This reaction mixture was dialyzed against ten times this volume of 0.05M carbonate buffered saline, pH 9.5 and stirred over-night in the cold. The dialyzate was placed on a column of G25 Sephadex equilibrated with 1 mM phosphate buffered saline (PBSA), pH 7.0. The fluorescently labeled protein which is completely separated from unconjugated FITC and Con A, is eluted first. Approximately 1 gm (wet weight) of amoebae growing in mid-log phase were washed free of nutrient medium and resuspended in 100 ml of PBSA. Approximately 1.0 ml of fl-Con A was added to 10 ml of the cell suspension and the mixture was agitated every 30 sec for 5 min. Unreacted fl-Con A was then removed by a 10 fold dilution with PBSA and centrifugation at 600 X g. The pelleted cells were resuspended in PBSA and examined immediately under a Zeiss Fluorescence Microscope using an FITC exciter filter, barrier filters 53/44 and non-fluorescing immersion oil. The fluorescently labeled Con A was protected from light and maintained at 0-4°C during the conjugation, purification and labeling of cells.

MATERIALS

Cyclic 3',5'-adenosine monophosphate, cyclic 3',5'-guanosine monophosphate, adenosine monophosphate, adenosine diphosphate, colchicine and procaine HCl were purchased from Sigma, St. Louis, Missouri; Griseofulvin from Aldrich, Milwaukee, Wisconsin, Cytochalasin B from I.C.I. Research Laboratories, Cheshire, England; Concanavalin A was obtained from Eli Lily and Fluoresceinisothiocyanate from Calbiochem, San Diego, California.

RESULTS

Amoebae taken from an early logarithmic phase culture were examined for specialized cell surface structures by conventional light, phase and electron microscopy. No specializations other than large pseudopodial structures were observed by light or phase microscopy; however, when cells were examined by standard transmission electron microscopy after mild fixation, occassionally amoebae with small filiform projections of the cell surface were observed (Fig.33). These filamentous structures called filopodia (25), were observed protruding from the cell surface when living amoebae were viewed under dark field conditions. In general, less than 10% of the cells contained filopodia which when present were not extensive (Fig.34). The surfaces of cells sampled from either middle logarithmic, late logarithmic or stationary phase (greater than 1.5 x 10^7 cells/ml) appeared identical. Treatment of logarithmically growing cells with cAMP (10^{-5} M) for five min resulted in the formation of filopodia on all of the cells. The induction was complete within ten min. Furthermore, the average length of the filopodia was greatly increased often reaching the equivalent of the cell's diameter (Fig. 35). Within the first few minutes after filopodia had been induced, the distance between cells appeared to become progressively shorter (Fig. 36) After ten minutes the majority of cells were present in large clusters.

The induction of filopodia appeared to be specific for cAMP at concentrations of at least 10^{-5} M. There was no

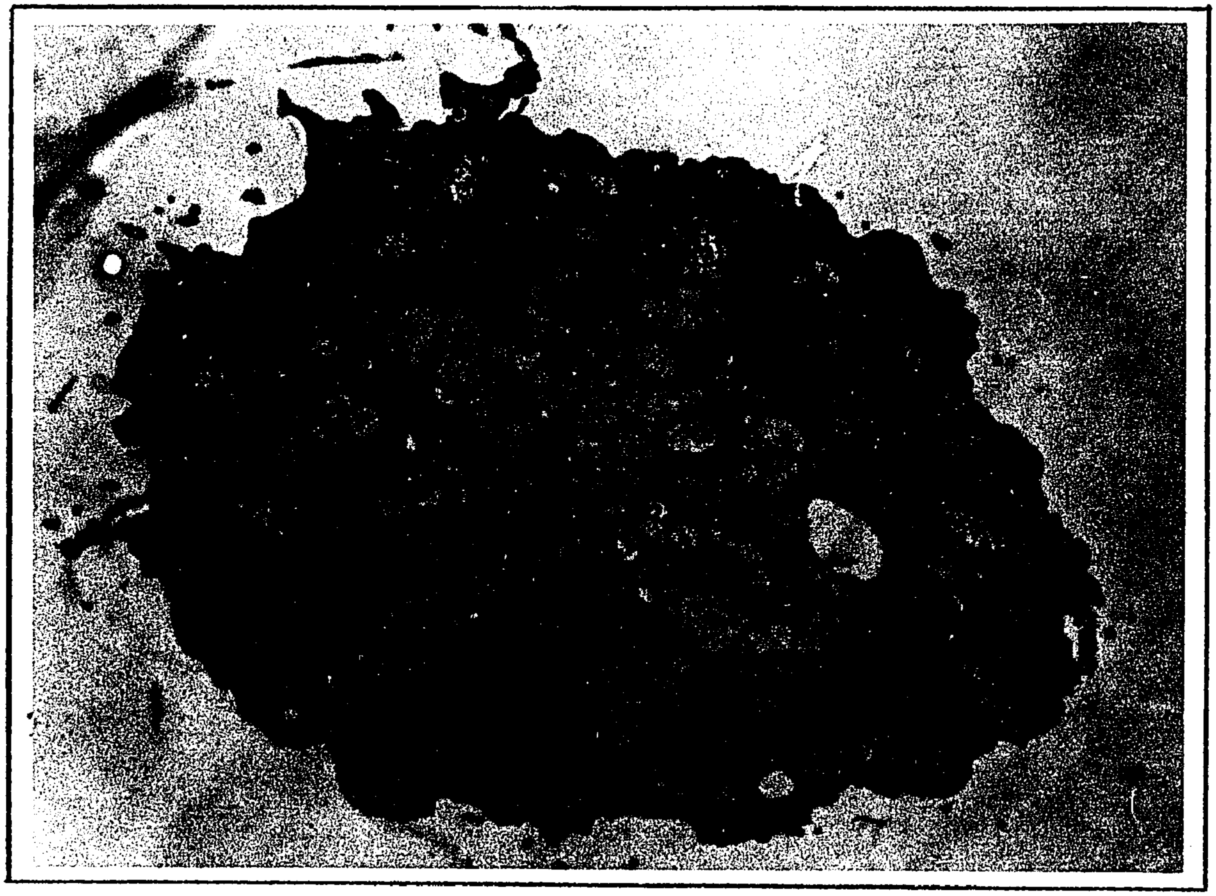


Figure 33. Transmission electron micrograph of an amoebae with small filiform projections of the cell surface.

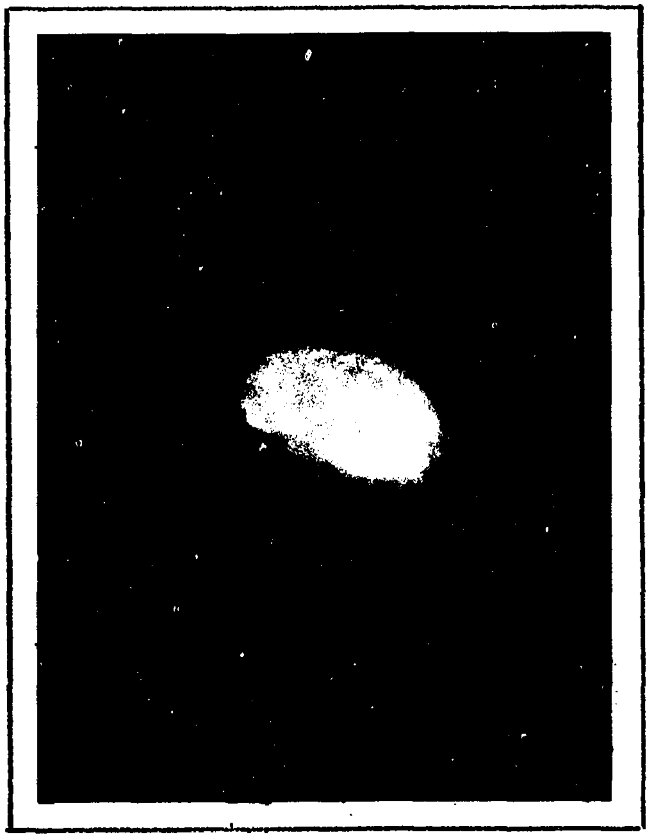


Figure 34. Dark-field photomicrograph showing a living cell bearing several filopodia.

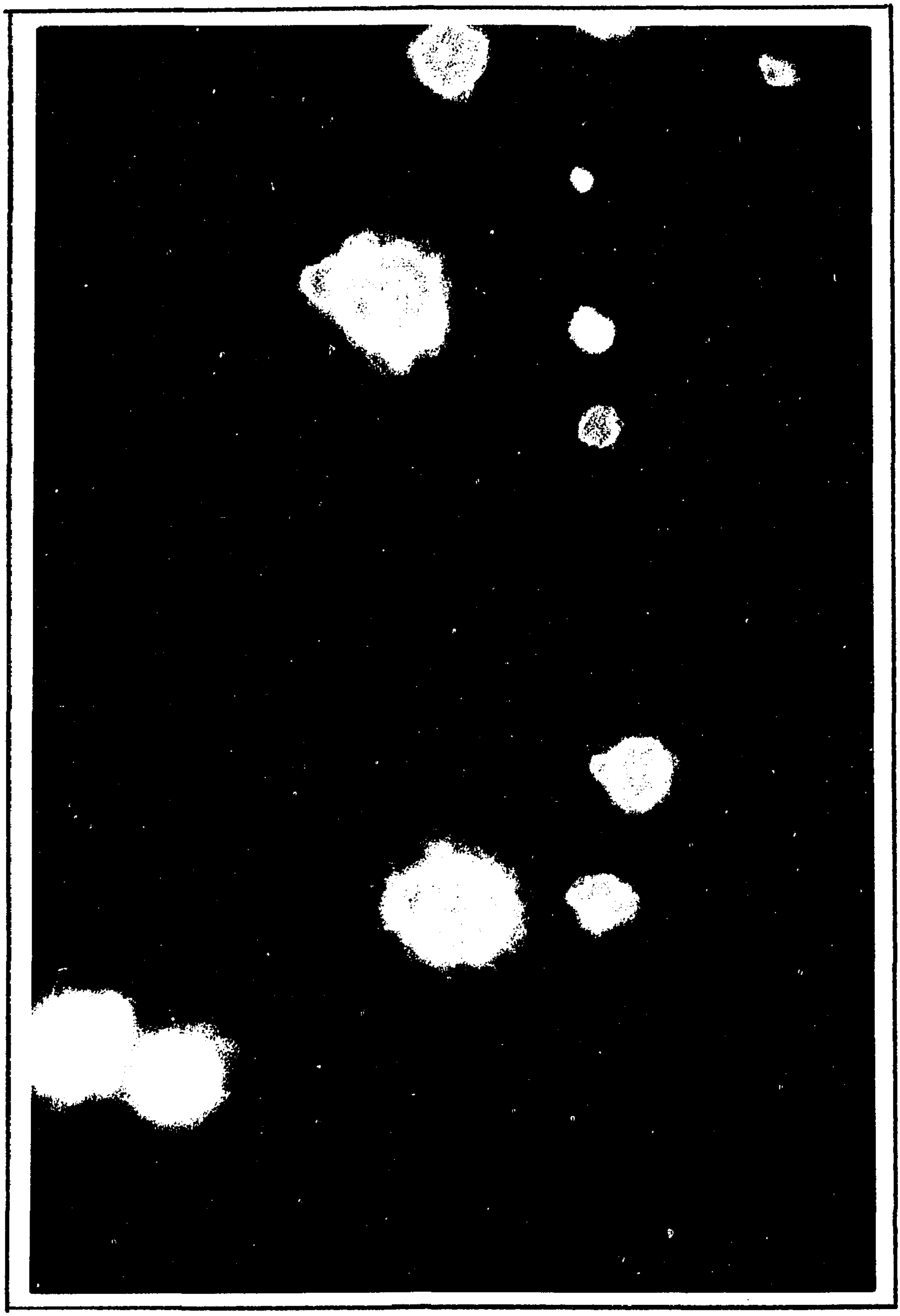


Figure 35. Dark-field photomicrograph of living amoebae which had been treated with cAMP (10^{-5}M) for 5 min.

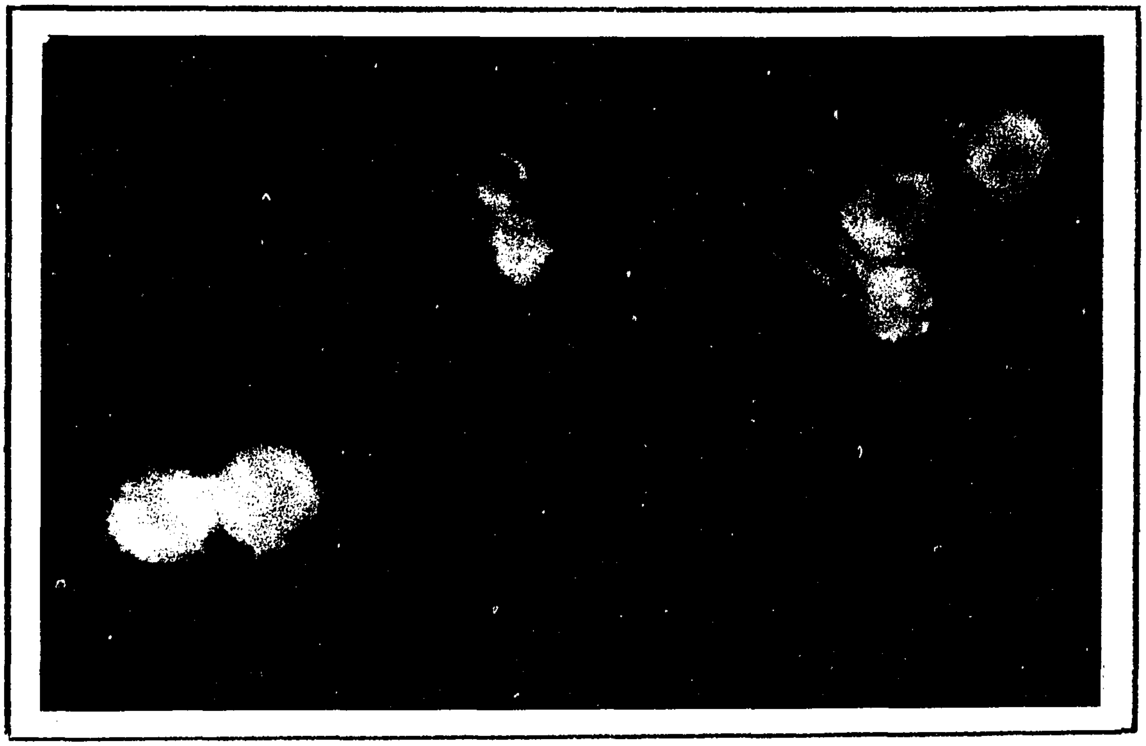


Figure 36. Dark-field photomicrograph showing cells interacting through their filopodia.

response to cAMP at 5 X 10^{-6}M. As seen in Table II, filopodia could not be induced by AMP, ADP or cyclic 3',5'-guanosine monophosphate at concentrations as high as 1.0 mM. The addition of 1.0 mM ATP caused induction of filopodia; however, the effect was not immediate and required incubation for at least 10 minutes. Presumably, the ATP acted after its conversion to cAMP by the membrane localized adenylate cyclase (26). Extensive clumping of cells was observed when either cAMP or ATP was used for induction. Amoebae were incubated with 0.1 mM 2-4 dinitrophenol, an uncoupler of oxidative phosphorylation, or with 1.0 mM sodium azide, a respiratory inhibitor, for 15 minutes followed by cAMP. In both cases, filopodia were induced in response to cAMP, indicating that a functional respiratory chain is not required for the formation of filopodia. Addition of these inhibitors without cAMP caused no induction. A previous report (27) had indicated that dinitrophenol did not prevent aggregation of slime molds.

The presence of filopodia similar to those found on the surface of transformed cells (28) prompted an investigation of the ability of Concanavalin A to promote agglutination. At a concentration of 0.5 mg/ml, Concanavalin A causes extensive clumping of cAMP induced slime mold cells. In the absence of cAMP induction, Con A agglutination was less pronounced and probably represented agglutination due to the pre-existing small filopodia.

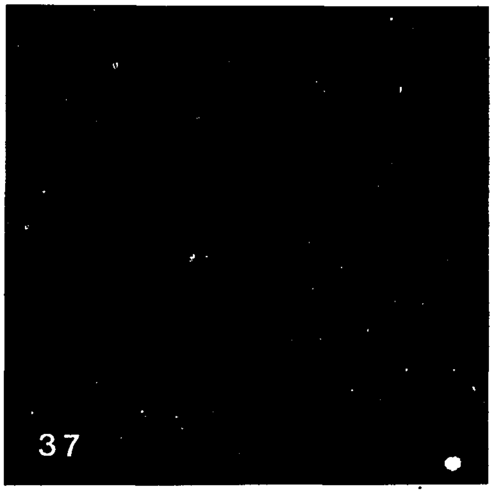
TABLE II

SPECIFICITY OF FILOPODIAL INDUCTION

Compound	Concentration	Extension of Filopodia *
Adenosine Monophosphate	1.0 mM	-
Adenosine Diphosphate	1.0 mM	-
Cyclic Guanosine Monophosphate	1.0 mM	-
Adenosine Triphosphate	1.0 mM	+
Cyclic Adenosine Monophosphate	10^{-5}M	+

* Cells were treated with the test compound at room temperature and after fifteen minutes were observed by dark field microscopy. A positive result indicates a significant increase in the number of cells bearing many long filopodia.

Binding of the lectin Concanavalin A to the cell surface was further investigated using fluorescently labeled Concanavalin A (fl-Con A). Cells which were labeled with fl-Con A and examined immediately, appeared uniformly fluorescent over their entire surfaces. After several minutes at room temperature however, the label was found in discrete patches on the cell surface(Fig. 37) and after 30-40 min the patches on the cell surface had coalesced forming a cap on one side of the cell (Fig. 38). This suggests that the Con A receptor sites are not topographically restricted within the plane of the membrane. Internalization of the labeled Con A by phagocytosis appeared to play no role in this phenomenon based on observations made with the dark field microscope. Fluorescent material was not observable within cellular vessicles.



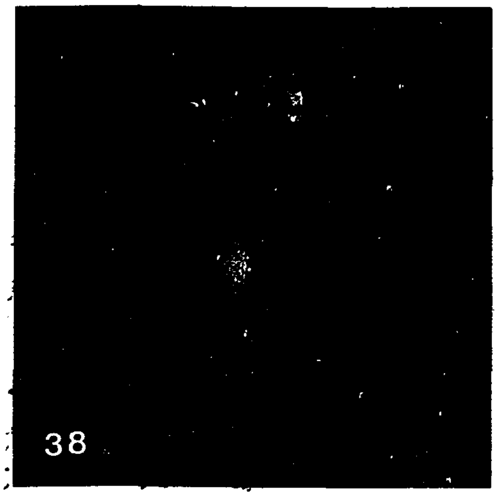


Figure 37. Amoebae treated with fluorescent Concanavalin A. Fluorescent label found in discrete patches within several minutes.

Figure 38. Approximately 40 min after labeling amoebae with fluorescent-Concanavalin A, the label appears in a cap on one side of the cell.

The cell surface was observed in finer detail by a scanning microscope analysis of the axenically grown amoebae. Cells from cultures in the early logarithmic phase of growth were generally spherical to ovoid in shape with a diameter approximately 15 µm; however, cells with a more irregular shape were also observed. The entire cell surface appeared to be highly ridged for cells of all shapes (Fig 39) while occasionally, small extensions were observed. Cells taken from a slightly older culture e.g. mid-log phase cells, appeared quite different, as filopodia were observed on the surfaces of most cells (Fig 40). Stationary phase cells appeared similar to the mid-log phase cells when examined by dark field microscopy although slightly larger in diameter. Scanning electron micrographs, however, reveal that stationary phase cells have a more complex surface with many ridges (Fig 41). Filopodia were present but in smaller number and of shorter length than those present on cells at the mid-log phase of growth. (Fig 42). Preaggregative amoebae obtained by washing mid-log phase cells free of nutrient medium and allowing them to develop on absorbent pads have extremely complex surfaces with extensive irregularities. Filopodia and pseudopodia of all sizes are observed on a highly cratered surface (Fig 43).

The induction of filopodia by cAMP was also examined by scanning electron microscopy. Addition of 1 mM cAMP to cell cultures in the early log-phase of growth for 5-10 min resulted in the extension of extremely long filopodia which often reached a length double that of the cell's diameter. The number of

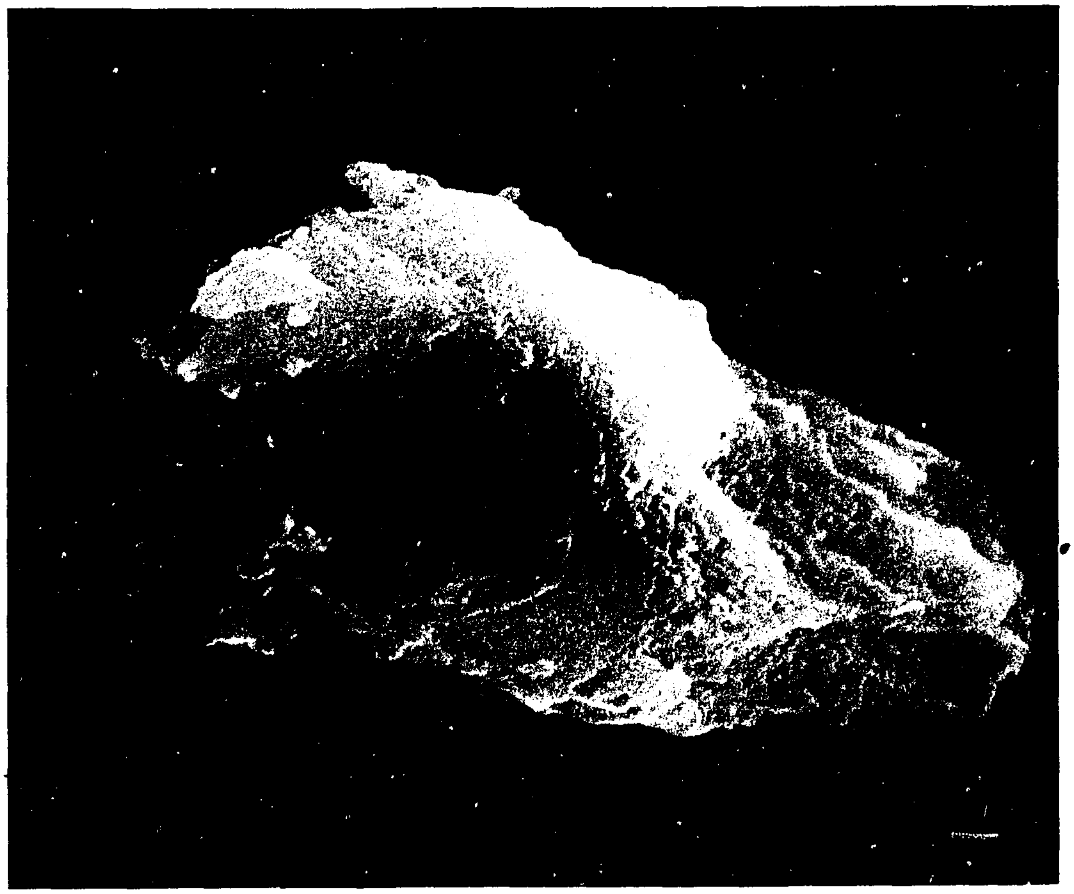


Figure 39. Scanning electron micrograph of an amoebae which had been growing in an early logarithmic phase culture.

Figure 40. Scanning electron micrograph of amoebae which had been growing in a mid-logarithmic phase culture.

Figure 41. Scanning electron micrograph of stationary phase amoebae

Figure 42. Higher magnification of Figure 41.

Figure 43. Scanning electron micrograph of preaggregative amoebae.

filopodia per cell was variable; however, they were ubiquitous (Fig 44).

Various parameters were next investigated to gain an insight into the biochemical mechanism of filopodial formation. The effects of the antimitotic agents colchicine and griseofulvin, as well as low temperature, were investigated to determine whether microtubules are involved in the process of filopodial extension and retraction. It has been established that microtubules become depolymerized at a temperature of 4°C (29) or in the presence of colchicine which binds specifically to tubulin, the microtubule subunit protein (29-31). D. discoideum amoebae growing in logarithmic phase, treated with 0.1 mM colchicine become blocked in cell division. Furthermore, it has been reported that microtubules of these cells are depolymerized under these conditions (32). The presence or appearance of the filopodia on mid-log phase cells was not affected when the cells were placed in a water bath at 3-4°C for 30 min. Treatment of the cells at 22°C with 0.1 mM colchicine for 30 min also had no effect on the filopodia. In addition, treatment of the cells with 10 mM griseofulvin, an antimitotic agent which interferes with microtubule function but not assembly (33) did not block the induction of filopodia. It is therefore unlikely that microtubules are necessary for the formation and maintenance of filopodia.

Slime mold amoebae contain microfilaments (34) as well as microtubules. Bundles of microfilaments can be seen in

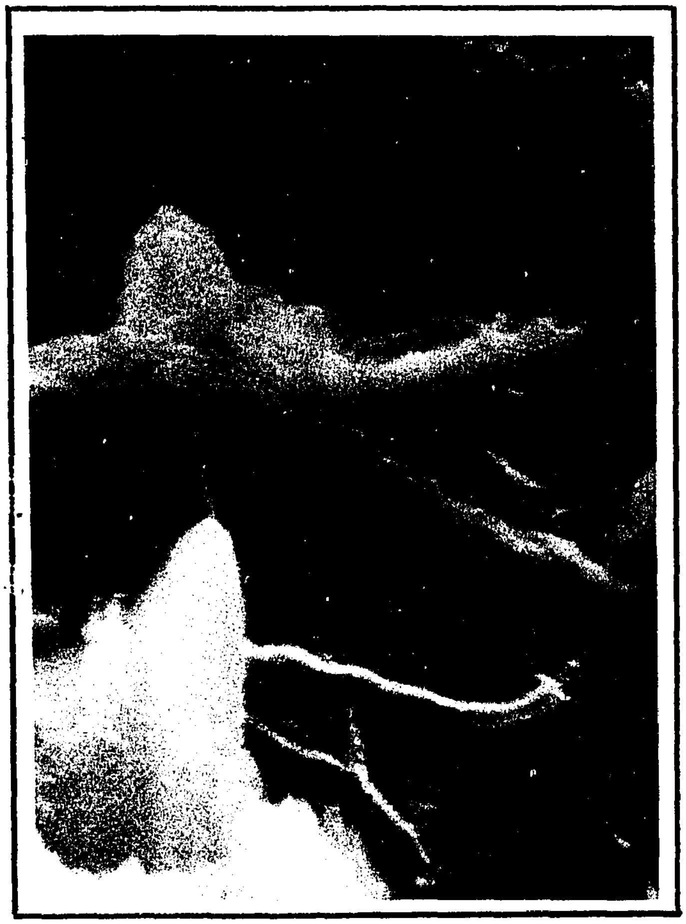


Figure 44. Scanning electron micrograph of cAMP treated amoebae which had been growing in the early logarithmic phase.

cross sections of filopodia from cells examined by transmission electron microscopy (Fig 45). The possibility that submembrane microfilaments were involved in maintaining the structure of filopodia was investigated by treating the cells with cytochalasin B, an antibiotic which exerts its effect by specifically interacting with microfilaments (35). Observation by dark field microscopy revealed that the addition of cytochalasin B (1.0 μg/ml) to cells in the mid-log phase of growth caused the immediate collapse of all filopodia. After this treatment, the cells resembled control cells (Fig 39). Similarly, treating the mid-log phase cells with procaine (10 mM) for 5 min caused the temporary collapse of long filopodia and, in addition, resulted in some blebbing of the cell surface (Fig 46). Local anaesthetics such as procaine have been shown to effect microfilaments as well as microtubules and to disrupt the subplasma membrane cytoskeletal organization in 3T3 cells (36).

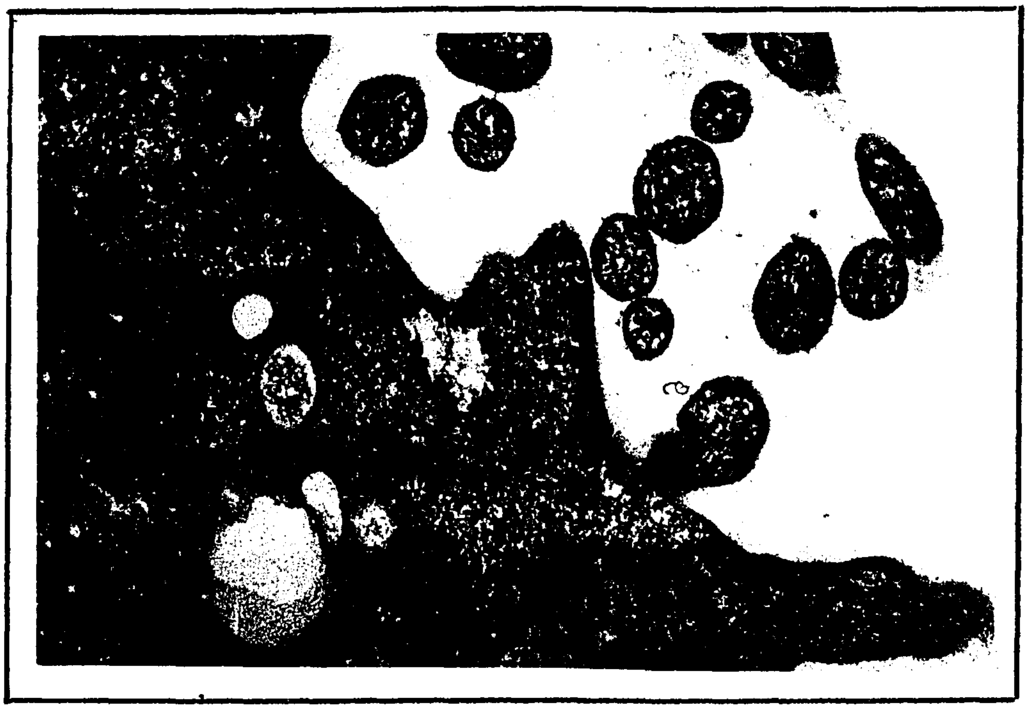


Figure 45. Transmission electron micrograph showing cross sections and a longitudinal section of filopodia.

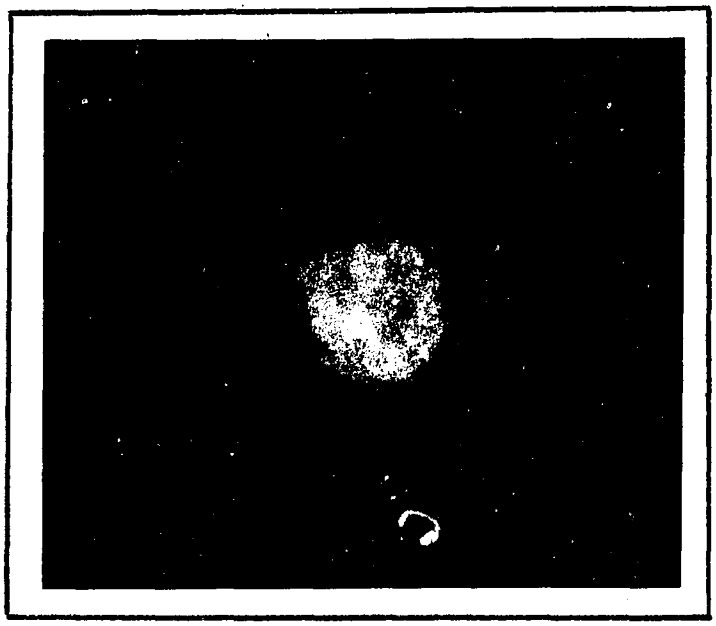


Figure 46. Amoebae from a mid-log phase culture which had been treated with procaine HCl (10 mM) for 5 min.

DISCUSSION

The present study reports the formation and extension of filopodia on the surface of logarithmically growing ameboid cells of D. discoideum in response to treatment with cAMP. The reaction is specific for cAMP and occurs at a cAMP concentration of 10^{-5}M. Filopodia have not been previously observed by dark field microscopy; however recently similar structures have been reported in studies with the scanning electron microscope (21).

The process of intercellular adhesion has been extensively studied in cell systems which undergo a natural aggregation phase such as limpet haemocytes (37,38), mammalian blood platelets (39), cultured lizard myoblasts (40) as well as D. discoideum (34). On a theoretical basis, it has been suggested that the first process in cell adhesion might involve the production of fine pseudopods extending from one cell surface to another (41,42). More recently, evidence to support this concept has accumulated as cell extensions have been observed on the cell surface during lizard myoblast aggregation (40), sea urchin development (43), sponge cell aggregation (44) and the aggregation of dissociated embryonic chick heart cells (45). Following treatment with 1.0 mM cAMP, the formation of elongated narrow processes on cultured myoblasts has been reported (46). Willingham and Pastan (28) have recently described the presence of microvilli on the surface of various transformed cells. By contrast, the surfaces of normal cell lines appeared free of these structures. These workers propose that transformed cells are more easily agglutinated with Con A due to the presence of these microvilli. After treatment with cAMP, the transformed cells apparently lose their microvilli and as a result become less sensitive to agglutination by Con A. It should be noted that these observations are in direct contrast to those observed with the slime mold, which forms and extends filopodia in response to cAMP treatment. Furthermore, when the filopodia are present in the extended state these cells are more easily agglutinated by Con A in confirmation of the hypothesis of Willingham and Pastan. Upon aggregation of the cells, the filopodia appear to retract. It has been reported that higher concentrations of Con A are required to agglutinate aggregating cells than exponentially growing cells (47) suggesting that the divalent Con A may cause agglutination by forming lectin bridges between cells by binding to carbohydrate containing sites on filopodia which are more extensive in growing cells.

This possiblity was investigated by labeling mid-log phase cells with fl-Con A and examining the topology of the fluorescence to determine if label could be found in higher concentration on filopodia. The low efficiency of fluorescence did not allow identification of the Con A receptor sites on the surfaces of filopodia, however, the observation that receptor sites are not restricted to specific regions on the plasma membrane as is true of filopodia supports the hypothesis that these sites are present on filopodia and therefore may be responsible for increased agglutination.

The cAMP sensitive induction of filopodia in *D. discoideum* suggests that the first contact made between two or more induced cells may be mediated through their filopodia. Such interactions might cause the two cells to become physically interdigitated and as a result to adhere to each other. Pre-aggregative cells have increased adhesiveness, presumably due to alterations of the cell surface. (11). Lipoproteins (48), surface antigens (16, 49) and carbohydrate associated proteins (50-52) have been implicated in the newly acquired adhesiveness. Based on electron microscopic observations of freeze-fractured membranes, it has recently been suggested that the adhesiveness may be due to structural alterations within the matrix of the plasma membrane (53,54). The results of this study suggest that cAMP may cause cells to become adhesive concomitantly with the induction of filopodia.

The scanning electron microscope analysis of the present study supports these findings and suggests that the morphology of the cell surface changes dramatically during growth and as the cells enter the developmental sequence. Furthermore, the higher resolution afforded by the scanning microscope reveals that cAMP treatment of early log-phase cells (axenic or wild type) which normally lack filopodia, caused their induction to lengths greater than observed by dark field microscopy.

Various substances with known biochemical action were tested in an attempt to learn something about the mechanism of filopodial formation. Lowering the temperature of the cells to 3°C, which causes disruption of microtubules had no effect on the filopodia in confirmation of the previous report of Garrod and Born (34). Addition of either colchicine or griseofulvin also had no effect on filopodia providing further evidence that microtubules are not required for the filopodial structure.

Electron microscopy has revealed the presence of thin filaments in the filopodia of *Dictyostelium* (34,55), in the microspikes of limpet haemocytes (38) and in many other cell types. Presumably, these thin filaments play a role in the contractile process (35). In this context, it should be noted that actin has been shown to make up a large fraction of the total soluble protein of non-aggregating cells of *D. discoideum* (56). The possible role of microfilaments in the maintenance of filopodial structure in slime mold amoebae was investigated. Addition of cytochalasin B which reversibly alters the morphology of microfilaments by causing the rapid disappearance of microfilament bundles thereby disrupting the supporting core of cell surface projections (38), caused the immediate and reversible collapse of filopodia. Furthermore, treatment of amoebae with procaine HCl, a local anaesthetic also resulted in the temporary collapse of existing filopodia. Tertiary amine local anaesthetics such as procaine are thought to alter the structural organization of membrane-associated microfilaments and microtubules (36). These results suggest that the maintenance of filopodial structure may depend, in part, on the integrity of bundles of microfilaments, presumably actin, within the core of the filopodia.

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CHAPTER 5

THE RESPIRATORY COMPETENCE OF THE SPORES OF DICTYOSTELIUM DISCOIDEUM

ABSTRACT

Analysis of the respiratory chain of spores of *Dictyostelium discoideum* which lack a cyanide-sensitive respiration indicates that cytochromes a-a_3, b and c-c_1 are present at levels identical to those found in the vegetative amoebae. In addition, the specific activities of enzymes of both the respiratory chain and the citric acid cycle are present in the 600 x g supernatant of sonicated spores in quantities at least as high as are found in similar preparations of amoebae. The activities of glutamic dehydrogenase and oligomycin-sensitive ATPase are reduced in the spores 30% and 56% respectively. Intact spores appear to lack a cyanide-sensitive respiration as a result of inadequate quantities of respiratory substrate and more importantly as a result of a lack of the cofactor NAD^+. The emergence phase of spore germination is sensitive to the antibiotic chloramphenicol which is a specific inhibitor of mitochondrial protein synthesis. It is concluded that germination requires the early synthesis of NAD^+ and generation of respiratory substrates and one or more mitochondrially synthesized proteins.

INTRODUCTION

The cellular slime mold *Dictyostelium discoideum* is an excellent organism for studies of cellular differentiation. The slime mold grows logarithmically as a unicellular amoeba with a generation time of 12-15 hr when supplied with adequate nutrients. Under controlled conditions, the amoebae aggregate into a multicellular mass and synchronously differentiate into either prestalk or prespore cells. The prespore cells undergo complex metabolic differentiation ultimately becoming a relatively dormant spore whose respiratory activity is extremely low. The compound N,N-dimethylguanosine which is the natural germination inhibitor has been ruled out as the causative agent of respiratory inhibition (1)

Presently, little is known to explain the low levels of oxygen consumption in the spores of the slime mold. In this section, the presence of a functional mitochondrial respiratory chain as well as citric acid cycle activities in these spores is reported. Furthermore, the results indicate that the insignificant oxygen consumption by spores apparently results from low levels of respiratory substrates and, more important, of the cofactor NAD^+.

MATERIALS AND METHODS

Dictyostelium discoideum, strain A-3 was a gift of William F. Loomis, University of Calif., San Diego and was grown axenically (9) in a medium containing 0.2 mg/ml of streptomycin sulfate as described previously (13). Strain NC-4 was grown in liquid culture together with *Klebsiela aerogenese*. Alternately, strain NC-4 amoebae were grown together with this bacteria on plates of a medium consisting of 10 gm Bactopeptone, 1.0 gm Yeast Extract, 10 gm dextrose, 2.05 gm $MgSO_4 \cdot 7H_2O$, 20 gm Difco agar and buffered with 10 mM potassium phosphate buffer, pH 6.4. Spores of strain NC-4 were harvested between three and five days post sorocarp formation by running a glass slide across the petri plate at a distance from the agar so as to make contact only with the sori. The spores are almost completely free of stalk cells, with no bacterial contamination as determined by inspection under a phase microscope. Approximately 0.5 gm (wet weight) of spores are harvested and washed free of germination inhibitor and other contaminants by repeated washing in 10 mM phosphate buffer pH 6.5 followed by centrifugation at 2000 X g. Similarly, amoebae from both strains A-3 and NC-4 were washed to remove nutrient medium and/or bacteria. All experiments were routinely performed using spores of the NC-4 strain and amoebae of the axenic strain since identical results were obtained in respiratory studies using A-3 or NC-4 amoebae. Furthermore, use of the A-3 amoebae eliminates the possibility of bacterial contamination biasing the results. Amoebae are resuspended at a final concentration of 10-15 mg/ml (wet weight), in a respiratory buffer consisting of 54.7 gm mannitol, 100 ml of 0.1 M Tris buffer, pH 7.5, 100 ml of 0.1 M KCl, 100 ml of 0.1 M potassium monophosphate and 25 ml of 0.1 M $MgCl_2$ per liter.

Sonication.

Amoebae were placed in a chilled metal cup and sonicated using four fifteen second bursts at 90 watts using a Branson Sonifier model W185D set at 20 Khz. Amoebae were then inspected by a Zeiss phase contrast microscope to determine the extent of cell lysis. Spores required more extensive sonication. Optimal disruption occurred when the concentration of spores was between 25-50 mg protein/ml and when sonication was performed 15 to 20 times with 15 sec bursts at 130 watts. During this treatment the spores were maintained at $0^{o}C$ by suspending the sonication cup in an ice-water-salt bath.

Respiratory studies.

Oxygen consumption was monitored polarographically using a Clark-type oxygen electrode. Three ml of sample (5-10 mg/ml) were placed in a cuvette sealed in a circulating water bath maintained at $25 \pm 0.5^{o}C$. All substrates or cofactors were added to a final concentration of 2-5 mM with potassium cyanide added to a concentration of 10 mM.

Enzyme assays.

Enzyme assays were performed on either a 600 x g supernatant or on a crude mitochondrial preparation which was prepared by washing the 600 x g supernatant twice in a medium containing 0.25 M sucrose, 0.01 M Tris buffer (pH 7.6) and 0.001 M EDTA followed by centrifugation at 17,000 x g. Succinic dehydrogenase, NADH dehydrogenase, succinate-cytochrome c reductase, and cytochrome c oxidase were each assayed at 23°C as described by Kim and Beattie (8). Glutamate dehydrogenase was assayed according to the method of Beaufay et. al. (3) with the exception that Triton X-100 was not used.

Spore germination.

Spores of strain A-3 were innoculated into axenic medium (9) in the presence or absence of chloramphenicol (4 mg/ml) and germination was monitored by periodic observation with a phase contrast microscope.

Spectral studies.

The room temperature difference spectrum of the 600 x g supernatant was determined in a Cary 15 u.v.-vis. recording spectrophotometer. The reference sample was oxidized with potassium ferricyanide and the experimental sample was reduced with sodium dithionite. The concentration of cytochromes in nanomoles was calculated using the extinction coefficients of Rieske (11).

Materials.

Proteose-Peptone, Bacto-Peptone and Yeast Extract were obtained from Difco Laboratories, Detroit Michigan. Streptomycin sulfate, grade B was from Calbiochem, San Diego, California. Malate, glutamate, pyruvate, isocitrate, α glycerophosphate, α ketoglutarate, succinate, ascorbate, tetramethylphenylenediamene, nicotinamide adenine dinucleotide (oxidized and reduced forms), phenazine methosulfate, dichlorophenolindophenol, grade 1, bovine serum albumin, cytochrome c (horse heart type III), oligomycin and chloramphenicol were purchased from Sigma, St. Louis, Mo.

RESULTS

The respiratory activity of intact amoebae and spores was investigated by measuring oxygen consumption with a Clark-type oxygen electrode. As seen in Table III, the amoebae have a relatively rapid rate of oxygen uptake which is inhibited 87% by cyanide, whereas spores have a negligible rate of oxygen uptake which is completely insensitive to cyanide. The cyanide-sensitive oxygen consumption of the amoebae, a measure of the mitochondrial respiratory chain, is vital for the amoeba. Amoebae cannot survive in liquid culture either in the presence of 1.0 mM cyanide or when the culture medium has been equilibrated with nitrogen. The cyanide-insensitive respiration of the spores, however, is apparently adequate to sustain life.

In an attempt to learn why spores have such low levels of cyanide-sensitive oxygen consumption, the mitochondrial respiratory chain was analyzed to determine whether one or more cytochromes was depressed or missing as has been reported for spores of the fungus *Botryodiplodia theobromae* (5). The difference spectrum of a 600 x g supernatant of both amoebae and spores suggests that the spores contain a normal complement of cytochromes $a-a_3$, b and $c-c_1$ (Figure 47 and Table IV). In addition, various partial reactions of the respiratory chain were also measured in the 600 x g supernatant obtained from both spores and amoebae. As seen in Table V, the

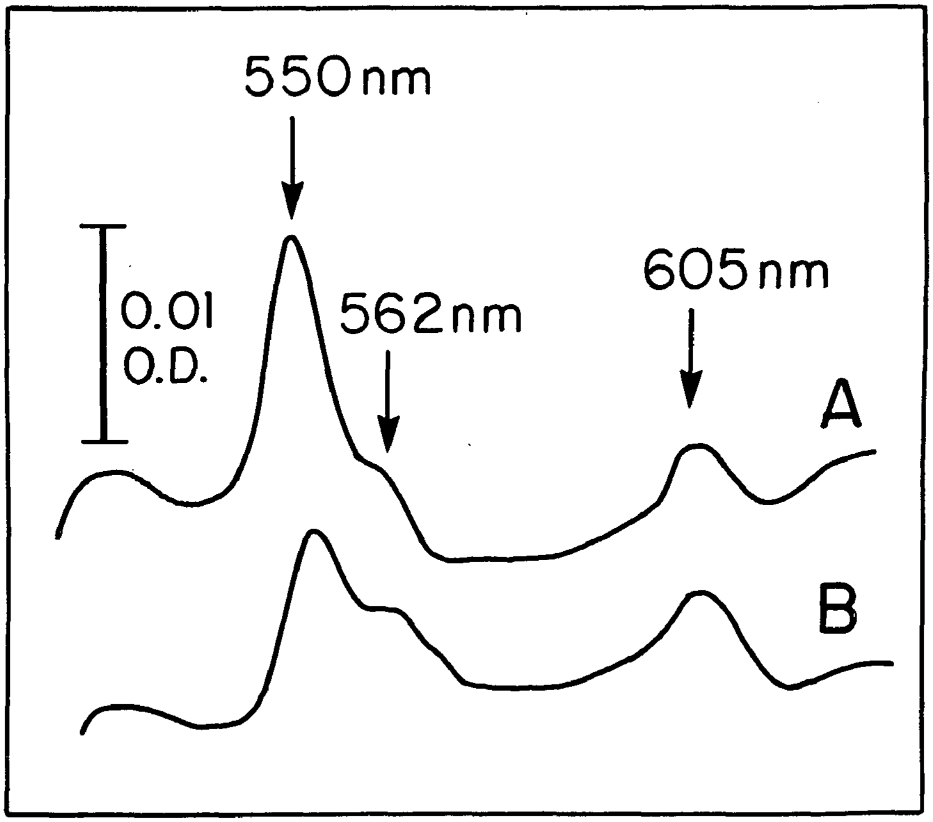

Figure 47. Room temperature difference spectra. Trace A: amoebae 600 x g supernatant, 4.05 mg protein/ml. Trace B: Spore 600 x g supernatant, 3.45 mg protein/ml. Reduction was with sodium dithionite and oxidation with potassium ferricyanide.

TABLE III

WHOLE CELL RESPIRATION

	Total Respiration	CN^- Sensitive Respiration	% CN^- Sensitivity
Amoebae	14.7	12.8	87
Spores	Negligible	0	0

Amoebae were washed free of nutrient medium and spores free of germination inhibitor and other contaminants and resuspended in respiratory buffer. Oxygen consumption was recorded polarographically and calculated as natoms oxygen consumed/min/mg protein.

TABLE IV

	DIFFERENCE SPECTRA OF CYTOCHROMES		
	$a-a_3$	b	$c-c_1$
Amoebae	.258	.440	1.248
Spores	.276	.440	1.412

Difference spectra were recorded on a Cary 15 spectrophotometer at room temperature. Sample was reduced with sodium dithionite and reference was oxidized with potassium ferricyanide. Protein was 4-6 mg/ml. Numbers are in nmoles/mg protein.

TABLE V

PARTIAL REACTIONS RELATED TO THE RESPIRATORY CHAIN

	Amoebae	Spores
A. 600 x g Supernatant		
Succinic dehydrogenase	11.5	29.3
Succinate-cytochrome c reductase	24.6	34.4
Cytochrome c oxidase	2.2	3.7
Malate dehydrogenase	19.9	31.7
Glutamate dehydrogenase	19.1	13.6
Isocitrate dehydrogenase		
NAD linked	11.6	12.3
NADP linked	24.2	60.4
B. Crude Mitochondria (17,000 x g pellet)		
Succinic dehydrogenase	55.7	93.7
NADH dehydrogenase	55.9	74.7
Succinate-cytochrome c reductase	54.9	77.6

Specific activities are calculated as nmoles substrate consumed/min/mg protein.

activities of succinic dehydrogenase, succinate-cytochrome c reductase and cytochrome c oxidase were increased 155%, 40% and 70% respectively in the spores. The high non-specific oxidation of NADH in the 600 x g supernatants made it necessary to prepare crude mitochondrial fractions to assay the NADH dehydrogenase complex. The specific activity of this enzyme was also elevated 34% in spore mitochondria relative to mitochondria prepared from amoebae. Furthermore, the activities of succinic dehydrogenase and succinate-cytochrome c reductase were also elevated 68% and 51% respectively in the mitochondria obtained from spores. Hence, it would appear that the spores are not deficient in any components of the respiratory chain as determined enzymatically, in agreement with the spectral data.

Several enzymes of the citric acid cycle were also studied (Table V). The specific activities of malate, glutamate and the NAD^+ linked isocitrate dehydrogenases were all present in the spores at the same or at a higher level than that of the amoebae. Since both the respiratory chain and certain enzymes of the citric acid cycle are present in the spores, I also investigated the oligomycin-sensitive ATPase activity, the enzyme complex responsible for the respiratory chain linked synthesis of ATP. The results of Table VI indicate that the oligomycin-sensitive ATPase activity of the spores is decreased to a level 44% that of the amoebae; however the proportion

TABLE VI

	ADENOSINE TRIPHOSPHATASE ACTIVITY		
	Total ATPase	Oligomycin-Sensitive ATPase	% Oligomycin Sensitivity
Amoebae	.0342	.0147	43.0
Spores	.0159	.0064	40.3

Assays were performed using 600 x g supernatants. Activities are recorded as μmoles P_i liberated/min/mg protein.

of the total ATPase activity which is oligomycin-sensitive is the same in both the spores and the amoebae.

To investigate the possibility that the lack of spore respiration results from the presence of an endogenous inhibitor, varying amounts of sonicated amoebae were added, in the absence of added substrate, to a constant volume of sonicated spores which have no cyanide-sensitive respiration by themselves. As seen in figure 48 , the specific activity of the combined cell extracts was actually greater than what would have been expected from the addition of the observed specific activities for each individual cell type. There appears to be a maximum rate obtainable when there is present approximately twice as much amoebae protein as spore protein. The stimulatory activity of the amoebae was lost when the amoebae were heated at 70°C for 10 min or were dialyzed against 10 mM phosphate buffer (pH 7.0) for six hr (Table VII). In addition, the cyanide-sensitive respiration of the amoeba decreased by more than 75% after dialysis. These results suggest that a relatively small, heat-sensitive substance (or substances) present in the amoebae which is lost upon dialysis, caused the stimulation of spore respiration. In confirmation, the cyanide-sensitive respiration could be completely restored in the dialyzed amoebae by the addition of both a respiratory substrate such as malate and the cofactor NAD^+ (Table VII).

These results suggested that NAD^+, which is both

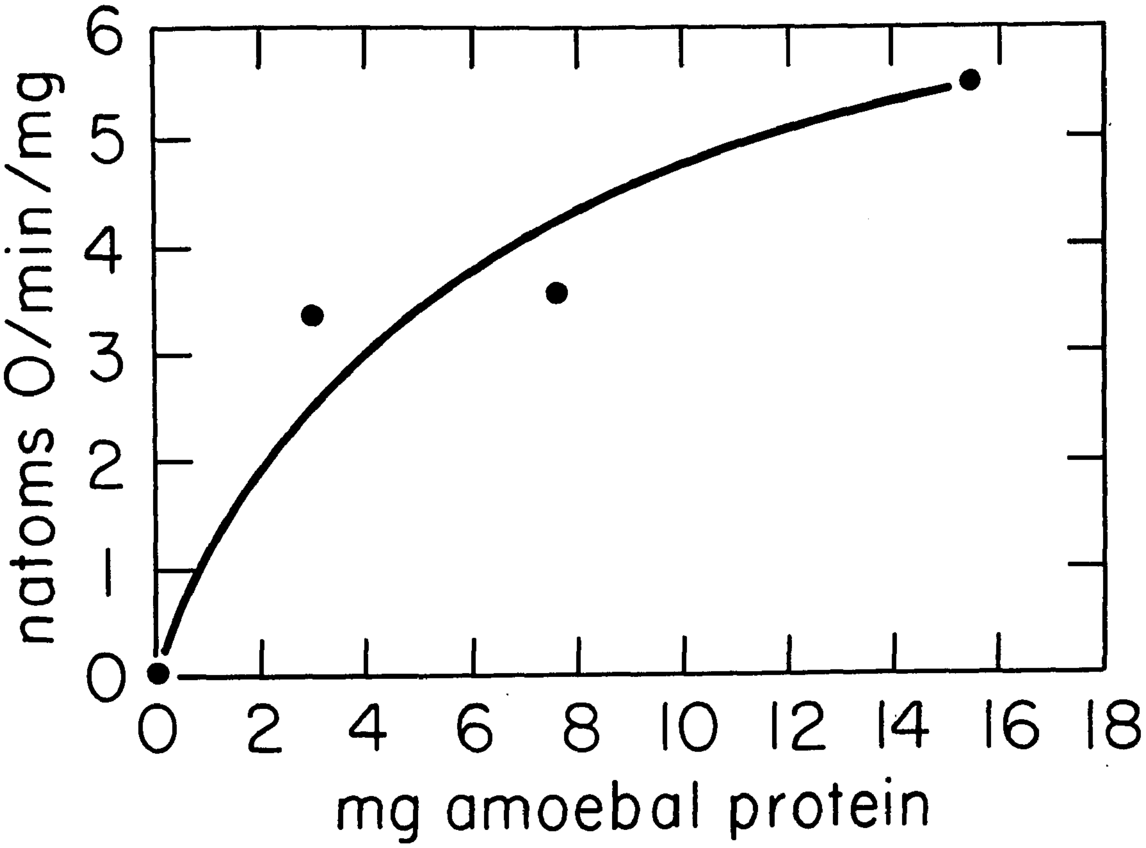

Figure 48. Varying amounts of amoeba 600 x g supernatant are added to a constant volume of spore supernatant containing 9.2 mg of protein. The ordinate represents the specific respiratory activity for the combination of spore and amoeba fractions.

TABLE VII

CYANIDE SENSITIVE OXYGEN CONSUMPTION IN 600 X g SUPERNATANT

	natoms O/min/mg
Amoebae	1.85
Spores	0
Amoebae + Spores (1:1)	2.45
Spores + Heat Treated Amoebae (1:1)	0
Spores + Dialyzed Amoebae (1:1)	0.35
Dialyzed Amoebae	0.42
Dialyzed Amoebae + NAD^+	0.81
Dialyzed Amoebae + NAD^+ + Malate	1.61
Amoebae + NAD^+ + Malate	2.25

Amoebae were either heated at 70°C for 10 min or dialyzed against 10 mM Phosphate buffer (pH 7.0) for six hr and then resuspended in respiratory buffer. Amoebae and spores are 12.6 and 6.0 mg protein/ml respectively and are mixed in equal volume. Oxygen consumption of 3.0 ml of this mixture is then recorded polarographically.

dialyzable and heat labile, as well as respiratory substrates may be lacking in spores and may be responsible for the low rates of oxygen consumption of the intact spores. The 600 x g supernatant of spores in the absence of added substrate or NAD^+ has no cyanide-sensitive respiration (figure 49 , trace A). The addition of malate alone does not stimulate oxygen uptake; however,the combination of malate and NAD^+ results in a rapid rate of oxygen uptake which is completely inhibited by cyanide (trace B). Addition of NAD^+ without substrate results in only a very slightly increased respiratory rate. The relatively high cyanide-sensitive respiratory rate of the amoebae in the absence of any addition can be seen in trace C. Addition of substrate has no effect on the rate of oxygen consumption, although addition of NAD^+ causes a slight stimulation. The 600 x g supernatants obtained from spores oxidize both NADH and succinate at a rate equal to or greater than that observed in the 600 X g supernatants from amoebae (Table VIII). The high rate of cyanide-sensitive oxygen consumption using ascorbate-TMPD as substrate provides further support to the spectral and enzymatic studies that spores contain a functional cytochrome c oxidase. Not surprisingly, the spores respire at high rates using citric acid cycle substrates provided that NAD^+ is present in the incubation medium.

To determine if there is a requirement for protein

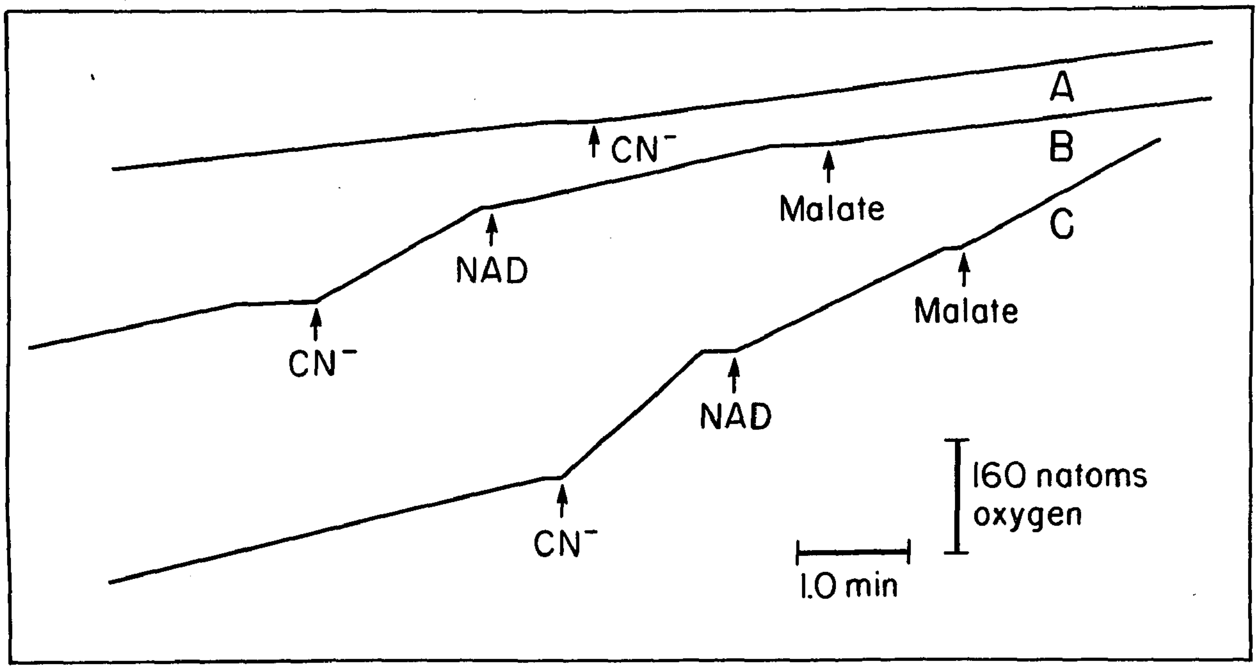

Figure 49. Tracing of the polarographically monitored oxygen consumption by 600 x *g* supernatants of A: spores, 6.1 mg/ml. B: spores, 6.1 mg/ml C: Amoebae, 9.5 mg/ml. Malate and NAD^+ were added to a final concentration of 2-5 mM. Cyanide was added to a concentration of 10 mM.

TABLE VIII

CYANIDE SENSITIVE RESPIRATION (natoms O/min/mg protein)

	SONICATED		600 x g Supernatant	
Addition	Amoebae	Spores	Amoebae	Spores
None	2.8	0.4	2.2	0
Malate	No increase	No increase	No increase	0.3
Glutamate	No increase	No increase	No increase	0
Pyruvate	No increase	No increase	No increase	0
Isocitrate	-	-	No increase	0
NAD$^+$	4.3	2.1	3.1	0
Malate + NAD$^+$	5.5	4.9	3.1	4.4
Glutamate + NAD$^+$	6.3	4.9	3.5	3.7
Pyruvate + NAD$^+$	4.1	2.1	2.5	2.7
Isocitrate + NAD$^+$	-	-	2.6	7.3
α-glycerophosphate	-	-	No increase	0
α- ketoglutarate	-	-	No increase	0
NADH	-	-	6.6	12.9
Succinate	-	-	4.8	4.7
Ascorbate-TMPD	-	-	5.5	4.8

synthesis on mitochondrial ribosomes during spore germination, I attempted to germinate A-3 spores in axenic medium in the presence of chloramphenicol which has been shown to be a specific and potent inhibitor of mitochondrial protein synthesis (2). Unlike the untreated spores which after six hr were observed to have germinated with 70-80% efficiency, the spores innoculated into axenic medium containing 4 mg/ml chloramphenicol were unable to complete germination. These spores became activated and swollen but no emergence took place. This inhibition was shown to be completely reversible if spores were washed free of antibiotic following the six hr incubation and re-innoculated into fresh medium. These spores germinate within two hours (Figure 50).

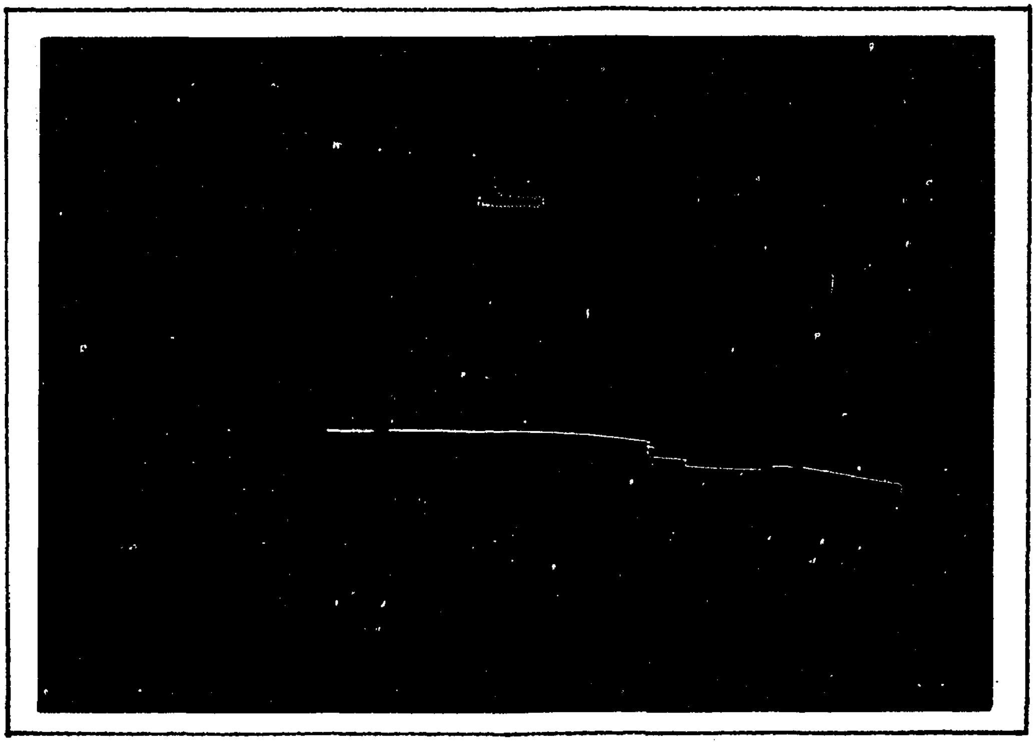

Figure 50. Phase contrast micrograph of 2 amoebae which have just emerged. The spores were treated with chloramphenicol (4 mg/ml) for six hr, washed free of the antibiotic and re-innoculated into fresh medium.

DISCUSSION

The results of this paper have demonstrated that cell-free extracts prepared from spores of D. discoideum can oxidize a variety of respiratory substrates provided that NAD^+ is added to the incubation medium suggesting that intact spores lack a cyanide-sensitive respiration because of a deficiency of both endogenous respiratory substrates and NAD^+. These results are in marked contrast to those obtained with spores of the fungus Botryodiplodia theobromae which also lack cyanide-sensitive respiration. The spores of this organism do not contain detectable amounts of cytochrome a-a_3, and hence, presumably cytochrome c oxidase activity (5). During the germination process, the content of cytochromes a-a_3 increases concomitantly with the increasing rate of respiration. The ability of the spores of D. discoideum to oxidize exogenous respiratory substrates in the presence of NAD^+ reflects the fact that the cytochrome content and the activities of the partial reactions of the respiratory chain as well as certain enzymes of the citric acid cycle are present in the spores in quantities equal to or greater than those of the amoebae. No significance is attached to the observation that higher specific activities for several enzymes were found in the spores, since purified preparations were not used. Furthermore, the degree of contamination of the crude mitochondrial preparations with other subcellular fractions may differ in the spores and amoebae.

It should be noted, however, that the activities of two mitochondrial enzymes, glutamic dehydrogenase and oligomycin-sensitive ATPase, are both decreased in the spores. It was previously reported that glutamic dehydrogenase activity of cells from the young sorocarp is 34% lower than that of amoebae obtained from the early aggregation phase of development (16). Perhaps, the decreased level of glutamic dehydrogenase is a means by which the large intraspore glutamate pool (16) is protected from catabolism. Similarly, it has been found that spores of *Saccharomyces cerevisiae* have lowered levels of this enzyme (4) suggesting that this decrease is not restricted to slime mold spores but rather may be a significant event in a cell which has become metabolically inactive.

The entire spore germination process of *D. discoideum* is dependent on mitochondrial respiration unlike spore germination of the facultative aerobe *S. cerevisiae* which can occur in the absence of cytochrome *c* oxidase activity (14). Cotter and Raper have reported that spore germination of D. discoideum is strictly aerobic and is inhibited at any stage by oxygen deprivation or respiratory poisons such as azide (6). Since respiration increases almost immediately after activation of the spores, it would appear that NAD^+ must be synthesized and respiratory substrates generated during the early part of the activation process so that the necessary energy can be generated by the respiratory chain already present in the spores.

Furthermore, germination would appear to require increased levels of oligomycin-sensitive ATPase which is reduced 56% in spores relative to amoebae. This enzyme complex which catalyzes the terminal reactions of ATP synthesis by oxidative phosphorylation has been shown to contain protein synthesized both on cytoplasmic and mitochondrial ribosomes (15). The observation that chloramphenicol prevents spore germination, may result, in part, from the inhibition of the intramitochondrial synthesis of components for this enzyme. As a consequence, ATP production may be inadequate to allow germination to proceed.

Protein synthesis on cytoplasmic ribosomes is also necessary for spore germination in _D. discoideum_. Cotter and Raper (7) reported that the antibiotic cycloheximide specifically and reversibly prevents emergence while not affecting spore activation or swelling. We have observed a similar effect of chloramphenicol, the specific inhibitor of mitochondrial protein synthesis, on spore germination. Hence it can now be stated unequivocably that protein synthesis on both cytoplasmic and mitochondrial ribosomes is required for the emergence phase of spore germination in _D. discoideum_.

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