1979

The expression of trehalase, alkaline phosphatase and lysosomal hydrolases during Dictyostelium discoideum spore germination.

Louis Stephen. Tisa
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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS RECUE
THE EXPRESSION OF TREHALASE, ALKALINE PHOSPHATASE AND lysosomal HYDROLASES DURING Dictyostelium discoideum SPORF GERMINATION

by

Louis Stephen Tisa

A Thesis submitted to the Faculty of Graduate Studies through the Department of Biology in Partial Fulfillment of the Requirements for the Degree of Master of Science at The University of Windsor

Windsor, Ontario, Canada

1979
ABSTRACT

The spore germination process in *Dictyostelium discoideum* consists of four stages: activation, postactivation lag, swelling and emergence. Several lysosomal acid hydrolase activities are not coordinately controlled during heat-induced strain NC4 spore germination. For example, the level of \( \beta \)-glucosidase activity increases significantly during the emergence stage of germination. The activity of acid phosphatase decreases after activation, but increases in activity during post-emergence. Both \( \alpha \)-glucosidase and N-acetyl-\( \beta \)-glucosaminidase remain relatively constant in activity until post-emergence, when they increase slightly. The expression of \( \alpha \)-mannosidase activity decreases during all stages of germination. \( \beta \)-galactosidase activity increases slightly, but not significantly during spore swelling, falls below the level found in time zero spores, and increases slightly during post-emergence. The expression of all of these enzyme activities, except \( \beta \)-galactosidase, may require protein synthesis. Spores in the lag phase of germination which are exposed to severe environmental stress are deactivated and exhibit reduced levels of acid phosphatase, \( \alpha \)-glucosidase, \( \beta \)-glucosidase and N-acetyl-\( \beta \)-glucosaminidase. Prolonged heat activation treatments reduce the level of lysosomal acid hydrolase activities in the activated spores at time zero, but do not change the enzyme patterns during spore swelling and emergence stages of germination.

Three different resting stages of the *Dictyostellia*ceae (spores, microcysts, and macrocysts) are found to possess low levels of trehalase activity. Upon germination, a significant increase in the expression of trehalase occurs. The increase in activity is dependant on protein
synthesis.

Alkaline phosphatase activity is found to increase slightly during the spore swelling stage and this enzyme activity is found to increase greatly during the emergence stage of germination. The initial increase in alkaline phosphatase activity does not require protein synthesis; however, the second increase in activity does require protein synthesis. Spores in the lag phase of germination which are exposed to severe environmental stress deactivate and exhibit an alkaline phosphatase activity level similar to spores at time zero. Prolonged activation treatments reduce the level of alkaline phosphatase activity in activated spores at time zero similar to the effect with lysosomal enzymes.
ACKNOWLEDGEMENTS

I would like to express my sincere appreciation of the support and assistance provided to me by a number of individuals: my friends, colleagues and co-workers at The University of Windsor, faculty and staff within the Biology Department, members of my Masters committee, and my family. Special thanks are expressed to Dr. David A. Cotter for his constant support and direction throughout both the experimental and writing phases of this thesis.

Finally, I wish to dedicate this work to late mother, who taught me the real meaning of courage; and my father, who gave me constant moral support.
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<tr>
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<tr>
<td>POPOP</td>
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<td>NAG</td>
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<td>B-Glc</td>
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<td>B-Gal</td>
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<td>Mann</td>
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I. INTRODUCTION

A. History and General Overview of the Life Cycle of the Cellular Slime Mold, Dictyostelium discoideum

The cellular slime mold Dictyostelium discoideum was first discovered by Raper (1935) in North Carolina. The organism is normally found in nature as a soil amoeba in forest detritus. A unique characteristic of this organism is the series of morphogenic events which occur upon depletion of its bacterial food source. Thus, D. discoideum is a prime system for detailed studies of the molecular basis of differentiation.

The life cycle of D. discoideum is shown in Fig. 1 (Jacobson & Lodish, 1975). Cells undergoing vegetative growth feed on bacteria in decaying fallen leaves and other matter, and divide by binary fission. The process of development is triggered by starvation. The cells collect in large streaming patterns to form groups containing up to $1 \times 10^5$ cells. This aggregation phase is oriented by a chemotactic response to cAMP (see Newell, 1977, 1978 for review).

Aggregating amoebae become intergrated by deposition of a surface sheath covering the whole mound of cells. The aggregate topples over and is now referred to as a pseudoplasmodium, grex, or slug. The slug is capable of migration in search of additional nourishment, and is both thermotactic and phototactic. At this stage, the homogenous cell population begins to differentiate into two cell types. The anterior third of the slug is committed to differentiate into stalk cells, while the posterior portion is committed (but reversibly) to differentiate into spore cells. Prespore cells contain a characteristic "prespore vesicle" (Hohl & Hamamoto, 1969).

After a variable period of migration, the slug halts and the
Figure 1. The Life Cycle of *Dictyostelium discoideum* (redrawn from Jacobson & Lodish, 1975).
anterior tip begins to move vertically in preparation for terminal differentiation. At this point in "culmination" (Fig. 1), cellulose is synthesized by cells near the tip of the mass, forming a sheath. Prestalk cells within this sheath expand and vacuolize to form the stalk. Once the stalk sheath makes contact with the underlying support on the agar surface, all further expansion results in extending the stalk vertically. As the mass rises, the peripheral cells begin to encapsulate and form spores. The formation of the fruiting body (sorocarp) is complete when all of the cells have differentiated into stalk or spore cells. The stalk cells are no longer viable after vacuolization, but mature spores may germinate, releasing amoebae (Raper & Fennell, 1952).

It is not the purpose of this thesis to extensively review the life cycle of D. discoideum. A more thorough treatment of the subject may be found in the excellent reviews of Bonner (1967) and Loomis (1975).

**B. Lysosomal Acid Hydrolase Activities During the Development of D. discoideum**

At present only a small percentage of the total enzymatic complement of D. discoideum has been analyzed at any stage in development. Many enzymes have been found to accumulate during discrete stages of development. At present, there are thirteen stage-specific enzymes which have been studied in detail. In the present study, the activities of four of these marker enzymes were examined during spore germination: N-acetyl-\(\beta\)-glucosaminidase (NAG), \(\beta\)-glucosidase (B-Glc), \(\alpha\)-mannosidase (Mann) and alkaline phosphatase. Alkaline phosphatase and \(\beta\)-glucosidase will be discussed later in this literature review. This portion of the review will be concerned with the remaining lysosomal acid hydrolases which were examined.
Loomis (1970) initially found that α-mannosidase (EC 3.2.1.24) accumulated soon after the start of development (preaggregation) but did not reach a maximal activity until 20 hours of development (culmination). The accumulation of the enzyme requires protein synthesis. The enzyme has been found to be stable in the absence of protein synthesis, so the increase in specific activity during development implies de novo synthesis of the enzyme (Loomis, 1970, 1975). Quance and Ashworth (1972) established that the enzyme undergoes both qualitative and quantitative changes during development, depending on the medium in which the myxamoebae are grown. The specific activity of the enzyme is due to the presence of two isozymes. The minor isozyme, Mann-2, accounts for 10% of the total activity in the fruiting body (Free & Loomis, 1974).

Mutant strains which specifically lack Mann-1 have been isolated, but the loss of this isozyme does not disrupt the normal biochemical sequence of differentiation (Free & Loomis, 1974). However, Free et al. (1978) recently reported on the characterization of D. discoideum strain M31, which accumulates only 1/3 of the normal amount of Mann-1 during development (Free & Loomis, 1974). They discovered that this mutant strain also had approximately 1/3 of the NAC activity compared to the level found in wild type cells (Free & Schinke, 1978; Free et al., 1978). Furthermore, the alterations resulting from the mutation bestow different properties upon many glycosidases (Free et al., 1978).

The Mann-2 isozyme accumulates only during the culmination stage of development. Studies of inhibitors of protein and RNA syntheses indicate that both protein and RNA syntheses are required for the increase in α'-mannosidase-2 activity (Free & Loomis, 1974; Free et al., 1976).

The isozymes appear to be located in two different compartments inside the cells. Mann-1 is located in the lysosomes, while Mann-2
appears to be particulate, and probably membrane bound (Ashworth & Quance, 1972; Free & Loomis, 1974; Free et al., 1976). The isozymes differ with respect to their pH optima and Km values. Furthermore, Mann-2 can be completely inhibited by 0.01 M cysteine or glutathione, a tripeptide containing cysteine, while the other isozyme is unaffected by these compounds (Free et al., 1976). Every and Ashworth (1973) isolated two electrophoretically distinct Mann isozymes from spent growth medium.

N-acetyl-β-glucosaminidase (EC 3.2.1.30) is another acid hydrolase located in the lysosome which has been extensively studied (Ashworth & Quance, 1972). Loomis (1969a) initially showed that the specific activity of NAG increases greater than ten fold after ten hours of development. The specific activity of the enzyme remains essentially constant until after spore germination; the level is then reduced to the normal vegetative level. The increase in the specific activity during development requires protein synthesis. RNA synthesis is also required two hours after the start of development (Loomis, 1969a, 1975). Quance and Ashworth (1972) demonstrated that the specific activity pattern of NAG during development could be altered both quantitatively and qualitatively depending on the medium upon which the myxamoebae were grown. This is similar to the effect on Mann activity. Loomis (1969a, 1975) found that mutant strains which were blocked slightly after the first step in morphogenesis synthesized the enzyme at the normal rate but for an extended period. Mutant strains which did not undergo the first morphogenic step did not exhibit an increase in specific activity. Loomis (1969a, 1975) concluded that the initiation and termination of NAG synthesis are controlled by the developmental program.
Every and Ashworth (1975) cited the following evidence to show that NAG activity accumulates as a result of de novo enzyme synthesis:

(1) Actinomycin D inhibits the accumulation of assayable NAG activity two hours after the drug is added to developing cells (Loomis, 1969a).

(2) Cycloheximide added during the first few hours of development stops the accumulation of enzyme activity (Loomis, 1969a). (3) No diffusible activators or inhibitors of enzyme activity are found during growth or differentiation (Loomis, 1969a; Every & Ashworth, 1975). (4) The enzyme is not found to be "unmasked" at any stage of development by \((\text{NH}_4)_2\text{SO}_4\) fractionation (Every & Ashworth, 1973). Wright and her co-workers have shown that trehalose-6-phosphate synthetase could be "unmasked" by \((\text{NH}_4)_2\text{SO}_4\) fractionation (Killick & Wright, 1972). (5) As assayable NAG activity increases during development, there is a exact proportional increase in the amount of specific anti-enzyme antibodies required to precipitate enzyme activity (Every & Ashworth, 1975). (6) Radioactively labeled amino acids are incorporated into enzyme molecules (Every & Ashworth, 1975).

Recently, Grabel and Loomis (1978) have reported that the accumulation of NAG appears to be dependant upon the action of a heat stable effector molecule excreted by the cells. The presence of the effector is required for at least three and one half hours during development. However, in the presence of cycloheximide and the effector, cells do not accumulate the enzyme. Therefore, the possibility that the effector is simply activating the enzyme, rather than inducing its synthesis can be eliminated.

NAG is excreted during both the stationary growth and slug migration phases of the life cycle (Ashworth & Quance, 1972; Dimond & Loomis, 1974).
The enzyme appears to be essential for slug migration (Dimond et al., 1973; Loomis et al., 1976). Since mutant strains lacking the enzyme are unable to migrate normally and have an altered component in the sheath surrounding the slugs (Dimond et al., 1973; Freeze & Loomis, 1977).

NAG purified from the spent medium of strain AX2 has a pH optimum of 5.0. The active enzyme has an apparent molecular weight of 168,000 daltons, with subunits of 65,000 and 55,000 daltons (Every & Ashworth, 1973; Dimond et al., 1973). A single gene appears to account for 99% of the activity (Dimond et al., 1973); the remaining 1% is a minor isozyme with distinct properties (Dimond & Loomis, 1974).

\( \beta \)-galactosidase (EC 3.2.1.23), which accumulates during the differentiation process, has not been studied as extensively as the other thirteen marker enzymes. Oohata (1976a) found two peaks of activity for B-Gal during the aggregation phase which occurs eight hours after the start of development. The activity of the enzyme decreases slightly, followed by a further increase in specific activity, reaching a maximum during culmination. However, Kilpatrick and Stirling (1976), using fluorogenic assaying techniques, detected only a slight increase in specific activity during most of development. The mature fruits were found to have the highest activity. They suggested that the enzyme may function to degrade a spore wall component.

Oohata (1976b) believes one possible role of B-Gal is to degrade acid mucopolysaccharide contained in the prespore vesicles. Oohata reported finding the enzyme activity increasing after dedifferentiation of cells disaggregated from a slug. This increase in enzyme activity reached a peak three hours into dedifferentiation, and coincided with the decomposition of acid mucopolysaccharide contained in the prespore vesicles.
The enzyme was reported to completely disappear upon completion of dedifferentiation. Both B-Gal accumulation and dedifferentiation are dependant on protein synthesis (Oohata, 1976a). The isozyme accumulating during dedifferentiation has been reported to be electrophoretically different from three other isozymes present in D. discoideum cells (Oohata, 1976a,b).

Electrophoretic analysis of B-Gal activity has identified four different isozymes. One specific isozyme is located only in vegetative cells, while another is found to be common to all types of cells. A third band is recovered from all morphogenic stages. The last band is the one found only in disaggregated slug cells (Oohata, 1976a,b).

In Dictyostelium mucoroides, a related species, pre stalk cells from the pseudoplasmodium have a higher B-Gal activity than prespore cells (Oohata & Takeuchi, 1977). The enzyme activity is also composed of four isozymes, three of which are common to both types of cells. One isozyme is detected only in pre stalk cells. The fourth B-Gal isozyme and an isozyme of acid phosphatase which is only found in pre stalk cells are the only specific markers for pre stalk cells (Oohata & Takeuchi, 1977).

Another lysosomal acid hydrolase which has not been characterized extensively is α-glucosidase (EC 3.2.1.20). This enzyme is present throughout development and appears to display a balanced turnover during most of development. The specific activity of the enzyme drops fairly rapidly when protein synthesis is blocked by cycloheximide (Loomis, 1975). Thus, A-Glc activity may result from constitutive synthesis of the enzyme throughout the life cycle. There is a slight accumulation of the enzyme during aggregation as a result of de novo synthesis of the enzyme (Every & Ashworth, 1973, 1975). Only one enzyme species can be isolated from
all stages of development. Loomis suggests that the enzyme may be an acid amylase which functions (1) to hydrolyze bacterial glycogen during vegetative growth and (2) to remove stored glycogen during development (Loomis, 1975).

Acid phosphatase, an acid hydrolase associated with the lysosomes, (Wiener & Ashworth, 1970) can be detected in all stages of the life cycle (Solomon et al., 1964). The accumulation of the enzyme begins at the initiation of development and requires protein synthesis (Wiener & Ashworth, 1970; Loomis, 1975). Acid phosphatase activity is greatest during the culmination stage of differentiation, while mature spores have minimal activity (Solomon et al., 1964). The pattern of enzyme activity during development changes quantitatively, but not qualitatively, if the amoebae are grown axenically, as opposed to growth in association with bacteria (Quance & Ashworth, 1972).

Parish (1976) recently reported on a minor isozyme of acid phosphatase which is associated exclusively with the external surface of the plasma membrane of vegetative amoebae. The isozyme is not present in phagocytic vacuoles isolated with latex beads. This isozyme cannot be equated with any of the isozymes first reported by Solomon et al., (1964). The isozyme disappears from the cells removed from nutrient medium and does not reappear during development. Inhibitors of protein synthesis cause a rapid loss of the isozyme, although only a 25% reduction in protein synthesis is required for this effect (Parish, 1976).

C. The Expression of β-glucosidase Activity During the Development of D. discoideum

Preliminary results suggested that β-glucosidase activity could be detected in D. discoideum cells during all stages of morphogenesis (Ros-
ness, 1968). However, this activity is the result of two isozymes of B-Glc in strain NC4. The first isozyme, B-Glc-1, shows a peak activity four hours after the removal of food from myxamoebae, after which the activity decreases. At culmination, about fourteen hours after the start of development, B-Glc-2 begins to appear, increasing in specific activity until a maximum is reached after culmination (Coston & Loomis, 1969).

The two isozymes of B-Glc are electrophoretically distinct; occasionally, a third peak is found (Coston & Loomis, 1969). Studies of developmentally defective mutant strains suggest a reduced or absent B-Glc-2 activity; these findings infer that the isozyme is developmentally controlled (Coston & Loomis, 1969; Loomis et al., 1976). The accumulation of B-Glc-2 during development requires both RNA and protein syntheses, as well as specific multicellular organization. However, accumulation of B-Glc-1 does not require RNA and protein syntheses (Coston & Loomis, 1969).

Ashworth and Quance (1973) showed that myxamoebae grown with host bacteria have a higher B-Glc activity than myxamoebae grown axenically with or without glucose. The presence of glucose has little or no effect on B-Glc-1 synthesis (Quance & Ashworth, 1972). However, Rosness (1968) found that 0.01 M D-glucose inhibited 50% of the enzyme activity; B-Glc activity is also inhibited by purines (Parish, 1977).

Myxamoebae of strain AX2 grown axenically show an enzyme pattern during development which is qualitatively similar, but quantitatively different from myxamoebae grown on bacteria (Quance & Ashworth, 1972). B-Glc activity initially falls rapidly during development due to the excretion and degradation of the enzyme (Every & Ashworth, 1975).
B-Glc is an acid hydrolase associated with the lysosome in a manner similar to other acid hydrolases (Ashworth & Quance, 1972; Wiener & Ashworth, 1970). The pH optimum for intracellular β-glucosidase activity was originally determined to be pH 4.0-4.5 (Rosness, 1968). However, Coston and Loomis (1969) reported the peak range to be pH 4.0-5.5. Recently, Free and Schimke (1978) studying a post-translational modification mutant strain, M31, suggested that the pH optimum is 2.8. The pH optimum for purified extracellular enzyme from spent medium of strain AX2 is 3.0-4.2 (Every & Ashworth, 1973).

Intracellular enzyme from an early stage of development undergoes a 40% reduction in activity when stored at 4°C for one week. However, the isoynzyme from mature sorocarps does not decay in activity when stored similarly (Rosness, 1968). Purified extracellular enzyme is stable when stored at 4°C in water or dilute buffer at the pH optimum (Every & Ashworth, 1973).

The two isoynzymes of B-Glc are similar in many characteristics, differing only in Km values for substrate paranitrophenol-β-glucoside (Coston & Loomis, 1969). Mutant strains lacking B-Glc-1 fail to produce B-Glc-2, suggesting that the two isoynzymes share a common subunit. Antibody work further supports this idea. The purified B-Glc-1 has been found to consist of two dissimilar subunits with apparent molecular weights of 88,000 and 68,000 daltons (Dimond & Loomis, 1976).

Functionally, several roles have been postulated for the enzyme. Rosness (1968) assumed that the function of the enzyme in D. discoideum was to hydrolyze cellobiose and cellotriose. Coston and Loomis (1969) have suggested that the enzyme may function during germination to open and utilize the spore coat. The spore would have the two isoynzymes
stored inside the protoplast; the isoymes might be located in some special cellular compartment, presumably with a distinct location for each isozyme. The accumulated B-Glc-2 would become physiologically active in some form during germination (Coston & Loomis, 1969). However, in D. mucoroides, B-Glc activity was found to be higher in prestalk cells than prespore cells (Ochata & Takeuchi, 1977). Mutant strains derived from strain AX3 which lack or have reduced B-Glc activity produce macrocyst-like spheres in the absence of the opposite mating type. This suggests that β-glucosidase may be required to prevent "selfing" and insure that macrocysts will form only when two mating types are present (Dimond & Loomis, 1976).

D. The Expression of Alkaline Phosphatase Activity During D. discoideum Development

Alkaline phosphatase (EC 3.1.3.1) activity increases late in development, some 18 and 26 hours after the initiation of differentiation. The increase in enzyme activity requires the multicellular state provided by the culminating fruiting bodies (Loomis, 1969b, 1975). The enzyme consists of two electrophoretically distinct isoymes. Alkaline phosphatase-2 is the isozyme found to accumulate during development (Solomon et al., 1964; Loomis, 1975). Because the accumulation of the enzyme occurs late in development, Loomis (1969b, 1975) has suggested that the enzyme might have a role during germination.

Alkaline phosphatase has been reported to be plasma membrane bound and not associated with phagocytic vacuoles (Crean & Rossomando, 1977; Parish & Pelli, 1974). However, Quiviger et al., (1978) recently reported cytochemical evidence that alkaline phosphatase is associated with the contractile vacuoles. No reaction product was associated with the plasma
membrane or phagocytic vacuoles. However, this does not completely rule out the possibility of low enzyme activity present at the plasma membrane since negative results are considered ambiguous when using cytochemical studies.

E. The Expression of Trehalase Activity During the Life Cycle of D. discoideum

Ceccarini (1965) initially reported the presence of the hydrolytic enzyme, trehalase, in the cellular slime mold, D. discoideum. The purified enzyme has an optimal pH of 5.5 and temperature optimum of 45 C. It is generally agreed that the enzyme is present in almost all stages of the life cycle. However, the presence of trehalase in dormant spores has been debated. Wright and her co-workers (Killick & Wright, 1972; Wright & Killick, 1975) and Roth & Sussman (1966) have reported trehalase in all stages of the life cycle. Jefferson and Rutherford (1976) disagreed, and have reported that they found no trehalase activity in dormant spores, but high trehalase activity in stalk cells.

Ceccarini (1967) indicated that vegetative amoebae have a high level of trehalase activity, but preferentially release the enzyme into the medium at aggregation. Once the enzyme is released, de novo synthesis of the enzyme only occurs if the amoebae are fed E. coli.

The activity of trehalase increases 7-8 fold upon spore germination as compared to the activity level in dormant spores. This increase in activity is cycloheximide-sensitive (Cotter & Raper, 1970).

F. Microcyst Formation and Germination in the Dictyosteliaceae

In several species of the cellular slime molds, there is an alternative sequence of development other than the formation of fruiting bodies. After vegetative growth, cells round up and individually encyst to form
microcysts.

Microst formation in Polysphondylium pallidum has been shown to have developmentally regulated protein synthesis (Francis, 1976).

Microcysts germinate synchronously within a few hours after being placed in a sterile non-nutrient medium (Cotter & Raper, 1968c). Ultrastructural studies of P. pallidum microcyst germination show that the process involves the removal of a bilayered cell wall containing protein, lipid, cellulose and glycogen (Hohl et al., 1970; Toama & Raper, 1967).

Both protein and RNA syntheses occur during P. pallidum microcyst germination (O'Day, 1974, 1976; O'Day et al., 1976; Gwynne & O'Day, 1978; Ennis et al., 1978). Protein synthesis is required for the completion of P. pallidum microcyst germination (Cotter & Raper, 1968c; O'Day et al., 1976; O'Day, 1974). Recently, P. pallidum microcysts have been reported to contain poly(A)+ RNA that can stimulate the incorporation of radioactively labeled amino acids into protein in an in vitro wheat germ protein synthesizing system (Ennis et al., 1978). The incorporation of precursors and the appearance of polysomes indicates that protein synthesis starts immediately after the microcysts are incubated under permissive conditions (Ennis et al., 1978; Gwynne & O'Day, 1978).

Studies of enzyme activities during P. pallidum microcyst germination have revealed intracellular increases of two enzymes, alkaline phosphatase (O'Day, 1974) and acid protease (O'Day, 1976). An extracellular increase in nine lysosomal glycosidases has also been reported (O'Day, 1974). All of these increases in activity are cycloheximide-sensitive (O'Day, 1974; O'Day et al., 1976).
Recently, germinating *P. pallidum* microcysts have been reported to possess carboxymethyl cellulase activity. This CM-cellulase activity increases several fold intracellularly and is excreted into the medium. The intracellular increase in activity can be blocked by cycloheximide. The addition of the enzyme extracellularly enhances the emergence of myxamoebae from microcysts, suggesting a possible role in germination. Inhibition of B-Glc activity with D-gluconic acid lactone was also found to inhibit emergence of myxamoebae implicating the involvement of this enzyme in microcyst germination (O'Day & Paterno, 1979).

G. Spore Germination in *Dictyostelium discoideum*

1. Spore Germination in General

*D. discoideum* spore germination consists of four stages: activation of dormant spores, postactivation lag, swelling of activated spores, and the release of a single myxamoeba from each swollen spore (Cotter, 1975). Wild type strain NC4 spores are constitutively dormant and must be activated by some treatment in order to germinate (Cotter & Raper, 1966). Dormant spores can be activated by various techniques, including suspension in peptone or a mixture of tryptophan, methionine and phenylalanine (Cotter & Raper, 1966), by the application of mild heat at 45°C for 30 minutes (Cotter & Raper, 1966, '1966a,b'), gamma radiation (Khoury et al., 1970), 8 M urea and its derivatives (Cotter & O'Connell, 1976; Cotter, 1979), 20 % DMSO (Cotter et al., 1976) and polyethylene glycol (Cotter, 1977). Spores activated by these procedures proceed through the same sequence, but at slightly different rates.

Figure 2 represents the kinetics of heat-induced spore germination and a diagramatic representation of spore germination. The time between the removal of the activating stimulus and the first sign of
Figure 2. The Germination Sequence of *Dictyostelium discoideum* Spores
Heat Activated at 45°C for 30 Min. (redrawn from Cotter, 1975)

(○) percent spore swelling; (●) percent myxamoebae emerged; morphological changes are shown schematically above the germination curves.
spore swelling is called the postactivation lag, and can be divided into two almost equal sections: early and late lag phase (Cotter, 1975). The postactivation lag for heat-activated spores is normally 60 minutes (Cotter & George, 1975). The lag for spores activated with urea, DMSO or ethylene glycol is approximately 30 minutes in duration (Cotter, 1977; Cotter et al. 1976; Cotter & O'Connell, 1976; Ennis & Sussman, 1975). However, the kinetics of spore swelling under optimal conditions are essentially sigmoidal regardless of the activation treatment.

The swelling stage of germination begins with the appearance of a lateral protuberance in the spore wall which enlarges until the entire spore is swollen. This is accompanied by a loss in refractility, so that the swollen spores appear phase dark when observed under a phase contrast microscope. The later part of this stage is characterized by the appearance of granules and one or more contractile vacuoles (Cotter & Raper, 1966, 1968a,b; Cotter, 1975). The swelling stage ends with a longitudinal splitting of the outer two spore walls (Cotter & Raper, 1966, 1968b; Hemmes et al., 1972).

The emergence stage of spore germination has been defined as the dissolution of the innermost wall with the release of the myxamoebae (Hemmes et al., 1972; Cotter, 1975). In this stage the lipoidal bodies and proteinaceous crystals are degraded so that the true vegetative amoebae are devoid of these structures (Cotter, 1975).

The process of spore germination in D. discoideum can be affected by four environmental factors: oxygen tension, pH, osmotic pressure and temperature. Spores in the lag phase of germination are usually deactivated if exposed to extremes of the above environmental conditions; such spores do not swell but instead return to the dormant state.
Spores in the swelling and emergence stages may be killed if exposed to these same severe conditions (Cotter, 1975; Cotter & Raper, 1968a, b; Cotter et al., 1979).

2. Macromolecular Synthesis During D. discoideum Spore Germination

Studies using cytoplasmic inhibitors of protein synthesis and amino acid analogs have suggested that protein synthesis is required for the completion of spore germination (Cotter & Raper, 1970). These inhibitors of protein synthesis allow activated spores to swell, but block the emergence of myxamoebae (Cotter & Raper, 1970). Kobilinsky and Beattie (1977) reported that chloramphenicol, an inhibitor of mitochondrial protein synthesis, at a concentration of 4 mg/ml inhibits myxamoebae emergence.

The initiation of protein synthesis, as measured by the incorporation of radioactively labeled amino acids, was first investigated by Bacon and Sussman (1973), who reported that the incorporation of amino acids begins immediately after spore activation and increases in rate during the swelling stage. However, recent studies (Yaguri & Iwabuchi, 1976; Giri & Ennis, 1977) indicate that incorporation of labeled amino acids begins during early swelling stage, and increases rapidly in rate during the emergence stage of germination. Data on the appearance of polysomes supports the belief that protein synthesis initially occurs at the early swelling stage. Polysomes are not found in dormant spores, but appear only during spore swelling. The first appearance of polysomes is detected approximately at the same time in the germination sequence as the start of the incorporation of radioactively labeled amino acids (Giri & Ennis, 1977). In the case of Dictyostelium purpureum spore germination, polysomes also appear following the
initiation of spore swelling (Plet et al., 1971)

The incorporation of radioactively labeled uridine occurs at a very low rate during the swelling stage, but the rate rapidly increases during the emergence stage (Yaguri & Iwabuchi, 1976; Giri & Ennis, 1977). However, without knowledge of the size of the precursor pools in germinating spores, the exact time of the initiation of these macromolecular syntheses cannot be accurately determined (Giri & Ennis, 1977).

Three inhibitors of RNA synthesis have been reported to block the completion of spore germination. Two of the inhibitors, daunomycin and lomofugin, appear to block the emergence stage, and another, thiolutin, has been reported to block spore swelling as well (Giri & Ennis, 1977), suggesting that RNA synthesis is required for the completion of spore germination.

The regulation of synthesis of different types of RNA has recently been investigated (Yaguri & Iwabuchi, 1976; Giri & Ennis, 1977, 1978). Giri and Ennis believe that RNA is synthesized de novo and is developmentally regulated. They examined newly synthesized RNA from germinating spores labeled at hourly intervals with radioactively labeled precursors. The RNA was separated into two groups based on the ability to bind to an oligo(dT)cellulose column. The RNA fraction which bound to the column consists of poly(A)+ messenger-like RNA. The RNA fraction which did not bind to the column consists of 4s RNA (presumably tRNA), rRNA, and a small amount of mRNA. They reported that the amount of label in the fraction of poly(A)-RNA increases during spore germination. However, the amount of label in the poly(A)+RNA fraction decreases when comparing the 0-1 hour labeled RNA to the 2-3 hour labeled RNA sample, suggesting that the mRNA is made during the initial
stages of germination (Giri & Ennis, 1978). Sedimentation analysis of the poly(A)-RNA fraction by Giri and Ennis (1978) indicates that, during the initial stages of germination, the majority of the RNA synthesis is mRNA and tRNA, as previously suggested by Yaguri and Iwabuchi (1976), and the initiation of rRNA synthesis occurs during the later stages of germination.

Fractions of mRNA from dormant spores, swollen spores and emerged myxamoebae have been reported to have the ability to stimulate the incorporation of amino acids into protein in an in vitro wheat germ system. As spores germinate, the amount of mRNA increases. The poly(A)+ mRNA from dormant spores is less efficient and has a lower specific activity than poly(A)+RNA from swollen spores and emerged{myxamoebae (Giri & Ennis, 1978).}

Analysis of protein synthesized in vitro during spore germination on SDS polyacrylamide gel electrophoresis indicates four classes of proteins: (1) proteins labeled early (0-1 hour, postactivated lag and early swelling stages) but not labeled at later stages during germination, (2) proteins which are synthesized in larger quantities at early stages of germination than at later stages, (3) proteins which appear only after the first hour of labeling and, (4) proteins labeled throughout germination (Giri & Ennis, 1977, 1978).

The only enzymes that have been examined during D. discoideum spore germination are trehalase (Cotter & Raper, 1970) and cellulase (Jones et al., 1979). The expression of trehalase activity increases several fold upon germination (Cotter & Raper, 1970). Recently, Jones et al., (1979) reported that dormant spores contain cellulase activity. Heat-activated spores release the cellulase activity into the extra-
cellular medium as the spores germinate. This release of cellulase, which begins during the swelling stage of germination, is not blocked by the presence of cycloheximide, although the emergence of the myxamoebae is prevented.

H. Macrocyst Formation and Germination in the Dictyosteliaceae

Following vegetative growth in several species of the cellular slime molds, there is another alternative sequence of development other than fruiting body formation which is called macrocyst formation (Blaskovics & Raper, 1957). The first morphological event in macrocyst formation (as shown in Fig. 1) is the production of a giant cell (cytophagic cell) in the center of an aggregate of myxamoebae. Initially, this cell is a binucleate heterokaryon because of cell fusion. Although the size of the aggregate does not change significantly, the size of the giant cell does increase as the cytophagic cell engulfs surrounding amoebae. The engulfed amoebae become encased in a vacuole called an endocyte. Soon after the giant cell begins ingesting neighboring amoebae, the cell becomes uninucleate and diploid. At this time, the primary wall of the macrocyst is laid down. This wall is composed of a fibrillar material similar to the slime sheath that surrounds the pseudoplasmodium. As the giant cell grows, the endocytes break down and are eventually digested, remaining for a long time as characteristic granules in the cytoplasm of the mature macrocyst. At this time, the secondary and tertiary walls are made. The secondary wall is composed of a rigid cellulosic material, which is ultrastructurally similar to a microcyst wall, although considerably thicker. The tertiary wall is composed of three layers, and is considerably thicker and more flexible than the secondary wall. It is similar in structure
to that of the spore wall (Hohl, 1976; Hohl et al., 1970; Hennes et al.,
1972; Blaskovics & Raper, 1957).

The mature macrocyst has cytoplasm filled with numerous granules
representing the remnants of endocytes, lipoidal inclusions and numer-
os nuclei. The entire protoplast is surrounded by the three walls
(Hohl, 1976).

Erdos et al., (1972) has found evidence for the occurrence of
meiosis when most of the endocytes have been fragmented. Within the
nucleus of the giant cell, single or paired axial elements of the
synaptonal complex have been observed. Subsequently, the giant cell
becomes multinucleated and the nuclei return to normal size. The
sequence from binucleate to uninucleate, and finally to multinucleate
stage, is strong evidence for a sexual process.

Mickerson and Raper (1973b) indicated that macrocyst germination is
age dependant. Old cysts germinate more readily than young macrocysts.
With young macrocysts, light is necessary to stimulate germination.

Morphologically, germination begins with the swelling of the dark
contracted contents of the dormant macrocysts. At this time, the inner
dense layer of the tertiary wall is split into two separate parts.
There is gradual loss of color and reappearance of cells within what
previously appeared as homogenous protoplasmic mass. The cyst then
cleaves into large uninucleate segments which are larger than myxamoebae.
These pro-amoebae still contain many endocyte fragments. The number of
organelles increases during this time, and the cytoplasm becomes less
dense. The secondary wall seems less compact at this stage, and it,
together with the separated part of the tertiary wall, breaks away,
leaving the inner part of the tertiary wall still intact. The pro-
amoebae divide several times to form myxamoebae. Macrocysts germinating in the presence of 150 μg/ml of cycloheximide proceed only to this point. Finally, the previously intact portion of the tertiary wall ruptures, liberating the myxamoebae; from this point on they behave as normal trophic myxamoebae (Nickerson & Raper, 1973b; Erdos et al., 1973).

I. The Purpose of this Study

The purpose of this study is to investigate the regulation of protein synthesis during D. discoideum spore germination using specific enzymes as markers. The activities of six lysosomal acid hydrolases were examined: three of these enzymes (Mann, β-Glc and NAG) are considered to undergo developmentally significant changes during other stages of differentiation. Alkaline phosphatase, which has been reported to be developmentally significant during culmination stage of differentiation and suggested to have a role in germination was also examined. In addition, comparative changes in trehalase activity during the germination of all three resting stages of the Dictyosteliaceae were explored.
II. MATERIALS & METHODS

A. Media Used

Dictyostelium discoideum (Raper, 1935) was grown in conjunction with the bacterium, Escherichia coli strain B/r on glucose-salts agar (Adams, 1959). This medium consists of the following components: 1.0 g NH₄Cl, 0.13 g MgSO₄, 3.0 g KH₂PO₄, 6.0 g NaH₂PO₄ and 20.0 g Bacto agar (Difco) per liter of distilled water. A total of 4.0 g of glucose in solution was sterilized separately and added to the medium after autoclaving. The medium was cooled to 50°C and dispensed into 100 x 15 mm plastic petri dishes.

Macrocysts of Dictyostelium mucoroides strain DM7 were produced on Lactose-Peptone agar (L-P agar) which consists of the following components: 1.0 g lactose, 1.0 g peptone and 20.0 g Bacto agar per liter of distilled water (Nickerson & Raper, 1973a). The medium was autoclaved and dispensed into petri dishes.

The phosphate buffer used in these experiments consisted of 1.04 g KH₂PO₄ in 1 liter of distilled water; the pH of the 10 mM solution was adjusted to 6.5 with concentrated KOH prior to autoclaving.

B. Production and Germination of Dictyostelium discoideum Spores

Spores of Dictyostelium discoideum strain NC4 (diploid) were aseptically transferred from a stock culture to 20-60 ml of sterile distilled water yielding a spore density of 1 x 10⁶/ml. A loopful of E. coli strain B/r was also added to this suspension and mixed thoroughly; 1-2 ml of this suspension was transferred to the glucose-salts plates and spread over the surface. The cultures were shaken 24 h and 48 h after plating to ensure even synchronous fruiting of the slime mold. By the third day aggregation centers were apparent and on the fourth
day mature fruiting bodies had been formed. The fruiting bodies were allowed to age for an additional day before the spores were harvested in order to ensure that all spores used in experimental work had entered dormancy.

Spores of P. discoides strain NC4 (diploid) 1-3 days old were harvested by passing a glass slide several millimeters above the agar surface in such a way as to collect only the spores from the fruiting bodies. The spores were suspended in sterile distilled water and poured into 15 ml conical or round bottom centrifuge tubes. Spore clumping was eliminated by vortexing, and the suspension was subjected to centrifugation in a Damon/IRC clinical centrifuge (Model CL) at speed setting 6 for 5 minutes. The resulting supernatant was saved for possible autoinhibitor substance(s), while the pellet was resuspended in fresh 10 mM phosphate buffer, pH 6.5 (referred to as buffer hereafter). This suspension was centrifuged again in fresh buffer and the procedure was repeated once more for a total of 3 washings.

Spores harvested and prepared in this manner were normally activated by the application of a heat shock. The spores were suspended in 5 ml of phosphate buffer and placed at 45°C for 30 minutes. The heat-activated spores were incubated at 23.5°C to allow germination to occur (Cotter & Raper, 1966, 1968a,b).

Spores were also activated in 5 ml of phosphate buffer at room temperature with the following chemicals: 20% DMSO for 60 minutes (Cotter et al., 1977); 3 M ethylene glycol for 60 minutes (Cotter, 1977) and 8 M urea for 60 minutes (Cotter & O'Connell, 1976). In all cases of chemically induced activation, the spore suspension was freed of the chemicals by centrifugation. The supernatant was discarded and the
spores were resuspended in fresh phosphate buffer, a second centrifugation was performed. The pelleted spores were again suspended in fresh phosphate buffer. In all cases, time zero is considered to be the end of the activation treatment.

Activated spores were placed in 10 x 1 cm test tubes or 250 ml flasks at a concentration between 1 x 10^6 to 1 x 10^7 spores/ml. Spore suspensions were incubated at 23.5 C in a Braun Thermomix 1420 waterbath. The spores were stirred with magnetic stirring bars propelled by a submersible stirring unit (Tri-R micro-submersible magnetic stirrer). Large spore suspensions (100 ml in 250 ml flasks) were incubated in a CSB/Precision Scientific Co. waterbath at 23.5 C. The spores were shaken at 80 oscillations/min.

The percentage of spore swelling and myxamoebae released was monitored by placing approximately 0.03 ml of the suspension on a slide and counting the first 200 objects with a Zeiss phase contrast microscope at a magnification of 650 x. The objects were placed into three groups: dormant spores, swollen spores, and emerged myxamoebae.

C. Production and Germination of Dictyostelium mucoroides Macrocysts

Amoebae from a stock culture of Dictyostelium mucoroides strain DM7 were aseptically transferred to 200 ml of sterile distilled water in a 250 ml flask. Several loopfuls of E. coli B/r were added to the suspension and mixed thoroughly; aliquots (10 ml) of the mixed suspension were added to 0.1 % L-P agar plates. The plates were placed in the dark at room temperature and the organisms were allowed to grow. Macrocysts began to form after three days of growth. The macrocysts were allowed to age at least 20 days after formation to ensure reasonable germination (Nickerson & Raper, 1973a, b).
Macrocysts were harvested from the 0.1 % L-P agar plates with a glass microscope slide and poured into 5 ml conical or round bottom centrifuge tubes. The preparations were centrifuged for 5 min at low speed centrifugation. The pelleted macrocysts were washed twice in phosphate buffer in a manner similar to that described for D. discoideum spores. The final pellet was resuspended in phosphate buffer with 25 ug/ml of streptomycin sulfate (Calbiochem Co.). The suspension was heated at 35 C for 30 minutes to lyse any amoebae which may have been present. The heated macrocysts were pelleted again and resuspended in fresh phosphate buffer with streptomycin sulfate. The macrocysts were poured into glass petri dishes and allowed to germinate under direct light at 25 C. Macrocyst germination was monitored with an Olympus light microscope (Carsen Co.) under the 10 x power objective. Germination was scored as dormant macrocysts and empty macrocyst cases since the germination of a macrocyst results in the emergence of more than one myxamoeba.

D. Production and Germination of Polysphondylium pallidum Microcysts

Amoebae of Polysphondylium pallidum strain WS320 from a stock culture plate were transferred aseptically to 100 ml flasks containing 20-60 ml of sterile distilled water. A loopful of E. coli strain B/r was added to the flasks and the suspension was mixed thoroughly. The mixture was added to glucose-salts plates in 1 ml aliquots. The suspension was evenly spread over the surface of the plates and these plates were stored in the dark at room temperature. After three days of growth, the amoebae had produced microcysts. The microcysts were allowed to age at least 13 days before harvesting (Hohl et al., 1970).
Sterile distilled water was added to the plates containing mature microcysts. The microcysts were carefully dislodged from the agar using a glass microscope slide. The microcysts were collected and poured into 15 ml conical or round bottom centrifuge tubes, and centrifuged in a manner similar to that of D. discoideum spores. The supernatant was discarded and the microcysts were washed twice in phosphate buffer. The final pellet was resuspended in phosphate buffer with 25 ug/ml streptomycin sulfate.

Microcyst germination was monitored with a Zeiss phase contrast microscope. Germination was scored such that individual microorganisms were placed in the following categories: dormant microcysts and emerged myxamoebae (Hohl et al., 1970).

E. Incorporation of Radioactive Isotopes into D. discoideum Spores

The incorporation of radioactively labeled uridine and amino acids was measured according to a modification of the method of Yaguri and Iwabuchi (1976).

Protein and RNA syntheses during heat-induced spore germination were evaluated using [14C-U]-algal protein hydrolysate and [3H-C]-uridine (ICN). The uridine (specific activity 10.4 Ci/m mole) and algal protein hydrolysate (specific activity 0.8 mCi/mg) were used at 34.0 and 3.75 uCi/ml, respectively.

The incorporation of [14C-2]-DL-methionine (NEN), [3H-C]-phenylalanine (ICN), and [14C-2-ring]-tryptophan (ICN) which were reported absent in dormant spores (Bacon & Sussman, 1973) were compared to the incorporation of [14C-U]-leucine (Calbiochem Co.), an amino acid reported to be present in dormant spores. The methionine (specific activity 0.5 mCi/mg), tryptophan (specific activity 20 Ci/m mole) and leucine
(specific activity 222 mCi/mmole) were all used at 1 uCi/ml. Phenylalanine (specific activity 1.25 Ci/mmole) was used at 10 uCi/ml. After activation, the spores were adjusted to $1 \times 10^7$ spores/ml. The labeled precursors were added at the previously mentioned concentrations. Streptomycin sulfate was added to all spore suspensions at a final concentration of 25 ug/ml to inhibit any bacteria which might be present. Triplicate samples were removed from the spore suspension at intervals; 0.2 ml samples of the spore suspension were removed and added to 5 ml of 10% TCA - 5% acetone solution. The insoluble material was precipitated in hot (90°C, 20 min for amino acid incorporation) or cold (0°C, for uridine incorporation) TCA. The precipitated materials were collected on 24 mm glass fiber filters (Whatman GF/A) and washed with four 5 ml aliquots of cold 5% TCA including 1% peptone (Difco), 1 mM methionine, 1 mM tryptophan, 1 mM phenylalanine, 1 mM leucine, or 1 mM uridine. The filters were dried under an infrared lamp and the radioactivity was determined with a Nuclear-Chicago Mark II liquid scintillation counter set at the desired preset channels, using 0.6% PPO (ICN), 0.01% POPOP (ICN)/toluene scintillation solution. All results were standardized to $10^7$ spores/ml concentration and corrected for background for purposes of comparison.

F. Decryption and Cell Breakage Techniques

As many as 20 plates of 2 day old NC4 (diploid) spores were harvested and washed as previously mentioned. The spores were used in the dormant state or allowed to germinate at 23.5°C and examined as myxamoebae. Equal volumes of spores or amoebae in phosphate buffer were placed in 6 round bottom centrifuge tubes. The tubes were centrifuged and the supernatant was discarded. The following procedures
were used to decryptify enzymes or break cells.

A 0.1% solution of Triton X-100 (ICN) in buffer was added to one tube of pelleted cells, and vortexed vigorously. Cells were exposed to the nonionic detergent for 30 minutes with occasional vortexing. After this period of exposure, the cells were centrifuged as previously mentioned. The pellet was resuspended in 0.05 M acetate buffer, pH 5.0 and centrifuged again. This procedure was repeated once more. After the final washing, the pellet was resuspended in fresh acetate buffer and stored on ice until enzymatic assay.

Decryptification with 50% DMSO was by the same procedure as described above except that a solution of 50% DMSO in acetate buffer was used instead of Triton X-100.

Cold acetone (-10 C) was added to another tube of pelleted cells, and quickly vortexed. The suspension was poured onto a Millipore filter apparatus fitted with a Whatman GF/A or GF/C glass fiber filter pad. A vacuum was applied to the system and the filter was washed twice with cold acetone (-10 C). The glass filter was removed and air dried. The decryptified cells were removed from the filter and suspended in 0.05 M acetate buffer (pH 5.0). The resulting suspension was stored in ice until enzymatic assay.

To another tube of pelleted cells was added 5 g of glass beads, 0.25-0.32 mm, (Thomas/Scientific Apparatus); 1.4 ml of 0.05 M acetate buffer was added to the tube so that the buffer rose only 1 mm above the level of the glass beads and the tube was vortexed at high speed for 1 minute. More acetate buffer was added to the tube and the tube was briefly vortexed. The supernatant was removed and stored on ice until enzymatic assay (Van Etten & Freer, 1978).
The preferred method of cell breakage was application of a French pressure cell (Amicon Co.). The pelleted cells were resuspended in 0.05 M acetate buffer (pH 5.0); the suspension was added to the French pressure cell and broken by three passages at 20,000 PSI. The crude cell lysate was stored on ice until enzymatic assay.

The control involved suspending the pelleted cells in 0.05 M acetate buffer (pH 5.0) and storing the suspended cell on ice until enzymatic assay.

All cell suspensions were assayed for enzymatic activity as mentioned in the next section, except that the crude, unfrozen cell lysate were used. Therefore, only units of activity could be determined and the methods of cell breakage and decrypification could only be compared against each other with similar types of cells, since the decrypified cells release very little protein.

G. Enzyme Assay Procedures

1. Trehalase Activity

Enzyme extracts were obtained by pelleting samples of germinating spores by low speed centrifugation. The supernatant was discarded and the pellet was resuspended in 0.1 M citrate buffer, pH 5.5. The cells were broken by three passages through a French pressure cell as above. The resulting crude cell lysate was centrifuged immediately for 10 min at 8,200 g in a Sorvall RC-2B to separate debris from enzyme in the supernatant. The supernatant was decanted off into a clean test tube and stored on ice until assayed.

The enzyme, trehalase, was assayed according to the method of Ceccarini (1966) as modified by Cotter and Raper (1970). The complete procedure is as follows:
1. The incubation mixture contained
   0.6 ml citrate buffer (0.1 M), pH 5.5
   0.5 ml trehalose (50 μM/ml)
   0.1 ml enzyme extract
2. Incubate 30 minutes at 35 C
3. Stop reaction by boiling for 10 minutes
4. Cool and bring to pH 7.0 with 0.8 ml of 0.1 M potassium phosphate buffer
5. Add 2.0 ml of Glucostat Special reagent (Worthington Co.)
6. Incubate for 45 minutes at 35 C
7. Read in a Beckman DB spectrophotometer at 400 nm
8. One unit of activity is equivalent to 1 umole of glucose released in 30 minutes at 35 C

Controls included heated enzyme, which was used to determine the amount of glucose present in the enzyme preparation. Blanks without enzyme but containing all other components and standards with 1 umole of glucose were also used.

Protein was determined by the Bradford procedure (1976) using Bovine serum albumin as a protein standard. The specific activity was determined by dividing the milligrams of protein released into units of enzymatic activity.

2. Lysosomal Acid Hydrolase Activities

For studies of lysosomal acid hydrolase activities, 0.05 M acetate buffer (pH 5.0) was used in all steps instead of 0.1 M citrate buffer (pH 5.5). The cells were pelleted and broken in the same manner as for the trehalase assay, except that the crude cell lysate was frozen immediately. Upon thawing, the lysate was centrifuged at 8,200 g in a
Sorvall RC-2B for 10 minutes. The supernatant was removed, placed in a clean test tube and stored on ice until enzymatic assay.

All assays were preformed according to modified methods of previously reported procedures (Coston & Loomis, 1969; Loomis, 1969a, 1970, 1975). The reaction mixtures were incubated at 23.5°C instead of the previously reported incubation temperatures of 30 and 35°C since (1) the organism cannot survive at temperatures above 27°C and (2) 23.5°C is the temperature used to germinate activated spores. The assays were carried out in a total volume of 0.5 ml and all enzymes assayed (with the exception of NAG) were with equal volumes of substrate and enzyme extract, both in 0.05 M acetate buffer, pH 5.0. NAG was assayed using 0.05 ml enzyme extract. The enzymes, A-Glc, B-Glc, B-Gal, NAG and Mann were assayed using $1 \times 10^{-2}$ M p-nitrophenyl-α-glucoside, $1 \times 10^{-2}$ M p-nitrophenyl-β-glucoside, $1 \times 10^{-2}$ M o-nitrophenyl-β-galactoside, $8 \times 10^{-3}$ M p-nitrophenyl-N-acetyl-β-glucosaminide, and $5 \times 10^{-3}$ M p-nitrophenyl-α-mannoside as their respective substrates. The reaction mixture and the blank without substrate were incubated at 23.5°C and the reaction was stopped by the addition of 1.0 ml of 1 M Na₂CO₃. The optical density at 420 nm was measured with a Beckman DB spectrophotometer. The measurements were corrected for substrate hydrolysis with substrate controls. One unit of activity was defined as that amount of enzyme which released 1 nmole of p-nitrophenol per minute under these conditions. Enzyme activity was described in terms of specific activity, which was equivalent to units of activity per milligram of released protein.

3. Phosphatase Activity
For studies on phosphatase activity, 10 mM MES buffer, pH 6.5, was used in all steps instead of 10 mM phosphate buffer. The samples were pelleted by low speed centrifugation. The pelleted cells were resuspended in the appropriate buffers: 0.05 M acetate buffer (pH 5.0) and 0.01 M Tris-HCl + 0.01 M MgCl$_2$ (pH 8.5) for acid and alkaline phosphatase, respectively. The cells were broken by three passages through a French pressure cell. The acid phosphatase samples were frozen immediately, while the alkaline phosphatase samples were centrifuged, immediately, in a Sorvall RC-2B at 8,200 g for 10 min to remove cellular debris. The resulting supernatants were placed in clean test tubes and stored on ice until enzymatic assay.

The alkaline phosphatase was assayed according to a modification of the procedure of Loomis (1969b). The assay was carried out in a total volume of 0.5 ml with equal volumes of substrate and enzyme extract in 0.01 M Tris-HCl (0.01 M MgCl$_2$) buffer, pH 8.5. The reaction mixture was incubated at 23.5°C using 1 x 10$^{-2}$ M p-nitrophenyl-phosphate as the substrate. The reaction mixture and the blank without substrate were stopped by the addition of 1.0 ml of 1 M Na$_2$CO$_3$. The optical density was measured at 420 nm with a Beckman DB spectrophotometer. The measurements were corrected for substrate hydrolysis. Units of enzyme activity and specific activity are defined as previously described for the lysosomal acid hydrolases.

The acid phosphatase samples were thawed and centrifuged in a manner similar to the alkaline phosphatase samples. The resulting supernatant was composed of 0.05 M acetate buffer, pH 5.0. The reaction mixture was composed of 0.5 ml enzyme extract and 0.5 ml substrate (1 x 10$^{-2}$ M p-nitrophenyl-phosphate in acetate buffer).
The reaction mixture was incubated at 23.5 C and 0.2 ml aliquots were removed from the reaction mixture and added to a test tube containing 0.8 ml of 1 M Na₂CO₃ to stop the reaction. Aliquots were taken at 0, 5, 10 and 15 min after the initiation of the enzyme assay. The time 0 samples were used as blanks when measuring the optical density at 420 nm. Calculation of the specific activity was defined in the same manner as the other lysosomal acid hydrolases.

H. The Effect of pH on Enzyme Activity

Both dormant spores and emerged myxamoebae were pelleted by low speed centrifugation and resuspended in 5 ml of phosphate buffer or 10 mM MES buffer, pH 6.5. For studies on phosphatase activity, MES buffer was used in all steps instead of phosphate buffer. The cells were broken by three passages through a French pressure cell. The resulting supernatant was in most cases frozen immediately, but in some instances was centrifuged immediately. Both unfrozen and freeze-thawed crude cell lysate were centrifuged in a Sorvall RC-2B at 8,200 g for 10 min to remove cellular debris. The resulting supernatants were transferred to clean test tubes and stored on ice until enzymatic assay.

Two different buffers were used for these experiments. A buffer consisting of 10 mM MES, 10 mM Tris, 10 mM citrate, 50 mM acetate and 10 mM MgCl₂ was used in one set of experiments for a pH range of 2.0 to 9.5. In a second set of experiments, pH values of 3.5 to 7.0 were obtained using 100 mM acetate buffer and pH values 2.5 to 3.5 were obtained by using 0.1 M citrate buffer.

The enzyme extracts were diluted five fold with sterile distilled water. The substrates were suspended in the buffers at the various pH values. The enzyme activities were assayed by incubating 0.25 ml
enzyme extract with 0.25 ml of substrate. The reaction mixture was incubated at 23.5 °C and stopped by adding 1.0 ml 1 M Na₂CO₃ to the reaction mixture. A blank without substrate and substrate controls were also prepared and incubated. The optical densities at 420 nm were measured with a Beckman DB spectrophotometer. One unit of activity is defined as that amount of enzyme producing 1 nmole of p-nitrophenol per minute at 23.5 °C. For comparison purposes, the pH with the highest activity was considered to be 100% activity and all other activities at other pH values were compared to that value.
III. RESULTS

A. General Aspects of Macromolecular Synthesis During Germination of Dictyostelium discoideum Spores

1. The Incorporation of Radioactively Labeled Macromolecular Precursors During D. discoideum Spore Germination

RNA and protein syntheses during germination of heat-activated D. discoideum strain NC4 spores was examined by the incorporation of $^3$H-uridine and $^{14}$C-amino acids into TCA insoluble material extracted from whole cells. Incorporation of these radioactively labeled precursors during heat-induced spore germination is shown in Fig. 3. $^3$H-uridine and $^{14}$C-amino acids are incorporated at a low rate for the first 2 hours after activation. This rate increases rapidly as myxamoebae emerge from swollen spores, confirming previously reported results (Yaguri & Iwabuchi, 1976; Giri & Ennis, 1977).

Bacon and Sussman (1973) have reported that methionine, tryptophan, and phenylalanine are absent from dormant spores. The data in Fig. 4 show that the rates of incorporation of these three amino acids during heat-induced spore germination are similar to the rate of incorporation of $^{14}$C-leucine, an amino acid reported to be present in dormant spores (Bacon & Sussman, 1973).

2. The Effect of Inhibitors of Macromolecular Synthesis on the Incorporation of Macromolecular Precursors During Germination of D. discoideum Spores

Chloramphenicol, a specific inhibitor of mitochondrial protein synthesis, has been reported to block emergence of myxamoebae of D. discoideum strain AX3 (at a concentration of 4 mg/ml) (Kobilinsky & Beattie, 1977). However, the Merck Chemical Index (1976)
Figure 3. The Incorporation of $^{14}$C-Amino Acids and $^{3}$H-uridine into TCA-Insoluble Materials During Heat-Induced D. discoideum Strain NC4 Spore Germination

See Materials and Methods section for experimental details. Heat-activated spores were incubated for 5 hours in the presence of labeled precursors and samples were assayed at the designated time intervals. Symbols represent: (□), incorporation of $^{14}$C-algal protein hydrolysate; (△), incorporation of $^{3}$H-uridine; (○), percent spore swelling; and (●), percent of emerged myxamoebae. Incorporation of radioactively labeled materials is expressed as a percentage; the highest counts of radioactivity (CPM) per 1 x 10^7 cells was considered 100 %, and all other values were compared to that value.
Figure 4. Incorporation of Radioactively Labeled Amino Acids into TCA-Insoluble Materials During Heat-Induced D. discoideum Spore Germination

See Materials and Methods section for experimental details. Heat-activated strain NO4 spores were incubated for 5 hours in the presence of the radioactively labeled amino acids and samples were assayed at the designated time intervals. Symbols represent: (△), incorporation of 14C-leucine; (□), incorporation of 14C-methionine; (○), incorporation of 14C-tryptophan; (△), incorporation of 3H-phenylalanine; (○), percent spore swelling; and (●), percent myxamoebae emergence. Incorporation of radioactively labeled materials was expressed as a percentage; the highest counts of radioactivity (CPM) per 1 x 10^7 cells was considered 100%, and all other values were compared to this value.
reports that the saturation point for chloramphenicol is 2.5 mg/ml in water at 25 C. A slurry of 4 mg/ml chloramphenicol does not block the completion of heat-induced strain NC4 spore germination (Table 1). The kinetics of germination are similar to those found with heat-activated strain NC4 spores in the absence of the drug. At 400 µg/ml chloramphenicol inhibits 14C-algal protein hydrolysate incorporation by 29.7% (Table 1) and 3H-phenylalanine incorporation by 20.8% (data not shown), which might be expected since 25% of the total DNA in the organism is mitochondrial (Loomis, 1975). This would suggest that mitochondrial protein synthesis is not required for the germination of D. discoideum spores.

Thiolutin, a potent inhibitor of RNA synthesis (Tipper, 1973), at 100 µg/ml prevents heat-activated strain NC4 spores from swelling and blocks the incorporation of 3H-uridine by 90.7% (Table 1) confirming the results reported by Giri and Ennis (1977). It should be noted, however, that the effects are not reversible when the spores are washed free of the drug. It appears that spores exposed to thiolutin are deactivated; that is they do not swell but return to the dormant state (data not shown).

Aurin tricarboxylic acid (ATA), an inhibitor of protein synthesis (Stewart et al., 1971; Siegel & Apiron, 1971) at concentrations up to 1 mg/ml, has no effect on heat-induced germination of strain NC4 spores (Table 1) or spontaneous germination of strains SG1 and SG2 (data not shown). ATA has no inhibitory effect on 3H-phenylalanine incorporation, indicating that the chemical probably does not penetrate the spores.

Cycloheximide, an inhibitor of peptide chain elongation (Davis et al., 1973), has been shown to reversibly block the emergence stage
Table 1. The Effect of Inhibitors of Macromolecular Syntheses on 
*Dictyostelium discoideum* Spore Germination.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc. (µg/ml)</th>
<th>Effect on Germination</th>
<th>% Inhibition of Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>400</td>
<td>+</td>
<td>29.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thiolutin</td>
<td>100</td>
<td>-</td>
<td>90.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>ATA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>500</td>
<td>+</td>
<td>2.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Edeine</td>
<td>200</td>
<td>+</td>
<td>99.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>200</td>
<td>+</td>
<td>93.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Puromycin</td>
<td>1000</td>
<td>+</td>
<td>99.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>(+) denotes swelling or emergence took place in the presence of the drug; (-), no swelling or emergence was detected.

<sup>b</sup>Incorporation of <sup>14</sup>C-algal protein hydrolysate was determined 5 hours after activation.

<sup>c</sup>Incorporation of <sup>3</sup>H-uridine was determined 5 hours after activation

<sup>d</sup>Aurin Tricarboxylic Acid
of heat-induced spore germination (Cotter & Raper, 1970) and inhibits 93.5% of $^{14}$C-amino acids incorporation at 200 μg/ml with strain NC4 (Table 1). However, cycloheximide has no effect on $^{3}$H-uridine incorporation (Table 1) during heat-induced strain NC4 spore germination. This confirms the findings of Yaguri and Iwabuchi (1976) which indicate that strain NC4 spore germination has no stringent control of RNA synthesis.

Puromycin, an inhibitor which causes premature release of the peptide chain (Davis et al., 1973) has been shown to irreversibly block the emergence stage of strain NC4 spore germination at a concentration of 1 mg/ml (Cotter & Raper, 1970) and inhibits incorporation of $^{14}$C-amino acids by 99.6% (Table 1).

Edeine is an inhibitor of protein synthesis which blocks at the initiation step (Borwska, 1962; Szer & Browska, 1970, 1972; Odem et al., 1978). The drug at a concentration of 200 μg/ml blocks the emergence of myxamoebae from heat-activated spores, strain NC4, and inhibits incorporation of $^{14}$C-amino acids by 99.0% (Table 1). However, this effect is not reversible. Strain NC4 spores heat-activated in the presence of edeine swell but do not release myxamoebae even if washed immediately after activation to remove the drug (data not shown). With SC1 mutants, edeine blocks the emergence of myxamoebae and allows only 25% of the spore population to swell. Blockage of the emergence stage of germination can be accomplished by adding the drug at any point up to 1.5 and 2.0 hours after activation of SC1 and NC4 spores, respectively (data not shown).

Thus, the data suggest that protein and RNA syntheses are not
required for spore swelling, but protein synthesis is required for myxamoebae emergence. This required protein synthesis appears to be cytoplasmic since an inhibitor which blocks mitochondrial protein synthesis does not halt heat-induced spore germination.

B. The Expression of Trehalase Activity During Germination of the Resting Stages of the Dictyosteliaceae

1. The Expression of Trehalase Activity During D. discoideum Spore Germination

Cotter and Raper (1970) reported an increase in the specific activity of trehalase in emerged myxamoebae compared to the level found in dormant spores. When the expression of trehalase is examined during strain NC4 heat-induced spore germination, the enzyme activity is found to increase at the transition point between the spore swelling and myxamoebae emergence stages of germination (Fig. 5). Activation of spores with DMSO, urea or ethylene glycol produces a similar increase in the expression of trehalase activity equal to or greater than the increase detected in heat-activated spores (data not shown).

The expression of trehalase activity was found to be dependent on protein synthesis, that is the increase in trehalase activity is not expressed in spores incubated in the presence of edeine, cycloheximide and puromycin (Table 2). This would indicate one of the following possibilities: (1) de novo synthesis of trehalase; (2) synthesis of a protease which activates an inactive preformed pro-trehalase or (3) synthesis of an enzyme to remove an inhibitor of trehalase.

The expression of trehalase activity in an enzyme extract is
Figure 5. Trehalase Activity During Spore Germination.

Heat-activated spores were incubated at 23.5°C. Symbols represent: •, the percent swollen spores; ○, the percent myxamoebae and △, the ratio of trehalase specific activity compared to the specific activity of dormant spore trehalase.
Table 2. The Effect of Inhibitors of Protein Synthesis on the Expression of Trehalase Activity\textsuperscript{a} During Germination of the Three Resting Stages of the \textit{Dictyosteliaceae}

<table>
<thead>
<tr>
<th></th>
<th>D. discoideum</th>
<th>P. pallidum</th>
<th>D. mucrooides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spores</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Control (dormant)</td>
<td>1.29</td>
<td>1.07</td>
<td>1.13</td>
</tr>
<tr>
<td>Control (emerged)</td>
<td>6.83</td>
<td>7.75</td>
<td>6.76</td>
</tr>
<tr>
<td>Edeine (200 µg/ml)</td>
<td>1.43</td>
<td>1.55</td>
<td>0.44</td>
</tr>
<tr>
<td>Cycloheximide\textsuperscript{b} (400 µg/ml)</td>
<td>1.12</td>
<td>2.16</td>
<td>----</td>
</tr>
<tr>
<td>Puromycin (1000 µg/ml)</td>
<td>1.12</td>
<td>2.16</td>
<td>----</td>
</tr>
</tbody>
</table>

\textsuperscript{a}For comparison purposes, the values shown in the table represent the ratio of treated to dormant spore trehalase activity.

\textsuperscript{b}D. discoideum spores were treated with 200 µg/ml of cycloheximide.
affected by freeze-thawing of the soluble enzyme (Table 3). The expression of trehalase activity increases slightly after freeze-thawing. This would suggest some trehalase is freed from myxamoebae fragments, which otherwise would have remained cryptic after treatment with a French press.

2. The Expression of Trehalase Activity During Germination of Polysphondylium pallidum Microcysts

Microcysts of Polysphondylium pallidum strain WS320 aged for 6 days were allowed to germinate and were assayed for trehalase specific activity (Fig. 6). As the microcysts germinated, the expression of trehalase activity increases 7-8 fold when compared to the specific activity of dormant microcysts. The increase in the expression of trehalase activity occurs as the microcysts begin to release myxamoebae (Fig. 6).

Both the release of myxamoebae and the expression of trehalase activity are blocked when inhibitors of protein synthesis are introduced at the beginning of the incubation period (Table 2). The data suggest that protein synthesis is required for the expression of trehalase activity during microcyst germination.

3. The Expression of Trehalase Activity During Germination of Dictyostelium mucoroides Macroysts

When Dictyostelium mucoroides strain DM7 macrocysts are incubated for 15 days in direct light at 25 C, an average of 67% germination is observed. The expression of trehalase activity in germinating D. mucoroides macrocysts is 6-7 fold greater than the activity level in dormant macrocysts (Table 2). Both the release of myxamoebae from macrocysts and the expression of trehalase activity are blocked by
Table 3. The Effect of Freeze-Thawing on the Expression of Trehalase Activity in Emerged Myxamoebae of *D. discoideum*.

<table>
<thead>
<tr>
<th></th>
<th>Specific Activity of Trehalase</th>
<th>Ratio of Specific Activity to Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (No Freeze-Thawing)</td>
<td>0.175</td>
<td>1.00</td>
</tr>
<tr>
<td>Freeze-Thawed (1x)</td>
<td>0.250</td>
<td>1.44</td>
</tr>
<tr>
<td>Freeze-Thawed (2x)</td>
<td>0.228</td>
<td>1.32</td>
</tr>
<tr>
<td>Freeze-Thawed (3x)</td>
<td>0.239</td>
<td>1.38</td>
</tr>
</tbody>
</table>

Spores were incubated for 5 hours after activation and then sampled for trehalase activity. The emerged myxamoebae were broken by three passages through a French pressure cell at 20,000 PSI. The crude cell lysate was centrifuged at 8,200 g for 10 minutes. The resulting supernatant was assayed and frozen.
Figure 6. Trehalase Activity During *P. pallidum* Microcyst Germination.

Microcysts were incubated at room temperature. Symbols represent: (○), the percent emerged myxamoebae and (△), the ratio of trehalase specific activity compared to the trehalase specific activity of dormant microcysts.
inhibitors of protein synthesis introduced at the beginning of incubation (Table 2). The data suggest that protein synthesis is required for the expression of trehalase activity during D. mucoroides macrocyst germination.

C. The Expression of Lysosomal Acid Hydrolase Activities During the Germination of D. discoideum Spores

1. Cell Breakage and Decryptification of Lysosomes

Activities of lysosomal acid hydrolases have been previously assayed at 30 and 35°C (Loomis, 1969a, 1970, 1975; Oohata, 1976a; Coston & Loomis, 1968). This may be unreasonable, since the vegetative organism cannot survive at temperatures above 27°C (Loomis, 1975). The work presented here is a comparative study of the various lysosomal acid hydrolase activities during spore germination. An attempt was made to standardize the assay techniques used to measure lysosomal acid hydrolase activities. Therefore, enzyme activities in dormant spores and emerged myxamoebae were measured after a variety of breakage and decryptification methods in an attempt to obtain a single satisfactory procedure. Each breakage technique was applied to an equal number of cells, as described in the Materials and Methods section. The various treatments were compared on an activity per tube basis, since the decryptification treatments release no measurable amount of protein. In all cases, the crude cell lysates or decryptified cell preparations were used as enzyme extracts. After the sodium carbonate was added, the reaction mixture was centrifuged at low speed in a clinical centrifuge for 5 minutes to remove the debris and cells. The supernatant was measured at 420 nm with a Beckman DB spectrophotometer.
Tables 4 and 5 present the results of these experiments. With most of the enzymes assayed, the French pressure cell appears to result in the greatest amount of activity. Furthermore, this method of cell breakage was preferred because it produces measurable amounts of protein released so that specific activities of enzymes could be compared.

Untreated spores assayed in 0.05 M acetate buffer (pH 5.0) have measurable enzyme activities. This suggests (1) that these enzymes may be released from the dormant spores, or (2) that some of these enzymes may be located in association with the plasma membrane. The untreated spores incubated in acetate buffer and p-nitrophenyl substrates are viable since spores germinate normally after the removal of the buffer and substrates (data not shown).

2. The Effect of Freeze-Thawing and Centrifugation on the Expression of Lysosomal Acid Hydrolase Activities

In normal lysosomes, the intact membrane prevents both the entrance of substrates to and the exit of lysosomal enzymes from the lysosomes. Thus, the lysosomal enzymes to be measured must be released by the disruption of the lysosomal membrane.

The method of freezing and thawing the enzyme extract was the technique employed to disrupt lysosomal membranes after preliminary cell breakage. Tables 6-9 show the effect of freeze-thawing on the activities of lysosomal acid hydrolase enzymes. From these results, it can be observed that freeze-thawing increases or has little effect on the activities of enzymes assayed. Centrifugation at 8,200 g was performed so that protein released could be measured and specific activities could be compared. As in the case of freeze-thawing,
Table 4. Decryptification and the Expression of Lysosomal Acid Hydrolase Activities in Dormant *D. discoideum* Spores

<table>
<thead>
<tr>
<th>Treatment</th>
<th>A-Glc$^b$</th>
<th>B-Glc$^b$</th>
<th>B-Gal</th>
<th>Mann$^b$</th>
<th>NAC$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (No Treatment)</td>
<td>0.12</td>
<td>0.15</td>
<td>0.15</td>
<td>0.70</td>
<td>0.50</td>
</tr>
<tr>
<td>Acetone (-10°C)</td>
<td>0.18</td>
<td>0.11</td>
<td>0.21</td>
<td>0.06</td>
<td>0.73</td>
</tr>
<tr>
<td>50% DMSO</td>
<td>0.17</td>
<td>0.07</td>
<td>0.18</td>
<td>0.09</td>
<td>0.38</td>
</tr>
<tr>
<td>0.1% Triton X-100</td>
<td>0.08</td>
<td>0.10</td>
<td>0.18</td>
<td>0.05</td>
<td>0.23</td>
</tr>
<tr>
<td>Glass Beads</td>
<td>0.13</td>
<td>0.04</td>
<td>0.06</td>
<td>0.18</td>
<td>2.73</td>
</tr>
<tr>
<td>French Press</td>
<td>0.16</td>
<td>0.15</td>
<td>0.26</td>
<td>0.69</td>
<td>7.19</td>
</tr>
</tbody>
</table>

$^a$Units of enzyme activity are equivalent to moles of p-nitrophenol released per minute at 23.5°C.

$^b$Abbreviations: A-Glc = α-glucosidase

B-Glc = β-glucosidase

B-Gal = β-galactosidase

Mann = α-mannosidase

NAC = N-acetyl-β-glucosaminidase
Table 5. Decryptification and the Expression of Lysosomal Acid Hydrolase Activities in Emerged *D. discoideum* Myxamoebae

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzyme Activities&lt;sup&gt;a&lt;/sup&gt;</th>
<th>A-Glc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B-Glc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B-Gal&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mann&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NAG&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (No Treatment)</td>
<td></td>
<td>0.13</td>
<td>0.12</td>
<td>0.28</td>
<td>0.10</td>
<td>0.62</td>
</tr>
<tr>
<td>Acetone (-10°C)</td>
<td></td>
<td>0.0</td>
<td>0.15</td>
<td>0.45</td>
<td>0.08</td>
<td>0.0</td>
</tr>
<tr>
<td>50% DMSO</td>
<td></td>
<td>0.15</td>
<td>0.12</td>
<td>0.0</td>
<td>0.08</td>
<td>1.70</td>
</tr>
<tr>
<td>0.1% Triton X-100</td>
<td></td>
<td>0.06</td>
<td>0.03</td>
<td>0.0</td>
<td>0.0</td>
<td>0.97</td>
</tr>
<tr>
<td>Glass Beads</td>
<td></td>
<td>0.13</td>
<td>0.34</td>
<td>0.59</td>
<td>0.0</td>
<td>6.40</td>
</tr>
<tr>
<td>French Press</td>
<td></td>
<td>0.13</td>
<td>0.45</td>
<td>1.13</td>
<td>0.08</td>
<td>18.70</td>
</tr>
</tbody>
</table>

<sup>a</sup>Units of enzyme activity are equivalent to μmoles of p-nitrophenol released per minute at 23.5°C.

<sup>b</sup>Abbreviations: A-Glc = α-glucosidase
B-Glc = β-glucosidase
B-Gal = β-galactosidase
Mann = α-mannosidase
NAG = N-acetyl-β-glucosaminidase
Table 6. The Effect of Freeze-Thawing and Centrifugation on Lysosomal Acid Hydrolase Activities\textsuperscript{a} in Dormant \textit{D. discoideum} Spores

<table>
<thead>
<tr>
<th>Enzyme Activities\textsuperscript{a}</th>
<th>A-Glc\textsuperscript{b}</th>
<th>B-Glc\textsuperscript{b}</th>
<th>B-Cal\textsuperscript{b}</th>
<th>Mann\textsuperscript{b}</th>
<th>NAG\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Cell Lysate</td>
<td>0.29</td>
<td>0.62</td>
<td>0.71</td>
<td>0.58</td>
<td>10.07</td>
</tr>
<tr>
<td>Freeze-Thawed Crude Cell Lysate</td>
<td>0.29</td>
<td>0.62</td>
<td>0.71</td>
<td>0.98</td>
<td>10.27</td>
</tr>
<tr>
<td>8,200 g Supernatant of Freeze-Thawed Crude Cell Lysate</td>
<td>0.33</td>
<td>0.62</td>
<td>0.50</td>
<td>0.62</td>
<td>12.00</td>
</tr>
</tbody>
</table>

Dormant spores are washed, broken with three passages through a French pressure cell and assayed for enzymatic activities.

\textsuperscript{a}Activity is defined as moles of p-nitrophenol released per minute at 23.5 °C.

\textsuperscript{b}Abbreviations: A-Glc = $\alpha$-glucosidase
B-Glc = $\beta$-glucosidase
B-Cal = $\beta$-galactosidase
Mann = $\alpha$-mannosidase
NAG = N-acetyl-$\beta$-glucosaminidase
Table 7. The Effect of Freeze-Thawing and Centrifugation on Lysosomal Acid Hydrolase Activities in Heat-Activated Spores at Time Zero

<table>
<thead>
<tr>
<th>Enzyme Activities&lt;sup&gt;a&lt;/sup&gt;</th>
<th>A-Glc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B-Glc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B-Cal&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mann&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NAG&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Cell Lysate</td>
<td>0.29</td>
<td>0.14</td>
<td>0.50</td>
<td>0.45</td>
<td>9.40</td>
</tr>
<tr>
<td>Freeze-Thawed Crude Cell Lysate</td>
<td>0.23</td>
<td>0.14</td>
<td>0.50</td>
<td>0.45</td>
<td>9.90</td>
</tr>
<tr>
<td>8,200 g Supernatant of Freeze-Thawed Crude Cell Lysate</td>
<td>0.29</td>
<td>0.21</td>
<td>0.41</td>
<td>0.58</td>
<td>10.50</td>
</tr>
</tbody>
</table>

Heat-activated spores were broken immediately after activation and assayed for enzymatic activity.

<sup>a</sup>Activity is defined as nmoles of p-nitrophenol released per minute at 23.5°C.

<sup>b</sup>Abbreviations: A-Glc = α-glucosidase
B-Glc = β-glucosidase
B-Cal = β-galactosidase
Mann = α-mannosidase
NAG = N-acetyl-β-glucosaminidase
Table 8. The Effect of Freeze-Thawing and Centrifugation on Lysosomal Acid Hydrolase Activities in Heat-Activated Swollen Spores

<table>
<thead>
<tr>
<th>Enzyme Activities&lt;sup&gt;a&lt;/sup&gt;</th>
<th>A-Glc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B-Glc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B-Gal&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mann&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Cell Lysate</td>
<td>0.18</td>
<td>0.12</td>
<td>0.35</td>
<td>0.58</td>
<td>12.00</td>
</tr>
<tr>
<td>Freeze-Thawed Crude Cell Lysate</td>
<td>0.20</td>
<td>0.12</td>
<td>0.37</td>
<td>0.87</td>
<td>11.10</td>
</tr>
<tr>
<td>8,200 g Supernatant of Freeze-Thawed Crude Cell Lysate</td>
<td>0.22</td>
<td>0.12</td>
<td>0.35</td>
<td>0.87</td>
<td>13.30</td>
</tr>
</tbody>
</table>

Heat-activated spores were allowed to swell at 23.5°C until at least 90% of the spore population was swollen. The spores were broken with a French pressure cell and assayed for activity.

<sup>a</sup>Activity is defined as nmoles of p-nitrophenol released per minute at 23.5°C.

<sup>b</sup>Abbreviations: A-Glc = α-glucosidase  
B-Glc = β-glucosidase  
B-Gal = β-galactosidase  
Mann = α-mannosidase  
NAG = N-acetyl-β-glucosaminidase
<table>
<thead>
<tr>
<th>Enzyme Activities&lt;sup&gt;a&lt;/sup&gt;</th>
<th>A-Glc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B-Glc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B-Gal&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mann&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Cell Lysate</td>
<td>0.43</td>
<td>0.87</td>
<td>0.46</td>
<td>0.23</td>
<td>10.10</td>
</tr>
<tr>
<td>Freeze-Thawed Crude Cell Lysate</td>
<td>0.37</td>
<td>0.82</td>
<td>0.35</td>
<td>0.48</td>
<td>10.30</td>
</tr>
<tr>
<td>8,200 g Supernatant of Freeze-Thawed Crude Cell Lysate</td>
<td>0.52</td>
<td>0.80</td>
<td>0.27</td>
<td>0.23</td>
<td>11.70</td>
</tr>
</tbody>
</table>

Heat-activated spores were allowed to germinate for 5 hours at 23.5 °C. The myxamoebae were broken by a French pressure cell and assayed for activity.

<sup>a</sup>Activity is defined as nmol of p-nitrophenol released per minute at 23.5 °C.

<sup>b</sup>Abbreviations: A-Glc = α-glucosidase  
B-Glc = β-glucosidase  
B-Gal = β-galactosidase  
Mann = α-mannosidase  
NAG = N-acetyl-β-glucosaminidase
centrifugation increases most of the enzyme activities.

In other cell systems, lysosomal membranes are disrupted with Triton X-100, a nonionic detergent, or by sonication (Dean, 1977). However, in the case of D. discoideum spores, the addition of Triton X-100 to crude cell lysate before freeze-thawing does not significantly increase the enzyme activities in the freeze-thawed lysate (Table 10). Sonication for 40 seconds of the freeze-thawed crude cell lysate also does not significantly increase the enzyme activities (data not shown). Therefore, neither treatment with Triton X-100 or sonication increases breakage of lysosomes.

The pellets formed from the 8,200 x g centrifugation of the freeze-thawed crude cell lysate have little or no enzyme activities when resuspended in fresh acetate buffer (data not shown). Therefore, no residual enzyme activity is lost in the pellets.

In further experiments assaying lysosomal acid hydrolase activities, the following method of enzyme extract preparation was used: The cells were pelleted by low speed centrifugation and resuspended in 0.05 M acetate buffer, pH 5.0. The cells were broken by three passages through a French pressure cell at 20,000 PSI. The resulting crude cell lysate was quickly frozen. Upon thawing, the lysate was centrifuged at 8,200 x g to remove cellular debris. The resulting supernatant was removed, placed in a clean test tube, and stored on ice until enzymatic assay.

3. The Effect of pH on the Expression of Lysosomal Acid Hydrolase Activities in D. discoideum

The pH range for each of the lysosomal hydrolases was determined.
Table 10. The Effect of Triton X-100 Upon the Specific Activities of Lysosomal Enzymes of Dormant Spores

<table>
<thead>
<tr>
<th>Enzymes Activities&lt;sup&gt;a&lt;/sup&gt;</th>
<th>A-Glc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B-Glc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B-Gal&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mann&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NAG&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 % Triton X-100 (Control)</td>
<td>17.2</td>
<td>22.2</td>
<td>34.4</td>
<td>50.2</td>
<td>774.2</td>
</tr>
<tr>
<td>0.1 % Triton X-100</td>
<td>12.5</td>
<td>13.9</td>
<td>24.5</td>
<td>24.5</td>
<td>547.7</td>
</tr>
<tr>
<td>0.01 % Triton X-100</td>
<td>20.8</td>
<td>24.8</td>
<td>40.0</td>
<td>47.2</td>
<td>842.4</td>
</tr>
</tbody>
</table>

All spores suspensions were in 0.05 M acetate buffer (pH 5.0) with or without Triton X-100. The cells were broken with three passages through a French pressure cell at 20,000 PSI. The crude cell lysate was immediately frozen. Upon thawing, the lysate was centrifuged at 8,200 g, and the resulting supernatant was assayed for enzyme activities.

<sup>a</sup>Enzyme activity refers to units of activity per mg protein released, where one unit of activity is defined as nmoles of p-nitrophenol released per min at 23.5°C.

<sup>b</sup>Abbreviations: A-Glc = α-glucosidase  
B-Glc = β-glucosidase  
B-Gal = β-galactosidase  
Mann = α-mannosidase  
NAG = N-acetyl-β-glucosaminidase
as described in the Materials and Methods section. The data from these experiments are presented in Figure 7.

The sharp decrease in activity above pH 5.5 for all of the enzymes assayed indicates that they are all acid hydrolases. Extracts of both dormant spores and emerged myxamoebae were tested, but no significant differences in pH optima of the various enzymes were detected between the two cell types. No significant differences in activities were detected when enzymes were assayed using the two sets of buffers described in the Materials and Methods section.

All of the enzymes except B-Gal have a major peak of activity at pH 5.0 ± 0.2 (Fig. 7). B-Gal has a major pH peak at pH 5.5 and a minor peak at pH 3.0 (Fig. 7c). The values reported previously are pH 3.5 (Oohata, 1976a) and pH values 3.5-4.5 (Kilpatrick & Stirling, 1976), using sonicated cells from wild type strain NO4. These peaks of activity may be correlated to the minor activity peak found in Fig. 7c. The peak activity of B-Glc shown in Fig. 7b is similar to the 4.0-5.5 pH values reported by Coston and Loomis (1969). A-Glc has a peak of activity at pH 5.0 which is consistent with that reported by Loomis (1975). Mann has one peak of activity at pH 5.0 (Fig. 7d). This peak has been previously reported as a major peak of activity by Loomis (1970). However, the minor activity peak at pH 3.5 reported by Loomis (1970) is not present (Fig. 7d). NAC has a pH optimum at 5.0 (Fig. 7e) similar to the values reported by Loomis (1969a) and Wiener & Ashworth (1970). A minor peak of activity at pH 2.0 similar to one reported by Wiener and Ashworth (1970) is also present.

Although not all of the enzymes have a pH optimum at pH 5.0, this pH value was used in all further experiments for comparison studies.
Figure 7. The Effect of pH on *D. discoideum* Lysosomal Acid Hydrolase Activities

Both dormant spores and emerged myxamoebae were assayed according to the procedure described in the Materials and Methods section. The closed circles, (●), represent averaged percent of lysosomal enzyme activity, where the highest activity in each separate experiment was considered to be 100% and all other activities at the various pH values tested were compared to that value. Fig. 7a shows A-Glc activity; Fig. 7b shows B-Glc; Fig. 7c shows B-Gal activity; Fig. 7d shows Mann activity; and Fig. 7e shows NAG activity.
Furthermore, acetate buffers below pH 4.7 result in precipitation of protein, drastically affecting the activities of the enzymes. Once the enzymes precipitate out of solution, they are irreversibly denatured and cannot be measured, even if returned to their pH optima (data not shown).

4. The Expression of Lysosomal Acid Hydrolase Activities During Heat-Induced Strain NC4 Spore Germination

Lysosomal acid hydrolase activities have been previously assayed at 30 and 35°C by a variety of workers (Loomis, 1969a, 1970, 1975; Oohata, 1976a; Coston & Loomis, 1969). However, in this study, all lysosomal acid hydrolases were assayed at 23.5°C since (1) this is the temperature used to allow activated spores to germinate, and (2) the vegetative organism cannot survive at temperatures above 27°C. A comparison of the data in Fig. 8 with enzyme activities measured at the temperatures used by those previous workers (Loomis, 1969a, 1970, 1975; Oohata, 1976a; Coston & Loomis, 1969) suggest that no significant differences in the enzyme patterns exist (data not shown). However, the enzyme activities are generally greater upon incubation of the reaction mixture at 23.5°C. The data of Fig. 8 represent the expression of five lysosomal acid hydrolases during heat-induced strain NC4 spore germination. It can be seen from Fig. 8 that the expression of these enzymes is not coordinately controlled. That is, each enzyme is expressed independently.

In the case of each enzyme, specific activities in spores are lower immediately after the removal of the activating agent (spores at time zero) than in dormant spores. The effect of this heat-activation treatment was studied in more detail and will be discussed below.
Figure 8. The Expression of Lysosomal Acid Hydrolase Activities During Heat-Induced D. discoideum Strain NC4 Spore Germination

Symbols represent: (○), the percent spore swelling; (●), the percent emerged myxamoebae; (△), α-glucosidase activity; (▲), β-glucosidase activity; (□), β-galactosidase activity; (■), α-mannosidase activity; and (◆), N-acetyl-β-glucosaminidase activity. Specific activity refers to units of activity per mg protein, where one unit of activity is defined as nmols of p-nitrophenol released per minute at 23.5 C.
Extracellular enzyme activities were examined and found to be minimal as compared to intracellular enzyme activity. Extracellular enzyme activity is almost unmeasurable with some enzymes. Jones et al. (1979) also report that B-Glc activity remains inside germinating spores.

The most interesting enzyme in terms of quantitative change is β-glucosidase. This enzyme exhibits a significant increase in specific activity during the spore germination process. Emerged myxamoebae possess a 12 fold higher specific activity of B-Glc when compared to postactivated spores at time zero. This increase in the expression of B-Glc activity begins at the start of the emergence stage of spore germination (Fig. 8).

Another interesting enzyme pattern is that of B-Gal. The specific activity of this enzyme increases slightly, but not significantly, during the swelling stage of germination. The activity then rapidly falls below the level observed in spores at time zero; this drop is followed by a slight increase during post-emergence (Fig. 8). The specific activity of Mann decreases throughout the germination process, with dormant spores containing the highest activity (Fig. 8).

The specific activity of A-Glc remains relatively constant throughout spore germination. However, there is a slight increase in enzyme activity during post-emergence (Fig. 8). Similarly, NAG activity remains relatively constant throughout spore germination until post-emergence, when a slight increase in activity occurs (Fig. 8).

5. The Effect of Prolonged Heat-Activation Treatment on the Expression of Lysosomal Acid Hydrolase Activities
As mentioned previously, postactivated spores at time zero, that is, immediately after the removal of the activating agent, have reduced enzyme activities as compared to dormant spores (Fig. 8 & 9). Most of the lysosomal acid hydrolases undergo a reduction in specific activity as the duration of exposure to mild heat shock (45°C) is increased (Fig. 9). The specific activities of all enzymes except NAG are reduced to a basal value after 30 to 45 minutes of heat treatment. NAG is comparatively heat stable under these conditions.

Increased exposure to heat treatment (60 and 90 minutes) does not block emergence of myxamoebae, but increases the length of the post-activation lag stage of germination (Copth & George, 1975). The levels of lysosomal acid hydrolase activities in emerged myxamoebae after 60 and 90 minutes heat activation treatment are similar to the levels observed in emerged myxamoebae induced to germinate with 30 minutes of heat treatment (data not shown).

As observed above, the activities of the enzymes after removal from heat shock (time zero) are lower than the activities in dormant spores. For this reason, the activities of spores at time zero are used as control values for comparison studies of developmental changes during spore germination.

6. The Effect of Inhibitors of Protein Synthesis on the Expression of Lysosomal Acid Hydrolase Activities

When inhibitors of protein synthesis are added at the start of incubation (time zero), the completion of spore germination (Table 1) and the changes in the expression of lysosomal acid hydrolase activities are blocked (Table 11). The most striking example is B-Glc, which fails to undergo the significant increase in activity. The data
FIGURE 9. THE EFFECT OF PROLONGED HEAT TREATMENT ON THE EXPRESSION OF LYSOSOMAL ACID HYDROLASE ACTIVITIES IN POST-ACTIVATED SPORES OF Dictyostelium discoideum.

The specific activities of dormant spore enzymes were considered to be 100% and the specific activities of the heat treated spores were compared to the dormant spore level. The open triangles (△) represent α-glucosidase activity and the closed triangles (■) represent β-glucosidase activity. The open squares (□) represent β-galactosidase activity and the closed squares (■) represent α-N-acetyl-β-glucosaminidase activity. The closed hexagons represent α-N-acetyl-β-glucosaminidase activity (●).
Table 11. The Effect of Inhibitors of Protein Synthesis on the Expression of Lysosomal Acid Hydrolase Activities

<table>
<thead>
<tr>
<th>Enzyme Specific Activities&lt;sup&gt;a&lt;/sup&gt;</th>
<th>A-Glc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B-Glc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B-Gal&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mann&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NAG&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Time Zero Spores)</td>
<td>6.2</td>
<td>6.6</td>
<td>19.0</td>
<td>21.6</td>
<td>1559</td>
</tr>
<tr>
<td>Control (Emerged Myxamoebae)</td>
<td>10.8</td>
<td>81.2</td>
<td>18.8</td>
<td>9.6</td>
<td>1699</td>
</tr>
<tr>
<td>Edeine (200 µg/ml)</td>
<td>3.8</td>
<td>3.7</td>
<td>22.2</td>
<td>18.9</td>
<td>1160</td>
</tr>
<tr>
<td>Cycloheximide (200 µg/ml)</td>
<td>4.9</td>
<td>5.9</td>
<td>22.1</td>
<td>19.7</td>
<td>1417</td>
</tr>
<tr>
<td>Puromycin (1000 µg/ml)</td>
<td>2.9</td>
<td>6.8</td>
<td>20.9</td>
<td>20.9</td>
<td>1452</td>
</tr>
</tbody>
</table>

Spores were incubated for hours after activation and then sampled for lysosomal acid hydrolase activities.

<sup>a</sup>The specific activity of each lysosomal acid hydrolase was equal to the units of enzyme activity per mg protein, where one unit of activity was defined as mmoles of p-nitrophenol released per min at 23.5°C.

<sup>b</sup>Abbreviations: A-Glc = α-glucosidase
B-Glc = β-glucosidase
B-Gal = β-galactosidase
Mann = α-mannosidase
NAG # N-acetyl-β-glucosaminidase
suggest that protein synthesis is required for changes involved in the expression of the enzyme activities.

Heat-activated spores swell normally in the presence of 200 µg/ml of cycloheximide, but do not release myxamoebae. When the inhibitor is removed from the swollen spores, the myxamoebae begin to emerge one hour after the removal of the drug. Figure 10 represents the expression of lysosomal acid hydrolase activities during the relief of swollen spores from cycloheximide. Note that most enzyme activities are reduced in the presence of cycloheximide as compared to the levels in spores at time zero. The expression of the enzymes under these conditions is not coordinately controlled, similar to the normal situation of heat-activated spore germination in the absence of cycloheximide (Fig. 8 & 10).

B-Gal activity undergoes a slight increase in activity following removal of the drug, followed by a rapid decrease in activity, returning the activity to the level found in cycloheximide-held spores (Fig. 10). B-Glc activity increases significantly during myxamoebae emergence; however, the start of this increase in B-Glc activity occurs before the release of myxamoebae from swollen spores (Fig. 10). The slight increases in NAG and A-Glc activities which occur in the post-emergence stage are similar to the levels in spores germinating after heat-activation in the absence of cycloheximide (Fig. 8 & 10).

Swollen spores blocked by cycloheximide have a higher specific activity of Mann than emerged myxamoebae (Fig. 10). Once the swollen spores are washed and allowed to complete germination, Mann activity decreases to the level found in emerged myxamoebae.
Figure 10. The effect of the relief of spore spores from the injection of 200 ICU/rL. 
hyaluronidase and the expression of lipase and ribonuclease activity.

The hyaluronidase was removed 5 hours after heat activation. Open circles (O) 
represent α-amylase, open squares (□) represent β-amylase, closed squares (■) represent β-glucosidase, open squares (□) represent α-glucosidase, closed triangles (△) represent α-galactosidase, closed triangles (△) represent β-galactosidase, closed triangles (△) represent α-galactosidase, closed triangles (△) represent α-mannosidase, closed triangles (△) represent α-fucosidase, closed triangles (△) represent α-mannosidase, closed triangles (△) represent α-fucosidase.

The specific activity refers to units of activity per mg protein, where one unit of activity was defined as 1000 ml of P-nitrophenol released per mg at 23-2 C.
7. The Effect of Deactivating Conditions on the Expression of Lysosomal Acid Hydrolase Activities

When heat-activated spores are exposed to severe environmental stress during the early postactivation lag phase of spore germination, they do not swell, but return to the dormant state; that is they are deactivated (Cotter et al., 1979). Deactivating conditions are found to block both the initiation of spore germination and the changes in the expression of lysosomal acid hydrolase activities (Table 12).

D. The Expression of Phosphatase Activity During Germination of Dictyostelium discoideum Spores

1. The Effect of Freeze-Thawing and Centrifugation on the Expression of Phosphatase Activity

If germinating D. discoideum spores are broken by three passages through a French pressure cell, then soluble phosphatase enzyme activity is found in the 8,200 g supernatant. Phosphatase activity is detected in both acid and alkaline pH ranges (Tables 13 & 14). Freezing and thawing of the crude cell lysate immediately after fractionation increases the specific activity of acid phosphatase (Table 13). While protein concentrations decrease upon freeze-thawing, the enzymatic activity of the freeze-thawed samples increases. These data suggest that cellular debris may protect the enzyme during freeze-thawing, but freezing still disrupts the lysosomal membrane, increasing enzyme activity.

The protein concentration of alkaline phosphatase preparations is reduced by freeze-thawing. However, the enzyme activity is also reduced. The freezing and thawing drastically reduces alkaline phosphatase activity in the later stages of spore germination (Table
Table 12. The Effect of Deactivation Conditions on the Expression of Lysosomal Acid Hydrolase Activities

<table>
<thead>
<tr>
<th>Enzyme Specific Activities&lt;sup&gt;a&lt;/sup&gt;</th>
<th>A-Glc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B-Glc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B-Gal&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mann&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NAG&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Time Zero Spores)</td>
<td>7.6</td>
<td>4.8</td>
<td>12.1</td>
<td>21.2</td>
<td>1470</td>
</tr>
<tr>
<td>Control (Emerged Myxamoebae)</td>
<td>7.7</td>
<td>75.9</td>
<td>14.1</td>
<td>12.9</td>
<td>1763</td>
</tr>
<tr>
<td>2 mM Sodium Azide</td>
<td>1.9</td>
<td>8.0</td>
<td>23.4</td>
<td>24.7</td>
<td>1577</td>
</tr>
<tr>
<td>2 mM Potassium Cyanide</td>
<td>1.7</td>
<td>4.3</td>
<td>21.9</td>
<td>15.9</td>
<td>1226</td>
</tr>
<tr>
<td>0 C for 48 hours</td>
<td>0.0</td>
<td>2.3</td>
<td>20.0</td>
<td>22.4</td>
<td>1135</td>
</tr>
</tbody>
</table>

Spores were incubated for 5 hours after activation, except for the 0 C treatment, and then sampled for lysosomal activities.

<sup>a</sup>The specific activity of each lysosomal enzyme assayed was equal to the units of enzyme activity per mg protein, where one unit of activity was defined as nmoles of p-nitrophenol released per minute at 23.5 C.

<sup>b</sup>Abbreviations: A-Glc = α-glucosidase  
B-Glc = β-glucosidase  
B-Gal = β-galactosidase  
Mann = α-mannosidase  
NAG = N-acetyl-β-glucosaminidase
Table 13. The Effect of Freeze-Thawing and Centrifugation on the Expression of Acid Phosphatase Activity.

<table>
<thead>
<tr>
<th>Acid Phosphatase Specific Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Dormant Spores</th>
<th>Time Zero Spores</th>
<th>Swollen Spores</th>
<th>Emerged Myxamoebae</th>
</tr>
</thead>
<tbody>
<tr>
<td>8,200 g Supernatant of Crude Cell Lysate</td>
<td>655</td>
<td>448</td>
<td>785</td>
<td>811</td>
</tr>
<tr>
<td>Freeze-Thawed 8,200 g Supernatant of Crude Cell Lysate</td>
<td>1880</td>
<td>762</td>
<td>740</td>
<td>2194</td>
</tr>
<tr>
<td>8,200 g Supernatant of Freeze-Thawed Crude Cell Lysate</td>
<td>1641</td>
<td>1164</td>
<td>1395</td>
<td>2302</td>
</tr>
</tbody>
</table>

Heat-activated spores were allowed to germinate in 10 mM MES buffer, pH 6.5, at 23.5°C. The samples were broken by three passages through a French pressure cell. The broken cells were considered to be the crude cell lysate.

<sup>a</sup>Acid phosphatase activity was measured as described in the Materials & Methods section. Specific activity is equivalent to the units of activity per mg protein, where one unit of activity is defined as nmol of p-nitrophenol released per minute at 23.5°C.
Table 14. The Effect of Freeze-Thawing and Centrifugation on the Expression of Alkaline Phosphatase Activity

<table>
<thead>
<tr>
<th>Alkaline Phosphatase Specific Activity(^a)</th>
<th>Dormant Spores</th>
<th>Time Zero Spores</th>
<th>Swollen Spores</th>
<th>Emerged Myxamoebae</th>
</tr>
</thead>
<tbody>
<tr>
<td>8,200 g Supernatant of Crude Cell Lysate</td>
<td>15.6</td>
<td>6.3</td>
<td>21.4</td>
<td>66.7</td>
</tr>
<tr>
<td>Freeze-Thawed 8,200 g Supernatant of Crude Cell Lysate</td>
<td>20.1</td>
<td>6.7</td>
<td>22.0</td>
<td>48.5</td>
</tr>
<tr>
<td>8,200 g Supernatant of Freeze-Thawed Crude Cell Lysate</td>
<td>15.6</td>
<td>7.5</td>
<td>17.8</td>
<td>33.2</td>
</tr>
</tbody>
</table>

Heat-activated spores were allowed to germinate in 10 mM MES buffer, pH 6.5, at 23.5°C. The samples were broken by three passages through a French pressure cell. The broken cells were considered to be the crude cell lysate.

\(^{a}\) Alkaline phosphatase activity was measured as described in the Materials & Methods section, and is expressed in terms of specific activity which is equivalent to units of activity per mg protein. One unit of activity is defined as nmol of p-nitrophenol released per min at 23.5°C.
14). Since morphological changes must be considered during spore
germination studies, further studies of the soluble alkaline phosphatase
enzyme were conducted on the 8,200 g supernatant without the freeze-
thawing step.

2. The Effect of pH on Phosphatase Activity

The pH range for phosphatase activity was determined by the methods
described in the Materials and Methods section. The averaged data of
these experiments is shown in Figure 11. Phosphatase activity is
observed in both the acid and alkaline regions of the pH curve. The
alkaline phosphatase enzyme has a much lower activity when compared to
the activity of the acid region (Fig. 11). Extracts from dormant
spores and emerged myxamoebae were assayed for pH optima but showed no
significant differences between the two cell types. Freeze-thawing of
the 8,200 g supernatant reduces the total activity of alkaline
phosphatase, but does not change the pH optimum of the enzyme (data
not shown).

As can be seen in Fig. 11, the pH optima for alkaline phosphatase
activity is 8.5. This is consistent with alkaline phosphatase
activity at other stages of the life cycle of D. discoideum (Loomis,
1969b). Two peaks of phosphatase activity are found in the acid
region at pH values 2.5 and 5.0 (Fig. 11). Wiener and Ashworth (1970)
previously reported two peaks of activity for acid phosphatase at
pH values 3.0 and 4.0 using strain AX2 myxamoebae. The differences in
the results may be due to strain specific differences since the wild
type strain NC4 was used throughout these experiments. In all
subsequent phosphatase experiments, activity was measured at pH values
Figure 11. The Effect of pH on *Dictyostelium discoideum* Phosphatase Activity.

Both dormant spores and emerged myxamoebae were assayed according to the procedures described in the Materials and Methods section. Symbols: the closed circles (●) represent the averaged percent of phosphatase activity, where the highest activity in each separate experiment was considered to be 100% and all other activities at the various pH values tested were compared to that value.
5.0 and 8.5 for acid and alkaline phosphatase activity, respectively.

3. The Expression of Phosphatase Activity During the Germination of D. discoideum Spores

In order to assay phosphatase activity, 10 mM MES buffer (pH 6.5) was used in all steps instead of phosphate buffer. The spore germination kinetics resulting from the use of this buffer were similar to those obtained with phosphate buffer (Fig. 2 & 12).

When strain NC4 spores are induced to germinate by heat shock, the specific activity of alkaline phosphatase increases slightly during spore swelling and rapidly during the emergence stage of germination (Fig. 12). Emerged myxamoebae express a 6 fold higher specific activity of alkaline phosphatase when compared to postactivated spores at time zero (Fig. 12). These postactivated spores at time zero have a lower alkaline phosphatase activity than the level found in dormant spores suggesting that the heat treatment reduces the specific activity.

Due to this observation of the drop in specific activity of alkaline phosphatase, the effect of prolonged heat activation treatment at 45 C was examined. Spores heat shocked for 15 minutes at 45 C are not quantitatively activated, but 20 minutes of heat at 45 C will activate the majority of dormant spores (Cotter & Raper, 1968a,b). A heat shock extended longer than is necessary to activate the entire spore population will "damage" the activated spores. This damage can be observed as a lengthening of the postactivation lag time. Excessive heat activation at 45 C will kill spores and the time of exposure necessary to kill is dependant on spore age and the rate of thermal exchange (Cotter & George, 1975). However, spores are still viable after 90 minutes of exposure to 45 C, but undergo a 2 hour postactiva-
Figure 12. The Expression of Phosphatase Activity During Heat-Induced

*D. discoideum* Strain NC4 Spore Germination

Symbols represent: (○), the percent spore swelling; (●), the percent emerged myxamoebae; (△), acid phosphatase activity; and (▲), alkaline phosphatase activity. Specific activity refers to units of activity per mg protein where one unit of activity is defined as nmol of p-nitrophenol released per minute at 23.5°C.
ion lag before swelling. The effect of prolonged heat treatment on alkaline phosphatase activity is diagrammed in Figure 13. Enzyme activity was assayed immediately after release from the heat treatment. Alkaline phosphatase activity decreases after 15 minutes of heat shock at 45°C; this level of alkaline phosphatase activity remains constant until excessively long heat treatment causes a gradual reduction in the specific activity in the spores.

When heat-activated spores are assayed for alkaline phosphatase activity during germination in phosphate buffer, the enzyme pattern is both qualitative and quantitatively similar to the pattern found in Fig. 12.

Acid phosphatase activity remains relatively constant after heat activation until the late spore swelling stage of germination, then the activity drops slightly. Upon the initiation of the emergence stage, the acid phosphatase activity in emerging myxamoebae increases about 1.5 fold when compared to the level found in postactivated spores at time zero (Fig. 12). If heat-activated spores are allowed to germinate in phosphate buffer instead of MES buffer, the enzyme activity pattern for acid phosphatase is qualitatively similar to the pattern shown in Figure 12. However, this pattern is quantitatively different in that acid phosphatase activity is reduced in spores germinating in phosphate buffer (data not shown).

Prolonged heat treatment at 45°C did not significantly affect acid phosphatase activity in spores at time zero. This is the only lysosomal acid hydrolase assayed not affected by the heat shock treatment (Fig. 13).
Figure 13. The Effect of Prolonged Heat Treatment on the Expression of Phosphatase Activity in Postactivated Spores At Time Zero.

The specific activity of dormant spore enzymes were considered to be 100% and the specific activities of heat treated spores were compared to the dormant spore level. The open triangles (Δ) represent acid phosphatase activity and the closed triangles (▲) represent alkaline phosphatase activity.
4. The Effect of Inhibitors of Protein Synthesis on the Expression of Phosphatase Activity

Inhibitors of protein synthesis block the emergence stage of D. discoideum spore germination and prevents the incorporation of radioactively labeled amino acids (Table 1; Cotter & Raper, 1970; Yaguri & Iwabuchi, 1976; Giri & Ennis, 1977). The data in Table 15 show the effect of inhibitors of protein synthesis on the expression of phosphatase activity.

Heat-activated strain NC4 spores incubated with inhibitors of protein synthesis have specific activities of alkaline phosphatase equal to the activity found in swollen spores in the absence of the drugs (Table 15; Fig. 12). When heat-activated spores are incubated in the presence of 200 µg/ml of cycloheximide for 5 hours, the spores are held at the swelling stage. When swollen spores are relieved of the cycloheximide inhibition by washing, the swollen spores release myxamoebae one hour after the removal of the drug. A concurrent increase in alkaline phosphatase activity occurs as the myxamoebae are released (Fig. 14). This would suggest that protein synthesis is not required for the initial increase in alkaline phosphatase activity, but is required for further increases in enzyme activity during the emergence stage of spore germination.

Heat-activated strain NC4 spores incubated in the presence of inhibitors of protein synthesis do not exhibit the increase in acid phosphatase activity which normally occurs during the emergence stage of spore germination (Table 15; Fig. 12). After washing the swollen spores free of cycloheximide, the specific activity of acid phosphatase increases as the myxamoebae begin to emerge (Fig. 14).
Table 15. The Effect of Inhibitors of Protein Synthesis on the Expression of Phosphatase Activity

<table>
<thead>
<tr>
<th></th>
<th>Phosphatase Specific Activity$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acid$^b$</td>
</tr>
<tr>
<td>Dormant Spores</td>
<td>1540</td>
</tr>
<tr>
<td>Time Zero Spores</td>
<td>1531</td>
</tr>
<tr>
<td>Swollen Spores</td>
<td>----</td>
</tr>
<tr>
<td>Emerged Myxamoebae</td>
<td>2072</td>
</tr>
<tr>
<td>Edeine (200 ug/ml)</td>
<td>1297</td>
</tr>
<tr>
<td>Cycloheximide (200 ug/ml)</td>
<td>1572</td>
</tr>
<tr>
<td>Puromycin (1000 ug/ml)</td>
<td>1267</td>
</tr>
</tbody>
</table>

Heat-activated spores were incubated at 23.5°C in 10 mM MES buffer (pH 6.5). The inhibitors of protein synthesis were added at the start of incubation and sampled after 5 hours for phosphatase activity.

$^a$Specific activity refers to units of activity per mg protein, where one unit of activity is defined as mmoles of p-nitrophenol released per minute at 23.5°C.

$^b$Acid phosphatase activity was measured in 0.05 M acetate buffer (pH 5.0). Alkaline phosphatase activity was measured at pH 8.5 in 0.01 M Tris-HCl (0.01 M MgCl$_2$) buffer.
Figure 14. The Effect of the Relief of Swollen Spores from the Inhibition of 200 μg/ml Cycloheximide and the Expression of Phosphatase Activity.

The cycloheximide was removed 5 hours after heat activation. The symbols represent: (○), the percent spore swelling; (●), the percent emerged myxamoebae; (△), acid phosphatase activity; and (▲), alkaline phosphatase activity. Specific activity refers to units of activity per mg protein, where one unit of activity is defined as nmol of p-nitrophenol released per minute at 23.5°C.
that protein synthesis is required for the increase in the expression of acid phosphatase activity during emergence of myxamoebae.

5. The Effect of Deactivating Conditions on the Expression of Phosphatase Activity

When activated spores are exposed to severe environmental stresses, they are deactivated; that is spores do not swell, but rather return to the dormant state (Cotter et al., 1979). The data of Table 16 infer that both acid and alkaline phosphatase activities of deactivating spores are equal to or less than the specific activity of activated spores at time zero.
Table 16. The Effect of Deactivating Conditions on the Expression of Phosphatase Activity

<table>
<thead>
<tr>
<th>Phosphatase Specific Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Acid&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Alkaline&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time Zero Spores (Control)</td>
<td>1485</td>
<td>14.5</td>
</tr>
<tr>
<td>Swollen Spores (Control)</td>
<td>----</td>
<td>17.5</td>
</tr>
<tr>
<td>Emerged Myxamoebae (Control)</td>
<td>1081</td>
<td>55.5</td>
</tr>
<tr>
<td>2 mM Sodium Azide</td>
<td>1331</td>
<td>12.2</td>
</tr>
<tr>
<td>2 mM Potassium Cyanide</td>
<td>1129</td>
<td>16.7</td>
</tr>
<tr>
<td>0 °C for 48 hours</td>
<td>1419</td>
<td>14.7</td>
</tr>
</tbody>
</table>

Heat-activated spores were incubated at 23.5 °C in 10 mM MOPS buffer (pH 6.5) for 5 hours. The deactivating conditions began at the start of the incubation period and spores were sampled after 5 hours except for spores incubated at 0 °C which were sampled after 48 hours for phosphatase activity. The time zero control was sampled immediately after heat-activation and swollen spores were sampled 2 hours after activation.

<sup>a</sup>Specific activity refers to units of activity per mg protein where one unit of activity is defined as nmoles of p-nitrophenol released per min at 23.5 °C.

<sup>b</sup>Acid phosphatase was measured in 0.05 M acetate buffer (pH 5.0) and alkaline phosphatase in 0.01 M Tris-HCl buffer with 0.01 M MgCl₂ (pH 8.5).
DISCUSSION

Analysis of protein synthesis during the germination of *D. discoideum* spores could be approached in several ways: (1) analysis of data obtained by gel electrophoresis, (2) analysis of the rate of the incorporation of radioactively labeled amino acids, (3) *in vitro* translation of poly(A)+ mRNA, and (4) measurement of the specific activities of various enzymes.

Data have recently been accumulated using the first three methods mentioned above (Yaguri & Iwabuchi, 1976; Giri & Ennis, 1977, 1978). However, little is known about the changes in the specific activity of enzymes during germination of *D. discoideum* spores (Cotter & Raper, 1970; Jones *et al.*, 1979).

The incorporation of radioactively labeled amino acids into TCA insoluble materials provides limited data on protein synthesis during spore germination. The rate of incorporation of labeled amino acids during spore germination is similar regardless of the presence or absence of the amino acid in dormant spores (Fig. 3). However, protein turnover may be involved so that results obtained by incorporation methods may not give a true measure of protein synthesis during spore germination.

Inhibitors of macromolecular synthesis may have differential effects. Specific inhibitors of RNA synthesis block the emergence stage of germination and, in one case, the swelling stage of germination (Giri & Ennis, 1977).

Thiolutin was found to block both the swelling stage of germination and the incorporation of radioactively labeled uridine. Further work
indicates that spores are deactivated by the drug; that is, they return to the dormant state. Tipper (personal communication) has found that thiolutin is not a specific inhibitor of RNA synthesis, but also inhibits protein synthesis. The drug prevents the incorporation of labeled uridine, but does not affect adenosine incorporation. Thiolutin has also been found to inhibit respiration in vegetative cells of D. discoideum. The cells are killed within 60 minutes of exposure to the drug, and ATP levels fall by 80% (Cotter & Garnish, personal communication). This would suggest that thiolutin is acting as a respiratory inhibitor causing the deactivation of the activated spores.

Inhibitors of RNA synthesis have been found to block both RNA and protein syntheses. At present, no inhibitor which is specific to RNA synthesis has been found which can penetrate the spores; it is only nonspecific inhibitors which can penetrate the spores (Ennis, personal communication). Therefore, knowledge concerning the requirements for RNA synthesis during D. discoideum spore germination is limited.

Specific inhibitors of protein synthesis block the completion of D. discoideum spore germination. The spores swell, but do not release myxamoebae in the presence of these drugs. However, blocking protein synthesis with a specific inhibitor does not affect the incorporation of labeled uridine. Thus, stringent control of RNA synthesis is not in effect during germination of D. discoideum spores (Table 1). Further studies utilizing inhibitors of protein synthesis were limited to three drugs which affect the process at three different steps: (1) edeine, an inhibitor of the initiation stage of protein synthesis; (2) cycloheximide, an inhibitor of peptide chain elongation; and (3) puromycin, an inhibitor of peptide chain termination.
The fourth method of analysis of protein synthesis during *D. discoideum* spore germination involves the measurement of the specific activities of various enzymes. The specific activities of several hydrolases were examined during spore germination. These enzymes will be discussed separately in three sections: (1) trehalase activity; (2) lysosomal acid hydrolase activities; and (3) alkaline phosphatase activity.

It has been suggested that the genetic program to initiate macrocyst formation may involve the activation of gene sets which are involved in sheath, microcyst and spore formation. Ultrastructural analyses have shown the microcyst wall to be bilayered, while the spore wall contains an additional outer layer resembling the pseudoplasmodial slime sheath. The complex macrocyst wall appears to contain an outer layer resembling the slime sheath, a middle wall similar to the bilayered microcyst wall and an inner trilayered wall resembling the spore wall (Hohl, 1976; Hohl et al., 1970; Hemmes et al., 1972). It might be expected that the germination program would be the same once the various resting stages have been activated. This would imply that the organism is conservative in its use of genetic information. Macrocyst germination has been found to be age dependant (Nickerson & Raper, 1973b). Similarly, the ability to spontaneously germinate has been reported to occur in wild type strain NC4 *D. discoideum* spores which have been allowed to age in the intact fruiting body. Spores that have not aged are constitutively dormant and require an activation treatment to germinate (Dahlberg & Cotter, 1979). When the resting structures of the Dictyosteliaceae are incubated in the presence of cycloheximide, the innermost wall remains intact for all three
resting stages (Cotter et al., 1969; Hohl et al., 1970; Erdos et al., 1972).

At present, only spores have been reported to contain trehalose, the substrate for trehalase (Ceccarini & Filosa, 1965; Clegg & Filosa, 1961). However, the present study shows that all three dormant structures contain some trehalase activity (Fig. 5 & 6; Table 2). Trehalase has been reported to be absent in dormant D. discoideum spores, but localized in stalk cells (Jefferson & Rutherford, 1976). However, Rutherford (personal communication) believes that there is trehalase activity in the spore cells, but the activity is minimal compared to the activity of the stalk cells. The activity of this enzyme increases at least 6-7 fold during germination of all three resting stages of the Dictyosteliumae (Table 2; Fig. 5 & 6). The increase in expression of this enzyme specific activity is found to begin at the transition point between spore swelling and emergence of myxamoebae during D. discoideum spore germination (Fig. 5). Similarly the increase in trehalase activity during P. pallidum microcyst germination is initiated slightly before the release of myxamoebae (Fig. 6).

Inhibitors of protein synthesis block both the completion of germination and the increase in the expression of trehalase activity in all three resting structures (Table 2). This would indicate that protein synthesis is required for the increase in the expression of trehalase activity during germination.

The location of trehalase in slime molds might be significant. Although the role and location of the enzyme during germination are not yet known, they may be similar for all three resting stages. In Sacrophaga bullata flight muscle, trehalase activity has been
histochemically located in association with the inner mitochondrial membrane (Chang & Morrison, 1975). If this were found to be true for *D. discoideum* spores, this information would add support to the "multi-state" model for activation (Cotter, 1973; Cotter & George, 1975).

In the study of lysosomal enzymes, various techniques had to be developed or adapted to examine enzyme activity using the *D. discoideum* system of spore germination. Furthermore, various factors had to be considered during this study: (1) the enzyme assay temperature and the biological effect of the temperature on the organism; (2) effectiveness of different cell breakage techniques which enable the enzyme activities to be measured; and (3) morphological changes in the cells throughout the study.

Previous studies assayed the enzymes at temperatures above 27 C, the limit of survival of this organism. In fact, the enzymes were assayed at 30 and 35 C (Coston & Loomis, 1968; Loomis, 1969a, 1970, 1975). In this study the enzymes were assayed at 23.5 C because the organism does not survive at temperatures above 27 C, and this is the temperature used to allow activated spores to germinate. When the enzyme assays were conducted at both the higher temperatures and at 23.5 C, no significant change in any enzyme pattern was found. In some cases, the specific activity of some enzymes was higher when assayed at 23.5 C.

The morphological changes occurring during spore germination must be considered when discussing cell breakage and decryptification techniques. In earlier studies of these enzymes, a variety of techniques have been used on *D. discoideum* cells, including Triton X-100 permeabilization, sonication, and passage through a French pressure cell. Sussman (1966) recommends the use of a French pressure cell or a sonicator for cell
breakage. He noted, however, that sonication is less reproducible and less effective on mature fruits than a French pressure cell.

The use of cold acetone, Triton X-100 and DMSO to permeabilize cells has been reported to be successful in assaying enzyme activities in some microorganisms (Miczaki et al., 1978; Cotter et al., 1975). Recently, a new technique utilizing glass beads to break cells has been reported (Van Etten & Freer, 1978). Analysis of the effects of the above techniques on dormant spores and emerged myxamoebae suggest that the French pressure cell is the best method of cell breakage for comparison studies of these enzymes (Table 4 & 5). This method yields high enzyme activities and measurable amounts of protein released so that comparison studies during spore germination can be performed.

The glass bead method provides excellent breakage but nonspecific binding of protein to the beads affects enzyme assay values. The decryptification methods are not effective in "activating" enzyme activity and also result in the release of little protein; thus, comparison studies would be difficult to accomplish using decryptification methods.

Enzyme activities are increased by freeze-thawing the crude cell lysate immediately after cell breakage. Freeze-thawing reduces the protein concentration of the extract, but increases or has no effect on enzyme activity. The data suggest that cellular debris may protect the enzyme during freeze-thawing, but freezing still disrupts the lysosomal membrane increasing the enzyme activity. This procedure of freezing samples, immediately after cell breakage is also useful for comparison studies of spore germination stages since samples can be stored and then simultaneously assayed.
An interesting point in the cell breakage experiments is that several enzymes possess activities in the control similar to the values obtained by the French press technique. These unfractionated cells are still viable after enzyme assay. The presence of these assayable enzyme activities in dormant spores suggests that these enzymes may be located near, or bound to, the plasma membrane. When dormant spores are heat-activated at 45°C, the specific activities of all of these enzymes (except acid phosphatase) decrease. Prolonged heat-activation treatment causes a drop in activity, which finally reaches a basal level in superactivated spores, suggesting that some of these enzymes are located near the plasma membrane where the heat treatment will denature them. This further supports the idea that these enzymes may have more than one location in *D. discoideum* cells. Pugh and Cawson (1977) found that enzymes with optimal activity at acid pH are often found at more than one cell site and that the distribution of these enzymes varies during the life cycle of the fungi. In *Neurospora crassa* cells, B-Glc was found to be membrane bound, while some cellobiase activity was internal and cryptic. The aryl-B-Glc of whole cells has a higher activity than in the enzyme extract (Eberhardt & Beck, 1970). Similarly, acid phosphatase has been reported both in the lysosome and on the plasma membrane of *D. discoideum* cells. This membrane bound isozyme of acid phosphatase exists only during vegetative growth (Parish, 1976).

The expression of lysosomal acid hydrolase activities during heat-induced germination of *D. discoideum* strain NC4 spores is not coordinately controlled (Fig. 8). The increases or decreases in
lysosomal enzyme activities require protein synthesis, suggesting \textit{de novo} synthesis of the enzymes and/or \textit{de novo} synthesis of a protease or another enzyme to activate preformed pro-enzymes (Table 11). B-Gal is the only lysosomal enzyme which does not completely follow this pattern. This enzyme may be preformed and may be activated either by removal of an inhibitor or by activation by a protease; in any case, the activity rises slightly under conditions inhibitory to germination. However, the results do not rule out \textit{de novo} synthesis of B-Gal during spore germination.

NAG, acid phosphatase and A-Glc appear to undergo relatively little change in activity during spore germination. The slight increase in activities occurs after the majority of myxamoebae have been released (post-emergence), and may be involved in vegetative growth. However, the data from the experiments involving inhibitors of protein synthesis indicate that protein turnover may be involved in maintaining these relatively constant enzyme activities.

The increase in the expression of B-Glc activity requires protein synthesis. This increase appears to begin at the transition point between spore swelling and emergence. However, the increase probably occurs before emergence, since spores relieved from cycloheximide inhibition show this increase in activity before myxamoebae are released (Fig. 10).

The enzyme, B-Glc, has three of the four characteristics required to be a developmentally significant enzyme (Quance & Ashworth, 1972): (1) B-Glc undergoes a 12 fold increase in activity; (2) Inhibitors of protein synthesis block the increase in activity; (3) deactivating conditions block the expression of this increase in B-Glc activity.
"Mechanical" blockage of germination by UV irradiation may also affect this increase in activity; (4) At present, this increase in activity has not been shown to be altered in morphological mutants. However, this may be shown in future studies by assaying an abortive germination mutant, strain HE1, which swells, but does not release myxamoebae (Giri & Ennis, 1978).

At present, the roles of these enzymes during spore germination are not known and can only be speculated upon. It is possible that these enzymes are involved in the following functions: (1) degradation to provide energy or precursors for macromolecular synthesis, (2) direct involvement in the germination process itself, and/or (3) preparation for vegetative growth.

Mann may function during germination to provide energy or precursors for protein synthesis. The enzyme exhibits a loss in activity throughout spore germination, and is not released into the medium (Fig. 8). The loss in activity can be blocked by inhibitors of protein synthesis, possibly affecting proteases degrading the enzyme. The enzyme is not found in vegetative amoebae (Loomis, 1970).

B-Glc has been previously suggested to have a role in spore germination by opening and utilizing the spore wall (Coston & Loomis, 1969). Jones et al., (1979) have found that heat-activated spores release cellulase activity into the medium during spore swelling. They suggested that the cellulase acts to degrade the middle layers of the spore wall. The enzyme B-Glc probably hydrolyzes the products of the cellulase activity, cellobiose and cellotriose, in a manner similar to what Rosness (1968) predicted. B-Glc was found to remain associated with the germinating spores (Jones et al., 1979). Therefore, these
substrates must be transported to the enzyme inside the cells. The increase in D-Glc activity during the emergence stage may indicate that the enzyme is required to provide energy for this stage in germination. However, the enzyme may also be involved in phagocytic activity during vegetative growth. Thus, the increase in activity may be involved in preparation for vegetative growth.

The activity of alkaline phosphatase in heat-activated spores increases slightly during spore swelling and rapidly during myxamoebae emergence (Fig. 12). These data have been confirmed by another laboratory (Ennis, personal communication). The data suggest that protein synthesis is not required for the initial increase in alkaline phosphatase activity during spore swelling, but is required for further increases in enzyme activity (Table 16 & Fig. 14).

As in the case of lysosomal acid hydrolases, alkaline phosphatase undergoes a reduction in activity during heat-activation. However, alkaline phosphatase activity reaches its basal value in spores after only 15 minutes of heat shock at 45°C. This is less exposure to the heat treatment than the normal activation period of 30 minutes.

Quiviger et al., (1978) have demonstrated that alkaline phosphatase activity in D. discoideum cells is associated with the contractile vacuoles. The contractile vacuole appears to be absent in dormant spores, and first appears during spore swelling (Cotter, 1975). Thus, the increase in alkaline phosphatase activity during swelling stage of germination may further support Quiviger's proposed location. Inhibitors of protein synthesis still allow this initial increase in activity and the appearance of contractile vacuoles.
The results from trehalase, lysosomal enzymes and alkaline phosphatase studies answer some questions; however, they also provide new questions. Is the expression of the lysosomal enzyme activities the result of single isoforms? Are these enzyme activities the result of de novo synthesis of the enzymes in the case of trehalase, B-Glc, and alkaline phosphatase? The use of polyacrylamide gel electrophoresis and incorporation of labeled precursors might help answer these questions.

The requirement for the above enzymes during spore germination must also be examined in future studies. In Phycomyces blakesleeanus, the increase in trehalase activity during spore germination was found to be unnecessary for the breaking of dormancy (Van Assche et al., 1978). This may also be true in the germination of D. discoides spores, in that none of these enzymes may be required for any stage of germination. The isolation of temperature-sensitive mutants lacking activity for an enzyme would be a useful tool in studies of this subject. Examination of UV irradiated spores (which specifically do not release myxamoebae) for the expression of these enzyme activities could also be useful. The spontaneous germination mutant strains, SG1 and SG2, may have different enzyme activity patterns.

At present, little information is known about what energy sources are utilized during spore germination. An examination of the respiratory quotient would give an indication of what types of compounds are being utilized. The possibility that amino acids are being utilized for energy could be examined by measuring the ammonium ion concentration in the extracellular medium throughout germination. An increase in this ion may indicate that amino acids are being
utilized and that the cells are releasing the ion as a waste product.

The data on Mann activity suggest involvement of proteases in spore germination; work by Hemmes et al., (1972) also suggest the involvement of protease activity in spore germination. The expression of protease, amylase and esterase activity should be the next enzyme activities surveyed.
LITERATURE CITED


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