The metabolism of endogenous energy reserves during Dictyostelium discoideum spore germination.

Dennis Paul. Jackson
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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS REÇUE
THE METABOLISM OF ENDOGENOUS ENERGY RESERVES DURING
DICYTOSTELIUM DISCOIDEUM SPORE GERMINATION

by

Dennis Paul Jackson

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
1982
ABSTRACT

Multicellular differentiation in the cellular slime mold, Dictyostelium discoideum, is powered by endogenous protein and "free" amino acid reserves. This period is also marked by the accumulation of other endogenous reserves, namely lipid and trehalose. The final stage of development is the formation of a fruiting body. Atop a column of dead stalk cells sits a sorus containing dormant spores in a viscous matrix. Enzymes are abundant in both dormant spores and the extracellular matrix.

Analysis of crude extracts of the sorus demonstrates the presence of proteolytic enzymes. These include cathepsin B- and D-like proteases as well as leucine aminopeptidase. Further data concerning these enzymes plus a neutral protease during spore germination suggests that their involvement is not related to energy production either in maintaining the dormant state or during spore germination. These enzymes may serve other cellular requirements. Cathepsin B-like protease appears to be directly involved during emergence. By degrading peptide linkages in the cell wall this protease may be part of a group of enzymes responsible for the release of myxamoebae from the swollen spore.

Respiratory quotients indicate the involvement of lipid
and carbohydrate as the major endogenous energy reserves. Lipid is utilized to maintain dormant spores and throughout the entire germination process. Significant decreases in total lipid, neutral lipid, and lipase activity as well as a significant increase in free fatty acids during germination supports this idea. Carbohydrate, specifically the pool of trehalose, remains largely intact during heat-induced activation and also during post-activation lag. The initiation of spore swelling is accompanied by a decrease in the trehalose pool; the majority of trehalose is consumed before late spore swelling. Trehalose utilization dominates over the oxidation of lipid only during the swelling stage of germination.

A preliminary study of cellular metabolism through the use of inhibitors was initiated. Arsenate and fluoride could delay the emergence of myxamoebae but appear not to have any effect on spore swelling. This in part may be due to the impermeability of the spore itself. Closer examination of the effect of arsenate on respiratory quotients reveals a shift in metabolism to the sole oxidation of lipid. Oxygen utilization increased when monitored in the presence of arsenate. Energy production from lipid oxidation alone appears to be sufficient enough to complete germination albeit at a reduced rate.

Iodoacetamide and iodoacetic acid, while they can inhibit
spore swelling and the emergence of myxamoebae, are too general in their mode of action in *D. discoides* to draw any conclusions.
DEDICATION

This thesis is dedicated to the one person in my life who exhibits those unique qualities that I admire the most. These include kindness, unselfishness, understanding, and loyalty. The person I speak of is my mother.
ACKNOWLEDGEMENTS

I wish to express my gratitude to my colleagues, co-workers, and friends at the University of Windsor. I shall never forget their support, guidance, and above all their friendship. Thanks go to my committee members, Drs. Aiden Warner and Norman Taylor for their advice in preparing this thesis. I am also indebted to Dr. Hugh Fackrell for the use of his data processor. Finally, I wish to thank Dr. David Cotter, not only for his constant support and encouragement, but also for the opportunity to work with him.
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<tr>
<td>DAN</td>
<td>Diazocetyl-DL-norleucine methyl ester</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNPP</td>
<td>Dinitrophenylprotamine sulfate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HIP</td>
<td>Hexane:isopropylanol</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>Potassium phosphate</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulfate</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide dinucleotide</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Sodium phosphate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>Ammonium chloride</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>PSI</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TLC</td>
<td>N-{4-[(p-tosyl-L-lysine chloromethyl ketone HCl)</td>
</tr>
<tr>
<td>TPCK</td>
<td>1-chloro-4-phenyl-3-tosylamido-L-butan-2-one</td>
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<td>UV</td>
<td>Ultraviolet</td>
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A. History and Overview of the Life Cycle of the Cellular Slime Mold, Dictyostellium discoideum

The cellular slime mold, Dictyostellium discoideum NC4H, was first isolated by K. B. Raper (1935) from forest soil in North Carolina.

Figure 1 summarizes the life cycle of D. discoideum (Wright, 1963). Individual amoebae are found in bacteria-rich soils where they are indistinguishable from other soil amoebae. They feed on bacteria, either live or dead, in decaying forest matter and will continue to grow and undergo binary fission as long as food is available. Only when the bacterial food source is exhausted can multicellular development begin. The three stages of multicellular development are aggregation, pseudoplasmodial formation, and culmination. These series of events distinguish D. discoideum from other soil amoebae.

After a period of starvation, amoebae synthesize cAMP (Konijn et al., 1967; Bonner et al., 1969) and excrete the cyclic nucleotide in the form of pulses every few minutes (Gerisch and Wick, 1975; Shaffer, 1975). When amoebae are stimulated by a pulse of cAMP, they relay this pulse (Shaffer, 1962; Robertson et al., 1972; Ross et al., 1975; Shaffer, 1975). Such a relay mechanism permits the
Figure 1. The life cycle of *Dictyostellium discoideum* (after Wright, 1963).
SPORE → AMOEBA → GROWTH AND MULTIPLICATION → AGGREGATION → PSEUDOPLASMODIUM → CULMINATION → MATURE FRUITING BODY
coordinated aggregation of up to $10^5$ cells.

The aggregate becomes integrated by deposition of a surface sheath covering the entire mound of cells. This aggregate topples over onto the surface and migrates as a pseudoplasmodium, grex, or slug. The pseudoplasmodium is capable of migration in search of additional nutrients and is both thermotactic and phototactic. During migration, the cells differentiate into presumptive spore cells which become located in the posterior section and presumptive stalk cells which become located in the anterior section of the pseudoplasmodium.

During culmination the prestalk cells, surrounded by a cellulose sheath, turn into highly vacuolated dead stalk cells with a cellulose wall. Each prespore cell develops into a single uninucleate spore contained in the sorus at the apex of the stalk. This marks the end of culmination. To complete the life cycle, spores are released from the sorus and will germinate into vegetative amoebae under favourable conditions (see Newell, 1971, 1978; Loomis, 1975 for review).

B. Spores and Spore Germination in D. discoideum

The dormant spores of wild type strain NC4H are capsule shaped and vary in size; the average spore measures 6 to 9 um long and 2.5 to 3.5 um in diameter (Raper, 1935; Bonner, 1967). The cell wall of mature spores consist of four
distinct layers as determined by freeze-etching and replicating techniques (Hemmes et al., 1972). The outermost layer is composed primarily of mucopolysaccharide; the middle layer being constructed of two distinct cellulosic layers of fibrils which differ in their orientation. It is not clear whether cellulose is the only component in this layer, but both fibrillar layers are susceptible to cellulase. The innermost layer is composed of cellulose and protein.

Dormant spores of *D. discoideum* are contained in an extracellular viscous material, referred to as the matrix, at the apex of the sorocarp (Murata and Ohnishi, 1980). The matrix contains enzymes (Chan et al., 1981), complex carbohydrates (Ceccarini and Filosa, 1965), and discadenine, a spore germination inhibitor (Abe et al., 1976).

Spores are constitutionally dormant as they will not germinate into myxamoebae unless activated by physical or chemical treatments (Cotter and Raper, 1966; Sussman, 1976). Spore germination follows a sequential pattern and consists of four stages: activation of the dormant spore, postactivation lag, swelling of the activated spore, and the release of a single myxamoeba from each swollen spore (Cotter, 1975) (Fig. 2). Dormant spores can be activated by several techniques developed by Cotter and co-workers (see Cotter, 1981 for review). The activation techniques include
Figure 2. The germination sequence of heat-activated *Dictyostelium discoideum* strain NC4H spores (Cotter and Raper, 1968b).

Spores were heat-activated at 45°C for 30 min and incubated at 23.5°C for 5 hr. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae. The semidiagrammatic depiction of spore germination below the abscissa includes late lag, early swelling, mid swelling, late swelling, emerged amoebae, and post-emerged myxamoebae.
the following: suspension in 1% peptone for 30 min (Cotter and Raper, 1966); the application of mild heat at 45°C for 30 min (Cotter and Raper, 1966, 1968a, b); gamma irradiation (Hashimoto and Yanagisawa, 1970; Khoury et al., 1970; Hashimoto, 1971); 20% dimethyl sulfoxide (DMSO) for 60 min (Cotter et al., 1976); 8M urea (Cotter and O'Connell, 1976); 3M ethylene glycol for 60 min (Cotter, 1977); 6M guanidine HCl for 60 min, 2M dimethyl urea for 60 min, and 2M tetramethyl urea for 30 min (Cotter, 1979). Spores activated by these methods germinate in the same sequence but at slightly different rates.

The time between the removal of the activating stimulus and the first sign of spore swelling is called the postactivation lag, and can be divided into two almost equal sections: early and late lag phases (Cotter, 1975). The postactivation lag is normally 60 min after spores are activated by heat at 45°C for 30 min (Cotter and George, 1975) but for other activation methods the lag varies from 0.5 to 2.0 hr.

The swelling stage of germination begins with the appearance of a lateral protuberance and ends when the outer two wall layers have split longitudinally; the cell is retained by the innermost wall (Cotter et al., 1969; Hemmes et al., 1972). The protuberance enlarges until the entire spore is swollen. This is accompanied by the formation of
one or more contractile vacuoles and the loss of
refractivity, heat, and cold resistance (Cotter and Raper,
1966, 1968a, b; Cotter, 1975).

The last stage of spore germination, the emergence of
myxamoebae, involves removal of the innermost wall layer
(Cotter et al., 1969; Hemmes et al., 1972). The myxamoebae
possesses pseudopodia and are phase dark. In this final
stage, inhibition of RNA synthesis (Bacon and Sussman, 1973)
or protein synthesis (Cotter and Raper, 1966) prevents
emergence.

Over 95% of wild type, NC4H, spores will germinate to
release myxamoebae by the end of 5 hr after activation when
incubated at $10^6$ to $10^7$ spores/ml at 23.5°C in 10 mM phosphate
buffer (pH 6.5) with aeration (Cotter and Raper, 1968a, b).
As previously mentioned, freshly formed spores of NC4H are
constitutively dormant and require an activation treatment
in order to germinate. However, spores that age in the
intact sorocarp for more than 7 days, autoactivate when
washed and suspended in phosphate buffer. Spores that are
aged for 7 days will show approximately 1% swelling at 6 hr
and 17% at 8 hr while those that are aged for 10 days show
17% swelling at 6 hr and 86% at 8 hr. Washed spores that
have aged for 13 days will germinate to at least 95% in the
absence of activation treatments (Dahlberg and Cotter,
1978).
The spontaneous germination mutant, SG1, will germinate to more than 95% at a concentration of $10^7$ spores/ml in the absence of any exogenous activation treatments (Cotter and Dahlberg, 1977). Application of an activation treatment to dormant spores does not change the germination process.

Restrictive environmental conditions can inhibit the spore germination process in D. discoideum. These environmental factors include: reduced oxygen tension, high or low pH, high osmotic pressure, and high or low temperature. If spores in the lag phase of germination are exposed to any one of the above environmental extremes, then the spores will not swell, but return to the dormant state (deactivate). However, spores in the swelling or emergence stage die if exposed to some of the same conditions (Cotter and Raper, 1968a,b; Cotter, 1975; Cotter et al., 1979).

C. Spore Respiration

Oxygen consumption increases dramatically after activation of D. discoideum spores (Cotter and Raper, 1968b; Bacon and Sussman, 1973; Cotter and George, 1975; Cotter et al., 1976; Kobillinsky and Beattle, 1977). Several lines of evidence suggest that mitochondrial respiration is essential for the germination process. As previously stated, activated spores in the lag phase of germination are usually deactivated when exposed to severe environmental conditions. These conditions directly or indirectly restrict oxygen
utilization (Cotter et al., 1979). Activated spores incubated with respiratory inhibitors (azide, cyanide, fluoride, or malonate) will also deactivate (Cotter and Raper, 1968b; Bacon and Sussman, 1973; Cotter et al., 1976; Cotter et al., 1979).

The respiratory competence of D. discoideum was investigated by Kobilinsky and Beattie (1977). These authors report the presence of all the necessary structural components for mitochondrial respiration in dormant spores. Enzymatic analysis of dormant spores reveal that the respiratory enzymes either are equal to or greater than those in the vegetative amoebae. One exception is that the total oligomycin-sensitive ATPase activity is 56% lower in the dormant stage.

The addition of oxidized nicotinamide dinucleotide (NAD) and various respiratory substrates to sonically treated spores produce similar oxygen consumption rates when compared to sonically treated amoebae with respiratory substrates but lacking NAD (Kobilinsky and Beattie, 1977). The authors suggest that the basal level of respiration in dormant spores may be due to the lack of NAD. Earlier findings by Wright and Wasserman (1974) are contradictory. These authors found that the level of pyridine nucleotide in fruiting bodies and amoebae were almost identical. Compartmentalization of respiratory substrates may account
for these differences.

D. Endogenous energy reserves in D. discoideum

1. Protein Content

Exogenous nutrients are only essential during the vegetative stage when myxamoebae grow and multiply by binary fission (Solomon et al., 1964). Aggregation and multicellular development of D. discoideum is induced as a response to starvation, especially for amino acids (Marin, 1975, 1977). Therefore, under conditions of starvation and independent of exogenous nutrients, sporulation occurs in a closed system; the process must depend entirely on endogenous reserves to fuel macromolecular synthesis (Wright et al., 1968). Spores also rely on endogenous reserves; they contain their own sources of carbon and energy (Cotter and Raper, 1968a,b).

There are dramatic changes in vegetative amoebae after the onset of starvation. Most notable is a 50% reduction in the dry weight and a decrease in respiration (Gregg, 1950; Gregg et al., 1954; Gregg and Bronsweig, 1956; White and Sussman, 1961; Liddel and Wright, 1961). Even though D. discoideum cells have the ability to metabolize either carbohydrate or protein the decrease in dry weight is primarily due to a loss in protein content. The products of protein degradation are presumed to be used as an endogenous energy source (Gregg et al., 1954; Wright and Anderson,
1960; White and Sussman, 1961; Hames and Ashworth, 1974).

Wright and Anderson (1960) examined the protein levels at progressive stages of development. Their findings indicate that the "free" amino acids are utilized first as reflected by a 30% decrease during the transition from amoebae to the pseudoplasmodial stage. Ethanol-soluble protein and ethanol-insoluble protein are then oxidized respectively. Incorporation of $^{35}$S-methionine into protein indicates that the rate of protein synthesis does not change even though there is a net loss of over 50% of cellular protein during the multicellular developmental cycle (Wright, 1963). In starving E. coli and yeast endogenous proteins are also degraded with the early utilization of the amino acid pool.

Respiration in D. discoideum is maximal at the amoeba stage, when food supplies are abundant, declines to a plateau from the migrating pseudoplasmodium through to culmination, and drops sharply to a basal level at the sorocarp stage (Gregg, 1950; Liddel and Wright, 1961). The decrease in respiration observed during the plateau period coincides with the time when "free" amino acids are at a low level. It has been suggested that this period may mark a shift from utilization of "free" amino acids to the endogenous ethanol-soluble and insoluble proteins. The majority of proteolysis occurs between the early slug and
culmination stages (Gregg et al., 1954; Wright and Anderson, 1960).

2. Proteolytic Activity

Acid protease activity was initially reported to exist throughout the life cycle of D. discoideum (pH optima 2.3 and 2) (Sussman and Sussman, 1969; Weiner and Ashworth, 1970). Weiner and Ashworth (1970) indicate that the possible activity is localized in the lysosomes but the subcellular localization of individual proteases is not yet known.

Fong and Rutherford (1978) examined two acid proteases during multicellular development. These proteases have an optimal pH of 2.5-2.75 and 5.5 and are termed cathepsin D- and cathepsin B-like respectively. While both proteases decrease during multicellular development from amoebae to sorocarp formation, the pattern of decrease is different. Cathepsin D activity is similar in prestalk and prespore cells and in mature stalk cells and spores. Cathepsin B activity is higher in prestalk than prespore cells and much higher in stalk cells than spores. This is consistent in that spores retain higher levels of protein than stalk cells (Gregg et al., 1954).

Analysis by electrophoresis on polyacrylamide gels shows that there are no less than eight separate acid proteases using extracts of amoebae; an additional new
protease appears upon fructing body formation (North and Harwood, 1979). These proteases could be separated into two groups based on their electrophoretic mobility; the faster bands A, B, D, and E and the slower bands G and H. If the pH is increased from 2 to 5, then band C appears. The final protease, band F, which is active from pH 2-5 is not associated with early multicellular development but coincides with fructing body formation. The presence of this protease at this stage of multicellular development may be related to a need for proteolytic activity during germination (North and Harwood, 1979).

Proteases were characterized on the basis of their sensitivity to inhibitors. Acid proteases in D. discoideum were all found to be sensitive to HgCl (North, 1978; Fong and Rutherford, 1978; North and Harwood, 1979). However, exposure to phenylmethylsulfonylfluoride (PMSF), a serine protease inhibitor, did not affect enzyme activity. Inhibitors of aspartyl proteases, pepstatin and diazoacetyl-DL-norleucine methyl ester (DAN), have no effect on protease activity in D. discoideum according to North and Harwood (1979) but Fong and Rutherford (1978) found slight inhibition with pepstatin and complete inhibition by DAN. Thiol protease inhibitors, N-α-p-tosyl-L-lysine chloromethyl ketone HCl (TLCK), and 1-chloro-4-phenyl-3-tosylamido-L-butan-2-one (TPCK), have no
effect on protease activity but leupeptin reduces the activity of the faster bands as examined by North and Harwood (1979). Fong and Rutherford (1978) report inhibition by leupeptin, TLCK, antipain, iodoacetamide, and iodoacetate. Kost et al. (1981) now show that common multiple forms of acid proteases exist in related species including Dictyostelium mucoroides, Dictyostelium purpureum, Polysphondyllum pallidum, and Polysphondyllum violaceum. These authors are in agreement with the inhibition studies of North and Harwood (1979) except that their proteases, while not completely inactivated, are sensitive to thiol inhibitors. The difference in results may in part be due to differences in substrate type, inhibitor concentration, or enzyme assay. It appears then, that there are two major types of acid proteases in D. discoideum. One is of the aspartyl type (cathepsin D-like) and the other of the thiol type (cathepsin B-like). No protease activity has been detected above pH 6.

3. Amino Acid Catabolism

Slime mold metabolism, during multicellular development, appears to become increasingly efficient at using amino acids as an energy source and less efficient at using other endogenous reserves. Indeed, amino acid catabolism continues throughout multicellular development. This is evident from the release of ammonia and carbon
dioxide, a result of protein degradation and subsequent amino acid oxidation. Two end products of amino acid catabolism accumulate by the end of multicellular development. Pyruvate increases 10-fold to a final concentration of 0.5 mM (Cleland and Coe, 1969). Glutamate, formed from the transamination of amino acids to \(-\text{keto}\text{glutarate},\) increases throughout multicellular development and reached a final concentration of 1 mM (Wright, 1963).

Analysis of amino acid catabolizing enzymes further supported this idea. Threonine deaminase-1 activity is rapidly lost during multicellular development and eventually this biosynthetic enzyme is replaced by threonine deaminase-2. This enzyme increases 7-fold in activity to a maximum at the pseudoplasmodial stage after which it declines slightly (Pong and Loomis, 1973). Threonine deaminase-2 degrades threonine and serine. Alanine transaminase increases from 200 to 500 units/mg protein during the first 5 hr of multicellular development and remains constant (Flirtel and Brackenbury, 1972). Tyrosine transaminase is maximal at culmination after it increases 3-fold (Pong and Loomis, 1971). Three amino acid catabolizing enzymes, aspartate transaminase, glutamate dehydrogenase, and homoserine dehydratase remained at essentially the same specific activity throughout
multicellular development (Flrtei and Brackenbury, 1972). The activity of these enzymes may be high enough to meet the metabolic demands of multicellular development.

4. Carbohydrate Content

Total carbohydrate remains constant over the period of multicellular development, yet new stalk and spore cell-wall polysaccharide (cellulose) and a storage disaccharide (trehalose) are synthesized. The carbon source for these new saccharides comes from the reorganization of cellular glycogen and RNA (Clegg and Filosa, 1961; White and Sussman, 1963a, b; Wright, 1964; Sussman and Sussman, 1969). Since carbohydrate levels remain constant, it would appear that glycolysis and gluconeogenesis from amino acids are minimally involved (Cleland and Coe, 1968, 1969). Therefore amino acids produced by protein degradation are not used as a source of saccharide precursors but rather as a source of energy for the synthesis of new saccharides.

Trehalose, the storage disaccharide, varies throughout the life cycle of D. discoideum (Ceccarini and Filosa, 1965). In the vegetative amoebae and migrating pseudoplasmodia, the level of trehalose is very low and accounts for less than 0.5% of the total dry weight. The level of trehalose increases to 1.5% of the total dry weight at the culmination stage and further increases to a maximum of 5% in the mature spores. Trehalose and glucose are the
only water-soluble carbohydrates in the mature spores (Ceccarini, 1966, 1967). Indirect evidence suggests that trehalose is localized in vesicles within the spores (Muller and Hohl, 1975).

5. Expression of Trehalase activity

Dormant spores of *D. discoideum* contain a basal level of trehalase activity which is unaffected by heat activation (Cotter et al., 1979). This low activity remains through post-activation lag and early swelling stages (Cotter and Raper, 1970; Cotter et al., 1979; Tisa and Cotter, 1979b). By late swelling, approximately 15-30 min before the emergence of myxamoebae, the enzyme increases dramatically in activity and is released into the extracellular medium (Chan and Cotter, 1982; Jackson et al., 1982). The release of trehalase coincides with the synthesis of the newly-formed trehalase. The increase in this activity requires both RNA and protein syntheses as UV irradiation and the addition of cycloheximide (200 μg/ml) blocked the expression of trehalase activity during germination (Cotter and Raper, 1970; Tisa and Cotter, 1980; Demsar, personal communication).

6. Neutral Lipid and Fatty Acid Content

Neutral lipids vary the most during multicellular development and accounted for 40% of the total lipids (Davidoff and Korn, 1963). Up to the preculmination stage
they remained around 33 mg/10^{10} cells and increase to 45 mg/10^{10} cells in the sorocarp. Other neutral lipid components also increased in this period. These include the ubiquinones and the sterol esters (Long and Coe, 1974).

Fatty acid levels rise in Interphase amoebae suggesting a period of increased lipase activity. With the onset of aggregation fatty acids drop dramatically and increase once again at culmination (Long and Coe, 1974). Saturated fatty acids comprised 60% of the total of which stearic, 18:0, and palmitic, 16:0, acids predominate. Unsaturated fatty acids made up the other 40%. They include palmitoleic, 16:1(9), pelargonic and pelargonic in very small quantities, 16:1(7), vaccenic, 18:1(11), oleic, 18:1(9), 5,11-octadecadienoic, 18:2(5,11), and hexadecadienoic, 16:2 (5,9) acids (Davidoff and Korn, 1963). Fatty acids decrease slightly as spores age.

E. Purpose of this study

The purpose of this study is to survey the spore for (1) the source of the energy utilized during germination and (2) for the enzymes controlling energy utilization.
MATERIALS AND METHODS

A. Medium used

Each strain of Dictyostelium discoideum (NC4H, SG1, and SG2) used in this study was grown in conjunction with Escherichia coli B/r on glucose-salts agar (Adams, 1959). This medium consists of the following components: 1.0g NH₄Cl, 0.13g MgSO₄, 3.0g K₂HPO₄, 6.0g Na₂HPO₄, and 20.0g Bacto agar (Difco) per litre of distilled water. After autoclaving, 10 ml of 0.4g/ml of sterile glucose was added to the above medium. The medium was mixed, cooled to 50 C and dispensed into 100 x 15 mm plastic petri dishes.

Spores of D. discoideum were germinated in phosphate buffer consisting of 1.04g of KH₂PO₄ in 1 litre of double distilled water. The pH of the 10 mM solution was adjusted to 6.5 with concentrated KOH prior to autoclaving.

B. Formation of D. discoideum sorocarps

Spores of D. discoideum were transferred aseptically from a stock plate to sterile double distilled water or 10 mM phosphate buffer. A loopful of E. coli B/r was then transferred to the suspension and mixed thoroughly; 2 ml of the mixture was transferred onto the glucose-salts plates and incubated at 23.5 C. The culture was then shaken 24 hr after plating to ensure even growth and synchronous sorocarp formation. Myxamoebae were normally found the second day.

-21-
after plating. On the fourth day young spore-bearing sorocarps were observed on the plates. Sorocarps were allowed to age 2 days before the spores were collected in order to ensure that all spores used in germination experiments had entered dormancy.

C. Collection of matrix enzymes

Dormant spores of *D. discoideum* are contained within a viscous matrix of extracellular material at the apex of the stalk. Enzymes dominate the matrix. Collection of these enzymes from 2-13 day old sor1 was accomplished by passing a microscopic slide several millimetres above the agar surface. The sor1 which consisted of spores and extracellular material were suspended in 5-15 ml of sterile double distilled water or the appropriate buffer, depending on the enzyme to be assayed, and kept on ice. The spores were then removed from the extracellular material by low speed centrifugation in an IEC clinical centrifuge (setting 6). The supernatant containing the pigmented matrix material was stored on ice or at -21°C until assayed.

D. Germination of *D. discoideum* spores

Spores which had been washed once in double distilled water were washed twice again in 10 mM phosphate buffer (pH 6.5) to ensure that all spores were free of extracellular material.

Spores prepared in this manner were activated by the
application of a heat shock. The spores were suspended in 5 ml of 10 mM phosphate buffer (pH 6.5) and placed in a 45°C waterbath for 30 min. The heat-activated spores were diluted out with phosphate buffer so the final concentration was approximately $1 \times 10^7$ spores/ml. The spore suspension was incubated at 23.5°C with gentle shaking to ensure adequate aeration so as to allow germination to proceed. The number of spores/ml was calculated with a hemocytometer (Cotter and Raper, 1966, 1968a,b).

Spores were germinated after activation in one of two ways depending on the volume required. Small volumes of activated spores were placed in 10 x 1 cm test tubes and 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 submersible magnetic stirrer).

Large spore suspensions (100, 250, or 1000 ml) were introduced into large flasks and incubated in a GCA/Precision Scientific Co. waterbath at 23.5°C. The spores were shaken at 80 oscillations/min.

The percentage of swollen spores and released myxamoebae 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 320 X. The objects were placed into three categories:
unswollen spores, swollen spores, and emerged myxamoebae.

To study the effect of inhibitors (arsenate, fluoride, lodoacetamide, and lodoacetate) on germination, spores were prepared in the manner described above and heat-shocked in the presence of the inhibitor or with phosphate buffer. Both activated samples were divided into two equal aliquots and centrifuged (setting 6 IEC centrifuge). One pellet from each type of activation method was resuspended in phosphate buffer and the other two with the inhibitor under study. Germination was scored as described above; the phosphate-shocked sample resuspended in phosphate buffer served as the control.

E. Production and germination of Polysphondyllum pallidum microcysts

Amoebae of Polysphondyllum pallidum strain WS 320 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 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.

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).

F. The study of endogenous respiration

Standard manometric techniques were used to determine the endogenous respiration of dormant and germinating spores of D. discoideum and P. pallidum (Umbreit et al., 1957). Spores and microcysts of both species were harvested and activated as previously mentioned.

Endogenous oxygen consumption or carbon dioxide production was measured in Warburg flasks containing approximately $1 \times 10^7$ spores/ml in the main flask and 0.2 ml of 20% KOH or 0.2 ml of phosphate buffer in the centre well. The total volume never exceeded 3.0 ml.

To determine the effects of exogenous substrates on
endogenous spore respiration glucose, trehalose, or acetate was added to the main flask. The final concentration was 10 mM in each case. All respiratory experiments were conducted at 23.5 C in a Gilson differential respirometer.

G. Utilization of endogenous lipid reserves during spore germination of D. discoideum

1. Lipid extraction

A modification of the procedure of Hara and Radin (1978), using hexane:isopropanol (HIP), was employed to extract lipid from dormant and germinating spores of D. discoideum. At one hour intervals during the germination process, 10-ml samples were removed, washed free of phosphate buffer by low speed centrifugation at setting 6 in an IEC centrifuge, and resuspended in 5 ml HIP (3:2). The mixture was then passed through a French pressure cell (Amicon Co.) three times at 20,000 PSI, and the resulting crude cell lysate filtered through Whatman 1 filter paper. The pressure cell, funnel, and residue were washed with 2-ml portions of HIP and the filtrate combined in a 16 x 100 mm test tube. The test tube was immediately sealed with a hard rubber syringe stopper and paraflim; the HIP solvent being removed with a stream of N2 at 40 C. The tube remained sealed in the presence of N2 until assayed for lipid content.

2. Fractionation of neutral lipid
Crude lipid was separated into its neutral lipid fraction by employing acid-treated Florisil (Hanson and Phillips, 1981). Florisil was acid-treated by mixing 100g (60-100 mesh, BDH) with 350 ml of concentrated HCl in a 1 litre Erlenmeyer flask, and heated in a hood for 3 hr (all work was carried out in a fume hood to avoid exposure to liquid and vapour). The supernatant was decanted and the settled resin washed with 50 ml of concentrated HCl. Another 350 ml of concentrated HCl was added and the slurry heated overnight (12 to 16 hr) in a round-bottom flask fitted with a condenser at 100 C. The supernatant was decanted and the resin washed 3 x with distilled water. The resin was placed on a Buchner funnel and washed until the effluent was neutral. The resin was dried by applying a vacuum to the funnel. Added to the resin in succession were: 150 ml of methanol, 150 ml of methanol-chloroform (1:1 V/V), 150 ml of chloroform, and 150 ml of ether. The resin was dried overnight at 120 C in a glass petri dish being careful to remove as much ether as possible from the resin beforehand. To 12g of the acid-treated Florisil, chloroform was added to make a slurry. The slurry was degassed by vacuum for 10 min and poured into a column (1 x 30 cm, fitted with a solvent reservoir, glass-wool plug, and greaseless Teflon stopcock).

The total crude lipid (prepared as in section 1) was
redissolved in 3 ml of chloroform and vortexed. The solution was carefully applied to the column and the tube rinsed 2 times with chloroform. These chloroform washes were also added to the top of the column and the column was drained until the chloroform just entered the resin bed. An additional 5 ml of chloroform was added and drained until all the lipid entered the resin bed. Chloroform was added to the column and reservoir (50 ml) and was allowed to flow through the column at a rate not exceeding 1 ml/min. The effluent was collected in a glass vessel until the chloroform just entered the resin bed. The column was washed in succession with 60 ml of chloroform-acetone, 60 ml of acetone, 60 ml of methanol, and 60 ml of chloroform for re-use. The neutral lipid fraction was dried in a 12 x 75 mm test tube under a stream of nitrogen at 40 °C and sealed in the presence of N₂ until assayed for lipid content.

3. Microdetermination of lipids

Lipid content was assayed using the semimicro method of Pande et al. (1963) for both total crude lipid and neutral lipid. To each sample tube, which was devoid of all solvent, 3 ml of 2% potassium dichromate (W/V) in 98% (W/V) sulfuric acid was added. Each tube was vortexed thoroughly to ensure contact of all lipid with the dichromate reagent. The tubes were placed in a boiling water bath for 15 min and then cooled in water; 4.5 ml of distilled water was added,
the contents vortexed, and recooled. The absorbance was measured at 590 nm with a Spectro-Plus (MSE) spectrophotometer against a reagent blank containing 3 ml of acid-dichromate reagent and treated like the rest.

Lipid content was reported as mg palmitate/10⁷ spores using palmitic acid (Sigma Chem. Co.) as a standard.

4. Preparation of free fatty acid (FFA) extracts

Spores were activated as described in section D in order to analyze the levels of FFA during spore germination of D. discoideum. At hourly intervals during the germination process 20-ml samples were removed, centrifuged at low speed (setting 6, IEC centrifuge), and the pellet resuspended in 10 mM phosphate buffer (pH 6.5). A 5-ml aliquot of the supernatant was taken to check for release of FFA from the spores during germination. The samples were then passed through a French pressure cell three times at 20,000 PSI. The spore extracts were centrifuged at 8200 x g for 15 min at 4 C in a refrigerated Beckman J-21C centrifuge. The supernatants after centrifugation served as crude FFA preparations.

5. Measurement of free fatty acids

The method of Soloni and Sardina (1973) was used for the determination of FFA based on the estimation of copper in a chloroform extract of their cupric salts with oxalic acid bis-(cyclohexylidenehydrazide). To 0.1 ml of the FFA
preparations 0.3 ml of copper reagent was added and vortexed. To this solution 3 ml of redistilled chloroform was added and shaken at 300 RPM on an orbital shaker (Lab-line Jr. Orbit Shaker).

After 30 min the organic phase was transferred to a clean test tube containing a filter paper pad (Whatman 1) to absorb the remainder of the blue (aqueous) phase. To a second clean test tube 2 ml of the chloroform was transferred and 1.8 ml of the cuprizone reagent added with gentle mixing followed by the addition of 0.2 ml of NH₄ reagent. The solution was thoroughly mixed by vortexing and incubated for 20 min. The absorbance was measured at 620 nm with a Perkin-Elmer (Model 575) spectrophotometer against a reagent blank containing 0.1 ml phosphate buffer and treated like the rest.

The FFA content was reported as ug palmitate/10⁷ spores using palmitic acid (Sigma Chem. Co.) as a standard.

6. Preparation of lipase samples

Spores were activated as described in section D in order to analyze the level of lipase activity during spore germination of D. discoideum. At hourly intervals during the germination process 10-ml samples were removed, centrifuged at low speed (setting 6, IEC centrifuge) and the pellet resuspended in 10 mM phosphate buffer (pH 7.0). The samples were then passed through a French pressure cell
three times at 20,000 PSI. The spore extracts were centrifuged at 8200 x g for 15 min at 4 C in a refrigerated Beckman J-21 C centrifuge. The supernatant after centrifugation served as crude lipase preparations.

7. Assay of lipase activity

The method of Seligman and Nachlas (1965) was used to assay for lipase activity during spore germination of D. discoideum. The only exception was that all volumes were reduced by one-half to accommodate the test tubes.

A stock solution of 2-naphthol laurate (Sigma Chem. Co.) (1 mg/ml) was prepared by dissolving 100 mg of 2-naphthol laurate in acetone and by making it up to 100 ml. The stock solution was pre pared by allowing 5 ml of the stock solution to run into a mixture of 10 ml barbiturate buffer (0.1 M; pH 7.4) and 35 ml distilled water with stirring.

Each sample required two separate determinations. The samples were processed in such a way that one determination was made in the presence of sodium taurocholate (Sigma Chem. Co.) and the other was not. To one test tube 0.5 ml of the taurocholate solution (8 x 10^{-2}M) was pipetted while the other corresponding test tube contained 0.5 ml of distilled water in place of the taurocholate. The lipase preparation (0.1 ml) and substrate suspension (2.5 ml) were pipetted successively into both tubes. After the samples were
incubated for 5 hr at 37.5°C, the following components were
added to each tube: 0.5 ml tetrazotized 0-dianisidine (4
mg/ml; prepared just prior to use), 0.5 ml TCA (40% W/V),
and 5 ml ethyl acetate. The tubes were vortexed between
each addition. The samples were then centrifuged for 5 min
(setting 7, IEC centrifuge) and approximately 5 ml of the
purple coloured ethyl acetate layer used for the
colourimetric measurements. The absorbance was measured at
540 nm against a reagent blank containing distilled water
and substrate suspension and treated as described above.
Lipase activity was determined by subtracting the tube
without taurocholate from the tube containing taurocholate.

Enzyme activity was defined as mg 2-naphthol released
per hr at 37.5°C under the assay conditions described above.
Protein was measured by the method of Bradford (1976) using
a-globulin as a standard; units of specific activity were
defined as units of enzyme activity per mg protein.

H. Utilization of endogenous trehalose reserves during spore
germination of D. discoideum

1. Preparation of trehalose samples

The carbohydrate trehalose, was prepared by means of
ethanol extraction (Cotter and Niederpruem, 1971). The
spores were allowed to germinate as described above. At
half-hour or hourly intervals, 10-ml samples were removed
and centrifuged at low speed to pellet the spores or
myxamoebae. After one wash with 10 mM phosphate buffer the samples were extracted with two portions of ethanol (80% V/V) at 80°C for 30 min. This procedure will solubilize trehalose and glucose but will not solubilize glycogen. The ethanol extracts were then air dried at 45°C. The dry extracts were redissolved in small quantities of 0.1 M acetate buffer (pH 5.5). In all experiments the germination buffer used to suspend the spores was also checked for the presence of trehalose which may have leached from the spores.

2. Measurement of trehalose utilization

A continuous coupled glucosidase assay was used to detect the amount of endogenous trehalose consumed during spore germination of D. discoideum (Chan and Cotter, 1980). The trehalase was partially purified to 100-fold purity by means of ethanol precipitation (Ceccarini, 1966; Killick, 1979, 1980). In the assays, a 0.5 ml volume of the trehalose extract was added to 0.5 ml of partial purified trehalase enzyme (95 U/ml). To the above mixture 1.0 ml of glucose reagent was added and the tube was incubated at 23.5°C for 1 hr. An enzyme control contained 0.5 ml of trehalase enzyme (95 U/ml) and 0.5 ml of acetate buffer; and a substrate control contained 0.5 ml of the trehalose extract and 0.5 ml of acetate buffer. Before incubation for 1 hr each of the above control tubes received 1.0 ml of glucose reagent.
Absorbances were taken at 420 nm against a blank containing 1.0 ml of acetate buffer and 1.0 ml of glucose reagent. The amount of glucose present in the substrate control was subtracted from that present in the trehalose samples; a unit of trehalose is defined as 1 nmole of glucose released per min per 10^7 spores.

1. The study of proteolytic enzymes during spore germination of D. discoideum

1. Preparation of protease samples

Spores were activated as described in section D in order to analyze proteolytic activity during spore germination of D. discoideum. At hourly intervals during the germination process 10-ml samples were removed, centrifuged at low speed (setting 6, IEC centrifuge), and the pellet resuspended in the appropriate buffers: 0.05 M citrate, pH 2.75 for cathepsin D, 0.05 M acetate (with added activators, 4 mM Na₂ethylenediaminetetraacetic acid and 10 mM dithiothreitol), pH 5.5 for cathepsin B, and 10 mM HCl for neutral protease. The samples were then passed through a French pressure cell three times at 20,000 PSI. The resulting crude cell lysates were centrifuged immediately for 15 min at 8200 x g in a refrigerated Beckman J-21 C centrifuge to separate debris from enzyme in the supernatant. The supernatants served as crude protease extracts and were kept on ice until assayed.
2. Assay for acid protease activity

The method of Fong and Rutherford (1978) was used to assay for acid protease activity during spore germination of 
D. discoideum. To 475 ul of the enzyme sample 97 ul of 
azoalbumin (50 mg/ml) (Sigma Chem. Co.), pH 7.2 was added. 
Incubation at 37 C was 15 hr for cathepsin B and 20 hr for 
cathepsin D. The reaction was terminated by the addition of 
555 ul of cold 40% TCA. The samples were centrifuged at low 
speed (3000 RPM) for 15 min in a refrigerated Beckman J-21 C 
centrifuge and 550ul of the TCA-soluble fraction transferred 
into a clean test tube. An equal volume of 0.5 N NaOH was 
added, mixed, and the absorbance measured at 440 nm in a 
Spectro-Plus (MCE) spectrophotometer against a reagent blank 
containing 550 ul of 40% TCA and 550 ul of 0.5 N NaOH.

3. Assay for neutral protease activity

Neutral protease activity during spore germination of 
D. discoideum was assayed for by the method of Brown and 
Freedman (1973). The incubation mixture (0.2 ml) consisted 
of equal volumes of the crude enzyme preparation in 10 mM 
HCl (0.1 ml) and dinitrophenylprotamine sulfate (DNPP) (0.1 
ml) dissolved in 0.1 M sodium phosphate buffer, pH 7.5 (10 
mg/ml). Incubation was at 37.5 C. Aliquots (0.05 ml) were 
taken after 20 min and mixed with 3 ml of 0.1 M sodium 
phosphate buffer, pH 7.0. With vigorous mixing, 1 ml of 
fluorescamine (2 mg per 10 ml acetone dried over magnesium
sulfate) was added. Fluorescence was determined on a Turner Model 430 fluorometer at an extinction wavelength of 390 nm, and an emission wavelength of 470 nm. Neutral protease activity was expressed as relative fluorescence (R. F.)/10^7 spores.

4. Preparation of dinitrophenylprotamine sulfate (DNPP)

DNPP was synthesized according to the method of Brown and Freedman (1973) with minor modifications. A volume of 1-fluoro-2,4-dinitrobenzene in methyl cellosolve (0.63 ml diluted out to 50 ml in methyl cellosolve) was added to 5 volumes of a 1% solution of protamine sulfate in 0.01 M sodium tetraborate, pH 9.2. After incubation at 37 C for 1 hr, 20 ml of absolute ethanol was added and the resultant yellow precipitate collected by centrifugation (1000 x g for 10 min). The precipitate was washed once with 20 ml of absolute ethanol and twice with 20 ml of acetone. The DNPP was dried at room temperature. In each case the yield averaged 84% based on 100 mg protamine sulfate.

5. Preparation of aminopeptidase samples

Spores were activated as described in section D in order to analyze the level of aminopeptidase activity during spore germination of D. discoideum. At hourly intervals during the germination process 10-ml samples were removed, centrifuged at low speed (setting 5, IEC centrifuge) and the pellet resuspended in 0.05 M potassium phosphate buffer (pH
7.2). The samples were then passed through a French pressure cell three times at 20,000 PSI. The spore extracts were centrifuged at 8200 x g for 15 min at 4 C in a refrigerated Beckman J-21 C centrifuge. The supernatant after centrifugation served as crude aminopeptidase preparations. An additional 10-ml aliquot from germinating spores was centrifuged and the supernatant was used to check for the release of aminopeptidase.

To obtain aminopeptidase samples from the extracellular matrix, sori were harvested as described in section C; the spores and crude matrix material being chilled on ice. Spores and crude matrix material were separated by centrifugation at 1000 x g. Matrix material that was free of spores was again centrifuged at 8200 x g for 15 min in a Beckman J-21 C centrifuge at 4 C; the supernatant after this second centrifugation served as an aminopeptidase matrix preparation. All samples were kept on ice until assayed for aminopeptidase activity.

6. Assay of aminopeptidase activity

The method of Flirtel and Brackenbury (1972) was used to determine aminopeptidase activity during spore germination of D. discoideum. To 0.1 ml of crude cell extract 0.5 ml of 0.05 M phosphate buffer (pH 7.2) was added. This buffer contained 0.016 ml of L-leucine-p-nitroanilide (0.24 M) dissolved in absolute methanol. The reaction mixture was
Incubated at 30 C for 30 min and terminated by addition of 0.5 ml of 1 M sodium carbonate. The p-nitrophenyl produced was determined by reading the absorption at 420 nm with a Spectro-Plus (MSE) spectrophotometer against a reagent blank containing 0.6 ml of 0.05 M phosphate buffer and 0.5 ml of sodium carbonate.

A substrate control was utilized to correct for substrate degradation which might occur during incubation in the absence of enzyme. The control was prepared by adding 0.5 ml of substrate and was incubated as described above. After the incubation period 0.1 ml of enzyme extract together with 0.5 ml of sodium carbonate was added simultaneously to each substrate control tube and the absorbance was measured as described above.

One unit of enzyme activity was defined as 1 nmole of p-nitrophenol released per min at 30 C under the assay conditions as described above. Protein was measured by the method of Bradford (1976) using γ-globulin as a standard; units of specific activity were defined as units of enzyme activity per mg protein.

J. The study of the effect of various metabolic inhibitors on spore germination in D. discoideum.

Spores prepared as previously described in section D were divided into 2 equal aliquots, centrifuged in an IEC clinical centrifuge (setting 6) and the supernatant
discarded. One pellet was resuspended in 10mM phosphate buffer (pH 6.5) and the other in one of four inhibitors. The inhibitors included 10mM potassium arsenate (Sigma Chem. Co.), 10mM sodium fluoride (J. T. Baker Chem. Co.), 1mM iodoacetamide (Sigma Chem. Co.), and 1mM iodoacetic acid (Eastman Kodak Co.).

The spore suspensions were heat shocked for 30 min at 45°C. Each sample was further divided into 2 equal aliquots and washed three times in buffer or the appropriate inhibitor. Each aliquot was resuspended in the solution in which it had just previously been washed and incubated in a GCA/Precision Scientific Co. waterbath at 23.5°C. The spores were shaken at 80 oscillations/min. At half-hour intervals samples were removed and germination scored as described in section D.

K. Determination of dry weight during *D. discoideum* spore germination.

A gravimetric method was employed to determine the dry weight of dormant and germinating spores of *D. discoideum*. At one hour intervals during the germination process, $1 \times 10^7$ cells were pipetted onto predried and preweighed filter discs (Whatman GF/C). The spore suspension was added dropwise onto prewetted filters to ensure all the cells remained in the center of the filters. With the aid of forceps the filters were transferred to glass petri dishes
and placed in a drying oven (Blue M model) at 80 C. The filters were dried and weighed on an analytical balance (Mettler H54) to a constant weight. The dry weight was determined by measuring the difference in filter weights. Phosphate buffer (10 mM, pH 6.5) was pipetted onto filters and processed like the others to act as controls.
RESULTS

A. Determination of the respiratory quotients (R. Q.) during heat-induced spore germination of D. discoideum strain NC4H.

The respiratory quotients provided a means to determine the possible endogenous respiratory substrate(s) which were metabolized by dormant and heat-activated D. discoideum strain NC4H spores. Dormant spores possessed R. Q. values of 0.88 which indicated that lipids and/or amino acids were the main source of respiratory substrates at this time (data not shown).

The R. Q. pattern of heat-activated spores is shown in Fig. 3. After the post-activation lag period the R. Q. increased to 2.24. Spore swelling was marked by a rapid decrease in the R. Q. which remained greater than or equal to 1.00 until late swelling but prior to the emergence of myxamoebae. This indicated that the predominate substrate consumed was carbohydrate. During the emergence of myxamoebae the R. Q. decreased below 1.00 and continued to decrease to a point where at the end of 5 hr the R. Q. was 0.74 (Fig. 3). This again indicated that lipids and/or amino acids were the primary respiratory substrate(s).

Exogenous carbon energy substrates (each 1mM) were added to spore suspensions during germination in order to determine their effect on the R. Q. values. When compared
Figure 3. The pattern of respiratory quotient (R. Q.) values during heat-induced spore germination of D. discoideum strain NCH4.

Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (■), R. Q. values.
to the endogenous R. Q. exogenous glucose, trehalose, and acetate did not significantly alter the pattern of R. Q. values (Figs. 4, 5, and 6).

In the related species, Polysphondyllum pallidum, the R. Q. pattern was similar to that of D. discoideum (Fig. 7). The initial R. Q. was 1.30 and decreased to 1.00 prior to the emergence of myxamoebae. This again indicated that the predominate substrate consumed was carbohydrate. During the emergence of myxamoebae the R. Q. decreased below 1.00 and continued to decrease to a point where at the end of 3 hr, the time of maximum emergence, the R. Q. was 0.78 (Fig. 7). It appeared therefore, that in P. pallidum lipids and/or amino acids were the main respiratory substrate(s) for the emergence of myxamoebae.

B. Lipid utilization during heat-induced spore germination of D. discoideum strain NC4H.

1. Total crude lipid content

Dormant spores of D. discoideum contained a high level of lipid when based as a percentage of its dry weight (Long and Coe, 1973). When measured as mg palmitate/10⁷ cells, dormant spores also contained a high level; the amount of total crude lipid was greatest at this stage (Fig. 8). Under normal germination conditions the total crude lipid content decreased dramatically by late spore swelling. This was shown by the 7-fold decrease in the amount of total
Figure 4. The effect of exogenous glucose on the respiratory quotient values during heat-induced spore germination of *D. discoideum* strain NC4H. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (■), R. Q. values in the presence of exogenous glucose.
Figure 5. The effect of exogenous trehalose on the respiratory quotient values during heat-induced spore germination of D. discoideum strain NC4H. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (■), R. Q. values in the presence of exogenous trehalose.
Figure 6. The effect of exogenous acetate on the respiratory quotient values during heat-induced spore germination of D. discoideum strain NC4H. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (■), R. Q. values in the presence of exogenous acetate.
Figure 7. The pattern of respiratory quotient values during microcyst germination of *Polysphondyllum pallidum* strain WS 320.
Symbols represent: (●), percent emerged myxamoebae; (◼), R. Q. values.
Figure 8. Utilization of endogenous total crude lipid during heat-induced spore germination of D. discoideum strain NC4H. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (□), total crude lipid content in units. Units were expressed as mg palmitate/10⁷ spores.
crude lipid present in the cells by late swelling (Fig. 8). The emergence of myxamoebae was marked by a small decrease in the total crude lipid content.

2. Neutral lipid content

Cellular energy is derived from the neutral lipid fraction of the total crude lipid. The neutral lipid content was greatest in the dormant spores and comprised 24% of the total crude lipid content (Fig. 9). The pattern of neutral lipid utilization during heat-induced spore germination is shown in Fig. 9. The neutral lipid content decreased 10-fold during the germination process. The pattern of neutral lipid utilization differed compared to that for the total crude lipid in that the neutral lipid fraction decreased at a more constant rate. By the end of 5 hr, the time of maximum emergence, the neutral lipid content present in the cells was almost completely exhausted (Fig. 9).

3. Free fatty acid (FFA) content

In order to be utilized as an energy source neutral lipid must first be metabolized to fatty acids (FFA). The FFA content increased 5-fold during heat activation (Fig. 10). However, by late spore swelling the level of FFA had decreased slightly. With the emergence of myxamoebae FFA again increased to the levels initially produced after the heat-activation period (Fig. 10).
Figure 9. Utilization of endogenous neutral lipid during heat-induced spore germination of *D. discoideum* strain NC4H.

Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (■), neutral lipid content in units. Units were expressed as mg palmitate/10⁷ spores.
Figure 10. Free fatty acid (FFA) content during heat-induced spore germination of *D. discoideum* strain NC4H.

Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (■), FFA content in units. Units were expressed as ug palmitate/10^7 spores.
4. Expression of general lipase activity

Lipase activity is widely distributed in fungi. The enzyme hydrolyzes the glycerol esters of fatty acids (neutral fats) to fatty acids and glycerol (Stryer, 1981).

A 3.5-fold higher specific activity was observed in dormant spores than in emerged myxamoebae (Table 1). The high specific activity was contributed to by high enzymatic activity. Protein concentration remained constant.

The change in specific activity of general lipase during heat-induced spore germination of D. discoideum NC4H is shown in Fig. 11. The specific activity continuously decreased throughout germination. The pattern of general lipase activity closely followed the decline in the total crude lipid content.

C. Trehalose utilization during heat-induced spore germination of D. discoideum strain NC4H.

Trehalose and glucose have been shown to be the only water-soluble carbohydrates present in the dormant spores of D. discoideum (Ceccarini, 1966, 1967). In order to act as a carbon source, trehalose has to be first hydrolyzed into glucose.

Under normal germination conditions, trehalose was hydrolyzed into glucose before maximum swelling (Fig. 12). This was shown by the sharp decrease in the amount of trehalose present in the cells by the end of spore swelling.
Table 1. The specific activity of general lipase in dormant spores and fully emerged myxamoebae.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Enzyme activity $^{b}$ (Units)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity $^{c}$</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dormant spores</td>
<td>0.12</td>
<td>0.06</td>
<td>2.00</td>
<td>3.5</td>
</tr>
<tr>
<td>Emerged amoebae</td>
<td>0.04</td>
<td>0.07</td>
<td>0.57</td>
<td>1.0</td>
</tr>
</tbody>
</table>

$^{a}$Sori were harvested in phosphate buffer (10 ml, pH 6.5), centrifuged, and the pelleted spores retained. After two washes in phosphate buffer half the spores were resuspended in the appropriate enzyme assay buffer as described in the Materials and Methods. The remainder of the spores were resuspended in phosphate buffer and heat-shocked at 45°C for 30 min. The dormant spores and fully emerged myxamoebae were processed and the lipase activity assayed for as described in the Materials and Methods.

$^{b}$Units of enzyme activity are equivalent to mg 2-naphthol released per ml per hr at 37.5°C.

$^{c}$Units of specific activity are equivalent to units of enzyme activity per mg of protein.
Figure 11. Expression of general lipase activity during heat-induced spore germination of *D. discoideum* strain NC4H.

Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (■), general lipase specific activity. Specific activity refers to units of activity per mg protein where one unit of activity is defined as mg 2-naphthol released per hr at 37.5°C.
Figure 12. Utilization of endogenous trehalose during heat-induced spore germination of D. discoideum strain NC4H.

Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (□), trehalose content in units. Units were expressed as nmoles of glucose released per min at 23.5° C when incubated with 95 U/ml of partially purified trehalase.
In order to study the relationship between spore swelling and the hydrolysis of trehalose, spores were heat activated and then incubated at 37 °C. Spores under these conditions remained unswollen (Fig. 13). After 2 hr of incubation at 37 °C, the spore suspension was shifted to 23.5 °C which is the normal germination temperature (Cotter and Raper, 1968a). Spores under the above conditions swelled almost immediately in a highly synchronous manner. When the utilization of trehalose was monitored at this time, the disaccharide was found to be quickly hydrolyzed after the spores were transferred to 23.5 °C (Fig. 13).

Activated spores which were incubated under anaerobic conditions did not swell or hydrolyze their endogenous trehalose pool (Fig. 14).

D. Expression of proteolytic activity during heat-induced spore germination of D. discoideum strain NC4H.

1. Cathepsin B-like protease activity

In order to be utilized as an energy source, proteins must first be catabolized to small peptides and free amino acids. This process is initiated by the action of proteases.

North and Harwood (1979) have reported that cathepsin B-like protease may be of the thiol type. Fong and Rutherford (1978) reported a necessity for the presence of added activators (dithiothreitol and EDTA). The requirement
Figure 13. Effect of elevated incubation temperature on trehalose utilization after heat-induced spore activation of *D. discoideum* strain NC4H. Spores, after heat activation, were incubated at 37°C for 2 hr. The spores were then shifted to 23.5°C (arrow). Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (□), trehalose content in units. Units were expressed as nmoles of glucose released per min at 23.5°C when incubated with 95 U/ml of partially purified trehalase.
Figure 14. Effect of anaerobic conditions on trehalose utilization after heat-induced spore activation of *D. discoideum* strain NC4H. Symbols represent: (○), percent swollen spores; (□), trehalose content in units. Units were expressed as nmoles of glucose released per min at 23.5 °C when incubated with 95 U/ml of partially purified trehalase.
for added activators was tested on cathepsin B-like protease activity from emerged myxamoebae of *D. discoideum*. Enzymatic activity in the presence of the activators was enhanced 3-fold (data not shown).

There was an 11-fold increase in cathepsin B-like specific activity in emerged myxamoebae when compared to dormant spores (Table II). The increase in specific activity was contributed to by high enzymatic activity. Protein concentration remained constant.

The change in specific activity of cathepsin B-like protease during heat-induced spore germination of *D. discoideum* is shown in Fig. 15. Following heat activation but prior to the emergence of myxamoebae there was a basal level of activity. The sharp increase in cathepsin B-like protease activity coincided with the rise in the number of myxamoebae during emergence.

When cathepsin B-like protease activity was studied in more detail, it was discovered that the enzyme was released into the medium at late spore swelling. This coincided with the time of increase in specific activity of endogenous cathepsin B-like protease (Fig. 16). At the end of 5 hr, which was the time of maximum emergence, the rate of enzyme released into the external medium continued to increase.

2. Cathepsin D-like protease activity

Cathepsin D-like protease was similar to cathepsin
Table II. The specific activity of cathepsin B-like protease in dormant spores and fully emerged myxamoebae.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Enzyme activity(^b) (Units)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity(^c)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dormant spores</td>
<td>0.18</td>
<td>0.71</td>
<td>0.25</td>
<td>1.0</td>
</tr>
<tr>
<td>Emerged amoebae</td>
<td>1.84</td>
<td>0.68</td>
<td>2.71</td>
<td>11</td>
</tr>
</tbody>
</table>

\(^a\)Sori were harvested in phosphate buffer (10 mM, pH 6.5), centrifuged, and the pelleted spores retained. After two washes in phosphate buffer half the spores were resuspended in the appropriate enzyme assay buffer as described in the Materials and Methods. The remainder of the spores were resuspended in phosphate buffer and heat-shocked at 45°C for 30 min. The dormant spores and fully emerged myxamoebae were processed and the cathepsin B-like protease activity assayed for as described in the Materials and Methods.

\(^b\)Units of enzyme activity are equivalent to mg of azoalbumin degraded per hr at 37°C.

\(^c\)Units of specific activity are equivalent to units of enzyme activity per mg of protein.
Figure 15. Expression of cathepsin B-like protease activity during heat-induced spore germination of D. discoideum strain NC4H.

Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (■), cathepsin B-like protease activity. Specific activity refers to units of activity per mg protein where one unit of activity is defined as mg azoalbumin degraded per hr at 37°C.
Figure 16. Specific activity of endogenous and exogenous cathepsin B-like protease during heat-induced spore germination of \textit{D. discoideum} strain NC4H. Symbols represent: (▲), specific activity of endogenous cathepsin B-like protease; (●), specific activity of exogenous cathepsin B-like protease; (■), total specific activity. Specific activity refers to units of activity per mg protein where one unit of activity is defined as mg azoalbumin degraded per hr at 37°C.
B-like protease in that its activity decreased significantly during multicellular development (Fong and Rutherford, 1978). Unlike cathepsin B-like protease, cathepsin D-like protease activity decreased 4-fold in emerged myxamoebae when compared to dormant spores (Table III). This decrease in specific activity was contributed to by low enzymatic activity.

The change in specific activity of cathepsin D-like protease during heat-induced spore germination of D. discoideum is shown in Fig. 17. Following heat-activation the specific activity decreased throughout the germination process and reached its lowest point at the end of 5 hr, the time of maximum emergence.

3. Neutral protease activity

Since protease activity had previously never been detected above pH 6.0, an attempt was made to measure the activity of a neutral protease. There was no significant increase in the level of neutral protease activity in emerged myxamoebae when compared to dormant spores (Table IV).

The change in specific activity of neutral protease during spore germination of D. discoideum strain NCA4H is shown in Fig. 18. Following heat activation, the enzyme activity increased slightly but not to any significant level by the end of 5 hr.
Table III. The specific activity of cathepsin D-like protease in dormant spores and fully emerged myxamoebae.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Enzyme activity(^a) (Units)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity(^c)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dormant spores</td>
<td>0.25</td>
<td>0.14</td>
<td>1.79</td>
<td>4.0</td>
</tr>
<tr>
<td>Emerged amoebae</td>
<td>0.18</td>
<td>0.42</td>
<td>0.43</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^a\)Sori were harvested in phosphate buffer (10 ml, pH 6.5), centrifuged, and the pelleted spores retained. After two washes in phosphate buffer half the spores were resuspended in the appropriate enzyme assay buffer as described in the Materials and Methods. The remainder of the spores were resuspended in phosphate buffer and heat-shocked at 45°C for 30 min. The dormant spores and fully emerged myxamoebae were processed and the cathepsin D-like protease activity assayed for as described in the Materials and Methods.

\(^b\)Units of enzyme activity are equivalent to mg of azoalbumin degraded per hr at 37°C.

\(^c\)Units of specific activity are equivalent to units of enzyme activity per mg of protein.
Figure 17. Expression of cathepsin D-like protease activity during heat-induced spore germination of D. discoideum strain NC4H. Symbols represent (○), percent swollen spores; (■), percent emerged myxamoebae; (■■), cathepsin D-like protease activity. Specific activity refers to units of activity per mg protein where one unit of activity is defined as mg azoalbumin degraded per hr at 37°C.
Table IV. Neutral protease activity in dormant spores and fully emerged amoebae.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Enzyme activity</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dormant spores</td>
<td>26.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Emerged amoebae</td>
<td>28.8</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*aSealed, then harvested in phosphate buffer (10 mM, pH 6.5), centrifuged, and the pelleted spores retained. After two washes in phosphate buffer half the spores were resuspended in the appropriate enzyme assay buffer as described in the Materials and Methods. The remainder of the spores were resuspended in the phosphate buffer and heat-shocked at 45 °C for 30 min. The dormant spores and fully emerged myxamoebae were processed and the neutral protease activity assayed for as described in the Materials and Methods.

*bEnzyme activity was expressed as relative fluorescence/10⁷ spores.
Figure 18. Expression of neutral protease activity during heat-induced spore germination of D. discoideum strain NC4H.

Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (□), neutral protease activity expressed as relative fluorescence/10^7 spores.
4. Leucine aminopeptidase activity

The breakdown of peptides to free amino acids is a stepwise process catalyzed by several enzymes. Aminopeptidases remove amino acids from the end opposite the carboxyl terminal group. Leucine aminopeptidase was examined and a higher specific activity was observed in dormant spores than in emerged myxamoebae (Table V). This only represented a 2-fold decrease.

Following heat activation the enzyme decreased 3-fold in specific activity. The change in specific activity of leucine aminopeptidase during heat-induced spore germination is shown in Fig. 19. The enzyme increased but did not change significantly during the germination process. There was no detectable release of aminopeptidase into the extracellular medium during the germination process (data not shown).

E. Expression of proteolytic activity in matrix material and in dormant spores during aging of D. discoideum


When NC4H spores were allowed to age in the intact sorocarp for more than 7 days, autoactivation would occur upon suspension of washed spores in phosphate buffer (Dahlberg and Cotter, 1978). When cathepsin B-like protease activity was analyzed in 2, 7, and 13 day old sorocarps, the
Table V. The specific activity of leucine aminopeptidase in dormant spores and fully emerged myxamoebae.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Enzyme activity&lt;sup&gt;b&lt;/sup&gt; (Units)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dormant spores</td>
<td>40.2</td>
<td>0.25</td>
<td>160</td>
<td>2.0</td>
</tr>
<tr>
<td>Emerged amoebae</td>
<td>1.49</td>
<td>0.02</td>
<td>74.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Sori were harvested in phosphate buffer (10 ml, pH 6.5), centrifuged, and the pelleted spores retained. After two washes in phosphate buffer half the spores were resuspended in the appropriate enzyme assay buffer as described in the Materials and Methods. The remainder of the spores were resuspended in phosphate buffer and heat-shocked at 45°C for 30 min. The dormant spores and fully emerged myxamoebae were processed and the leucine aminopeptidase activity assayed for as described in the Materials and Methods.

<sup>b</sup>Units of enzyme activity are equivalent to mg of azoalbumin degraded per hr at 30°C.

<sup>c</sup>Units of specific activity are equivalent to units of enzyme activity per mg of protein.
Figure 19: Expression of leucine aminopeptidase activity during heat-induced spore germination of *D. discoideum* strain NC4H. Symbols represent: ( ○ ), percent swollen spores; ( ● ), percent emerged myxamoebae; ( ■ ), leucine aminopeptidase activity. Specific activity refers to units of activity per mg of protein where one unit of activity is defined as nmols of *p*-nitrophenol released per min at 30 C.
specific activity remained constant in both matrix material and dormant spores (Fig. 20).

The specific activity of cathepsin B-like protease was also measured in the spontaneous germination mutants SG1 and SG2 (Figs. 21, 22). In both cases there was a marked difference from the wild-type strains. Each mutant contained extremely low levels of activity in 2 day old matrix material and dormant spores. The specific activity increased sharply and was at a maximum after 7 days; the higher activity was located in the matrix material. The activity once again decreased in both strains by 13 days but remained high when compared to 2 day old matrix material and dormant spores.

2. Cathepsin D-like protease activity in D. discoideum strain NC4H.

When cathepsin D-like protease activity was analyzed in 2, 7, and 13 day old sorocarps, the specific activity was confined to the dormant spores in each case. The levels of enzymatic activity detected in the matrix material was insignificant (Fig. 23). The specific activity in the dormant spores decreased by one-half after 7 days.

3. Leucine aminopeptidase activity in D. discoideum strain NC4H.

A higher specific activity of leucine aminopeptidase was located in 2, 7, and 13 day old dormant spores than in
Figure 20. The specific activity of cathepsin B-like protease in matrix material and in dormant spores of *D. discoideum* strain NC4H.

□, specific activity in matrix material; ■, specific activity in dormant spores. Specific activity refers to units of activity per mg protein where one unit of activity is defined as mg azoalbumin degraded per hr at 37 C.
Figure 21. The specific activity of cathepsin B-like protease in matrix material and in dormant spores of *D. discoideum* strain SG1.

□, specific activity in matrix material; ■, specific activity in dormant spores. Specific activity refers to units of activity per mg protein where one unit of activity is defined as mg azoalbumin degraded per hr at 37°C.
Figure 22. The specific activity of cathepsin B-like protease in matrix material and in dormant spores of *D. discoideum* strain SG2.

☐, specific activity in matrix material; ■, specific activity in dormant spores. Specific activity refers to units of activity per mg protein where one unit of activity is defined as mg azoalbumin degraded per hr at 37°C.
Figure 23. The specific activity of cathepsin D-like protease in matrix material and in dormant spores of D. discoideum strain NC4H.

☐, specific activity in matrix material; ■, specific activity in dormant spores. Specific activity refers to units of activity per mg protein where one unit of activity is defined as mg azoalbumin degraded per hr at 37°C.
the respective matrix material (Fig. 24). When the enzyme activity was analyzed during the aging of spores in the sorocarps, leucine aminopeptidase in both dormant spores and matrix material was found to decrease but at levels which were not significant (Fig. 24).

F. **Effect of various metabolic inhibitors on heat-induced spore germination of D. discoideum strain NC4H.**

1. **Arsenate**

Arsenate affects glycolysis in such a way that it competes with phosphate in the formation of 3-phosphoglyceroyl phosphate. This reaction is catalyzed by glyceraldehyde 3-phosphate dehydrogenase (Lehninger, 1975). In the presence of arsenate no high-energy phosphate compound is generated. Even though glycolysis is not inhibited directly arsenate can uncouple oxidation from phosphorylation.

When oxygen was monitored during heat-induced spore germination the rate of O₂ utilization was greater in the presence of arsenate when compared to normal germination (Fig. 25). This increased rate occurred from the end of the post-activation lag period to the time of maximum emergence. This may represent the uncoupling action of arsenate.

Spores were heat-shocked in the presence of 10mM phosphate buffer (pH 6.5) and resuspended in 10mM arsenate. The spores swelled normally but only 30% of the amoebae had
Figure 24. The specific activity of leucine aminopeptidase in matrix material and in dormant spores of D. discoideum strain NC4H.

□, specific activity in matrix material; ■, specific activity in dormant spores. Specific activity refers to units of activity per mg protein where one unit of activity is defined as nmole of p-nitrophenol released per min at 30°C.
Figure 25. The effect of arsenate on endogenous spore respiration of *D. discoideum* strain NC4H. Symbols represent: (■), normal endogenous spore respiration; (●), endogenous spore respiration in the presence of 10 mM arsenate.
emerged by the end of 5 hr (Fig. 26). Spores were also heat-activated in the presence of 10mM arsenate. When resuspended in phosphate buffer germination was slightly varied but the emergence of myxamoebae was normal (Fig. 27). When arsenate heat-shocked spores were resuspended in 10mM arsenate, swelling was again normal but the level of emergence was only 50% at the end of 5 hr (Fig. 28).

The addition of 10 mM arsenate to heat-activated spores dramatically changed the respiratory quotient pattern (Fig. 29). By the end of the post-activation lag period the R. Q. was 0.96 and decreased further to a point where at the end of 5 hr the R. Q. was 0.74. This suggested that a change to lipid and/or amino acid utilization occurred during a time when carbohydrate was normally used.

2. Fluoride

Fluoride is an effective inhibitor of the glycolytic enzyme enolase at low concentrations and is capable of almost completely blocking glycolysis (Metzler, 1977). Fluoride has also been implicated as an inhibitor of alkaline phosphatase (West and Todd, 1962).

When spores were heat-shocked in 10 mM phosphate buffer and resuspended in 10 mM fluoride, they swelled normally but only 23% of the amoebae emerged after 5 hr (Fig. 30). An identical germination pattern was observed when spores were heat-shocked in the presence of 10 mM fluoride and
Figure 25. The effect of arsenate on heat-induced spore germination of *D. discoideum* strain NC4H. Spores were heat shocked in 10mM phosphate buffer (pH 6.5) at 45 C for 30 min, centrifuged, and resuspended in 10 mM arsenate. Germination was scored as described in Materials and Methods. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (□), percent swollen spores in the presence of arsenate; (■), percent emerged myxamoebae in the presence of arsenate.
Figure 27. The effect of arsenate on heat-induced spore germination of *D. discoideum* strain NC4H. Spores were heat-shocked in 10 mM arsenate at 45°C for 30 min, centrifuged, and resuspended in 10 mM phosphate buffer (pH 6.5). Germination was scored as described in Materials and Methods. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (□), percent swollen spores in the presence of arsenate; (■), percent emerged myxamoebae in the presence of arsenate.
Figure 28. The effect of arsenate on heat-induced spore germination of D. discoideum strain NC4H. Spores were heat-shocked in 10 mM arsenate at 45 °C for 30 min, centrifuged, and resuspended in 10 mM arsenate. Germination was scored as described in Materials and Methods. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (□), percent swollen spores in the presence of arsenate; (■), percent emerged myxamoebae in the presence of arsenate.
Figure 29. The effect of arsenate on the respiratory values during heat-induced spore germination of *D. discoideum* strain NC4H.

Spores were heat-activated in MES (2(N-Morphollino) ethane Sulfonic Acid, pH 6.5) buffer at 45°C for 30 min, centrifuged, and resuspended in 10 mM arsenate dissolved in MES buffer. Symbols represent: (●), normal R. Q. values; (■), R. Q. values in the presence of arsenate.
Figure 30. The effect of fluoride on heat-induced spore germination of *D. discoideum* strain NC4H.

Spores were heat-shocked in 10 mM phosphate buffer (pH 6.5) at 45°C for 30 min, centrifuged, and resuspended in 10 mM fluoride. Germination was scored as described in Materials and Methods. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (□), percent swollen spores in the presence of fluoride; (■), percent emerged myxamoebae in the presence of fluoride.
resuspended in the same inhibitor except that the maximum emergence was 33% after 5 hr (Fig. 31). If spores were heat-shocked in the presence of 10 mM fluoride and resuspended in 10 mM phosphate buffer, only the emergence of myxamoebae was retarded by one-half hour (Fig. 32).

3. Iodoacetamide

Iodoacetamide, an alkylating agent, may irreversibly inhibit enzymatic activity by modifying cysteine and other side chains (Stryer, 1981). Iodoacetamide has also been implicated in the inhibition of carboxypeptidase, an enzyme responsible for clipping amino acids adjacent to the carboxyl terminal group on peptides. Fong and Rutherford (1978) have shown that 1 mM iodoacetamide completely inhibited cathepsin B-like protease in D. discoideum.

When heat-shocked in 10 mM phosphate buffer and resuspended in 1 mM iodoacetamide, 40% of the spores swelled compared to maximum spore swelling in the control sample (Fig. 33). At no time did spore swelling ever exceed 50%. Emergence of myxamoebae was completely inhibited. A more dramatic effect was seen when spores were heat-shocked in 1 mM iodoacetamide and resuspended in either 10 mM phosphate buffer or 1 mM iodoacetamide. In both cases spore swelling as well as emergence of myxamoebae was completely inhibited (Figs. 34, 35).

4. Iodoacetic acid
Figure 31. The effect of fluoride on heat-induced spore germination of D. discoideum strain NC4H. Spores were heat-shocked in 10 mM fluoride at 45 °C for 30 min, centrifuged, and resuspended in 10 mM fluoride. Germination was scored as described in Materials and Methods. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (□), percent swollen spores in the presence of fluoride; (■), percent emerged myxamoebae in the presence of fluoride.
Figure 32. The effect of fluoride on heat-induced spore germination in D. discoideum strain NC4H. Spores were heat-shocked in 10 mM fluoride at 45°C for 30 min, centrifuged, and resuspended in 10 mM phosphate buffer (pH 6.5). Germination was scored as described in Materials and Methods. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (□), percent swollen spores in the presence of fluoride; (■), percent emerged myxamoebae in the presence of fluoride.
Figure 33. The effect of iodoacetamide on heat-induced spore germination of *D. discoideum* strain NC4H. Spores were heat-shocked in 10 mM phosphate buffer (pH 6.5) at 45°C for 30 min, centrifuged, and resuspended in 1 mM iodoacetamide. Germination was scored as described in Materials and Methods. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (□), percent swollen spores in the presence of iodoacetamide; ( ■ ), percent emerged myxamoebae in the presence of iodoacetamide.
Figure 34. The effect of lodoacetamide on heat-induced spore germination of *D. discoideum* strain NC4H. Spores were heat-shocked in 1 mM lodoacetamide at 45°C for 30 min, centrifuged, and resuspended in 10 mM phosphate buffer (pH 6.5). Germination was scored as described in Materials and Methods. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (□), percent swollen spores in the presence of lodoacetamide; (■), percent emerged myxamoebae in the presence of lodoacetamide.
Figure 35. The effect of iodoacetamide on heat-induced spore germination of *D. discoideum* strain NC4H. Spores were heat-shocked in 1mM iodoacetamide at 45°C for 30 min, centrifuged, and resuspended in 1 mM iodoacetamide. Germination was scored as described in Materials and Methods. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (□), percent swollen spores in the presence of iodoacetamide; (■), percent emerged myxamoebae in the presence of iodoacetamide.
The properties of iodoacetic acid are similar in many respects to iodoacetamide. When spores were heat-shocked in 10 mM phosphate buffer and resuspended in 1 mM iodoacetic acid the pattern was similar as for iodoacetamide (Fig. 36). Spore swelling only reached 55% as compared to maximum spore swelling in the control sample and remained constant throughout the remainder of germination. Emergence was completely inhibited. The results were different when spores were heat-shocked in 1 mM iodoacetic acid and resuspended in 10 mM phosphate buffer (Fig. 37). The germination pattern closely followed that of the control sample.

G. Determination of dry weights during heat-induced spore germination of D. discoideum strain NC4H.

It is important to note that in many fungal systems spore germination is marked by an increase in dry weight. This is accomplished through cellular outgrowth. As previously described, spore germination in D. discoideum is completed by the release of a single myxamoeba. This process does not involve cellular outgrowth but rather cellular degradation.

The dry weight determinations are shown in Table VI. After heat-activation but prior to maximum spore swelling the dry weights were greatest. The dry weights decreased when spore swelling was at a maximum but remained constant
Figure 36. The effect of iodoacetic acid on heat-induced spore germination of *D. discoldeum* strain NC4H. Spores were heat-shocked in 10 mM phosphate buffer (pH 6.5) at 45°C for 30 min, centrifuged, and resuspended in 1 mM iodoacetic acid. Germination was scored as described in Materials and Methods. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (□), percent swollen spores in the presence of iodoacetic acid; (■), percent emerged-myxamoebae in the presence of iodoacetic acid.
Figure 37. The effect of iodoacetic acid on heat-induced spore germination of *D. discoideum* strain NC4H. Spores were heat-shocked in 1 mM iodoacetic acid at 45°C for 30 min, centrifuged, and resuspended in 10 mM phosphate buffer (pH 6.5). Germination was scored as described in Materials and Methods. Symbols represent: (○), percent swollen spores; (●), percent emerged myxamoebae; (□), percent swollen spores in the presence of iodoacetic acid; ( ■ ), percent emerged myxamoebae in the presence of iodoacetic acid.
Table VI. Macromolecular content during heat-induced spore germination of *D. discoideum* strain NC4H:

<table>
<thead>
<tr>
<th>Time (hr after activation)</th>
<th>Dry weight (ug/10⁶ cells)</th>
<th>Total Protein^a^ (ug/10⁶ cells)</th>
<th>RNA^b^ (ug/10⁶ cells)</th>
<th>DNA^c^ (ug/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dormant*</td>
<td>240</td>
<td>195</td>
<td>17.3</td>
<td>2.1</td>
</tr>
<tr>
<td>0.0</td>
<td>254</td>
<td>-</td>
<td>17.3</td>
<td>-</td>
</tr>
<tr>
<td>1.0</td>
<td>247</td>
<td>-</td>
<td>17.5</td>
<td>-</td>
</tr>
<tr>
<td>2.0</td>
<td>227</td>
<td>92</td>
<td>17.5</td>
<td>-</td>
</tr>
<tr>
<td>3.0</td>
<td>213</td>
<td>-</td>
<td>17.0</td>
<td>-</td>
</tr>
<tr>
<td>4.0</td>
<td>226</td>
<td>-</td>
<td>10.3</td>
<td>-</td>
</tr>
<tr>
<td>5.0</td>
<td>222</td>
<td>99</td>
<td>10.5</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* non-activated

^a^from Dernar (M. Sc. thesis, 1981)

^b^from Hamer (M. Sc. thesis, 1982)

thereafter until the end of the germination process. When compared to dormant spores this loss of dry weight never exceeded 10%.
DISCUSSION

Respiration studies were initially utilized to determine the possible endogenous energy source(s) consumed during spore germination of D. discoideum. Respiratory quotients (R. Q.) indicated that dormant spores oxidized lipid and/or amino acids (data not shown). With activation carbohydrates were catabolized until late spore swelling but prior to the emergence of myxamoebae (Fig. 3). Cells entering the emergence stage once again oxidized lipid and/or amino acids as the primary energy source. Added exogenous substrates had no effect on the R. Q. pattern (Figs. 4, 5, 6). In the related species, Polysphondyllum pallidum, the R. Q. was similar to that for D. discoideum (Fig. 7). Prior to the emergence of myxamoebae a carbohydrate was consumed but during the emergence stage the metabolism shifted to the utilization of lipid and/or amino acids. In the filamentous fungus, Neurospora tetrasperma, a similar pattern of substrate utilization was found during ascospore germination (Sussman, 1961). Respiration of dormant ascospores involved lipid reserves, whereas after heat-activation a carbohydrate fraction involving mainly trehalose supported the 20- to 40-fold increase in respiration. A changeover to lipid metabolism again occurred for germ tube emergence.
Since the primary goal of this work was to establish the energy source(s) utilized during germination further elucidation of the individual substrate(s) was necessary. The initial problem was to resolve the question of lipid/amino acid utilization during germination. The onset of sporulation in *D. discoideum* is marked by the starvation for amino acids; sporulation ultimately becomes a closed system (Wright et al., 1968). Wright and Anderson (1960a) have shown that protein and amino acid pool levels decreased throughout multicellular development. Therefore, proteins were used as the energy source during this period.

It follows then that in a closed system the cell must rely on endogenous substrate(s) not previously depleted and/or convert those present to usable forms. It is reasonable to suggest that lipid is the major endogenous energy source which maintains the dormant state. Preliminary evidence showed that lipid accumulated during multicellular development and was greatest at the culmination stage (Long and Coe, 1974). This was in agreement with Cotter et al. (1969) and Loomis (1975) who by means of electron micrographs showed the presence of large lipid "droplets" in dormant spores. The R. Q. values also indicated that lipid could be used as an endogenous energy source to maintain the dormant state (data not shown). When dormant spores were allowed to age the lipid content as well
as the level of free fatty acids decreased slightly (Long and Coe, 1974). The specific activity of lipase, the enzyme responsible in catalyzing crude lipid to neutral lipid, was greatest in dormant spores (Table I).

The data suggest that due to the actual depletion of the endogenous lipid reserves, lipid is probably the major endogenous reserve utilized for the production of energy. Lipid and/or amino acids were the primary energy substrate(s) for the emergence of myxamoebae as indicated by the R. Q. values (Fig. 3). The levels of total lipid, neutral lipid, and free fatty acids were measured in an effort to present a clear indication of lipid involvement during spore germination. Total lipid decreased 7-fold during germination; the majority of the decrease had occurred by late spore swelling (Fig. 8). Neutral lipid, which comprised 24% of the total lipid, is the fraction primarily used as an energy source. This substrate decreased 10-fold during germination with the period of utilization being more evenly distributed over the germination period (Fig. 9). The neutral lipid was almost completely exhausted by the time of maximum emergence. Concomitant with the decrease in lipid, free fatty acids increased 5-fold during the activation stage and did not change significantly throughout germination (Fig. 10). Lipase activity followed the decrease in lipid content.
during germination (Fig. 11).

Additional evidence supports this hypothesis. Electron micrographs showed a decrease in the number and size of lipid "droplets" during the germination process (Cotter et al., 1969). Cochrane (1963) suggested that even though protein could contribute to low R. Q. values (less than 1.0), analytical evidence indicated that lipid oxidation during germination of some spores contributed to the low R. Q. of those spores. Indeed, in germinating spores of *D. discoideum*, the total protein content and dry spore weight remained constant (Table VI). Shu et al. (1954) found that there was no significant change in protein content before and after germination ofuredospores while endogenous lipid decreased 59% over the same period.

Hence, endogenous lipid reserves not only provide energy to maintain the dormant state, but are probably used for energy in the entire germination process as well. This includes the period when carbohydrate hydrolysis predominates. An alternate function for lipid was proposed for spores of *Erysiphe graminis*. McKeen (1970) suggested that respiratory oxidation of the large lipid bodies during germination generated sufficient water for the spores to germinate. The oxidation of the lipid "droplets" in *D. discoideum* may have a similar role in spore swelling by means of water production.
Lipid was not the only endogenous reserve used. After activation lipid and carbohydrates are utilized during the swelling stage of germination. In *D. discoideum* spores the major storage carbohydrates are trehalose, glucose, and glycogen (Ceccarini and Filosa, 1965; Ceccarini, 1967; Emyanltoff and Wright, 1979). Nevertheless, the data indicate that the utilization of trehalose corresponds with the increase in R. Q. values in the swelling stage of germination (Fig. 12). In no case was the decrease in endogenous trehalose a result of sugar release into the medium.

*D. discoideum* is one of a number of organisms which use trehalose as an endogenous energy source during germination (Ceccarini, 1967). Sporangiospores of *Phycomyces* (Van Assche et al., 1978), ascospores of *Neurospora* (Sussman, 1961), and uredosporcs of rust fungi (Staples and Wynn, 1965) are similar in this respect. The experiments based on restrictive environmental conditions, such as incubation at 37°C (Fig. 13) or anaerobiosis (Fig. 14), support the data that activated spores utilize trehalose during early spore swelling to form glucose. The glucose may then be converted to energy and carbon via the glycolytic pathway. This energy may be required for the swelling process or the emergence of myxamoebae but due to the presence of other energy substrates (i.e., lipid) a conclusive role for
trehalose is yet to be determined.

Recently, it was proposed by Thevelein (Ph.D. thesis, 1981) that the induction of germination in fungal spores requires a carbohydrate, present either externally or internally. Furthermore, the response may be due to the carbohydrate's energy-producing function or allosteric effects brought on by its presence or breakdown. This may include the early synthesis of nicotinamide adenine dinucleotide and respiratory substrates as suggested by Koblinsky and Beattle (1977). Alternatively, it has been shown that the level of trehalose in young spores can be modulated by metabolic perturbation (Wright et al., 1979; Emyanitoff and Wright, 1979). By using this technique Emyanitoff and Wright (1979) showed that trehalose may increase the heat resistance of spores. Thus, trehalose in D. discoideum spores may serve a dual role. Initially, it may aid in maintaining the dormant state of the spore by increasing its resistance. But, when spores are activated the trehalose may then be utilized for the germination process.

The presence of trehalose in addition to lipid may be due to a large demand for cellular energy throughout the germination process. This is especially true during the swelling and emergence stages of germination. This was not only reflected in the depletion of the endogenous energy
reserves but in the decline of ATP levels as well. ATP comprised 25% of the total acid soluble nucleotides in dormant spores (Hamer, personal communication). During the swelling stage of germination there was a ten-fold decrease in the cellular ATP pool. Therefore, the demand for ATP during this period may require a large depletion of endogenous reserves as seen with lipid and trehalose.

A preliminary study of the proteolytic enzymes was undertaken in order to further clarify the involvement of protein and/or amino acids. If proteins were involved in energy production it follows then that proteases must be active in order for proteins to be degraded to "free" amino acids. North and Harwood (1979) demonstrated the presence of acid proteases during multicellular development while Fong and Rutherford (1978) studied cathepsin D- and cathepsin B-like proteases in detail. The activities of both these proteases decreased throughout multicellular development to a point where by culmination the enzymatic activities were at their lowest point.

These same two proteases were studied during spore germination of D. discoideum. The activities of the individual proteases did not significantly increase in the time frame of germination when cellular energy is required. It appears that these proteases do not have an important energy-related role in either the maintenance of dormancy or
spore germination. Experiments based on spore aging showed cathepsin D-like protease declined (Fig. 23) while cathepsin B-like protease activity did not significantly change (Fig. 20). During the germination process cathepsin D-like protease activity continued to decline (Fig. 17). The specific activity of cathepsin B-like protease on the other hand remained at a basal level until the emergence stage when the enzyme increased significantly (Fig. 15). While the increase in activity seen during germination did not exactly parallel the pattern of emergence, total cathepsin B-like protease activity, exogenous plus endogenous, (Fig. 16) closely followed the emergence of myxamoebae. Neutral protease activity did not significantly increase in activity (Fig. 18). This is not to say that other proteases may exist which could have an energy-related function. North and Harwood (1979) found a total of eight acid proteases during multicellular development; an additional acid protease appeared at culmination. This last acid protease as well as alkaline proteases not yet examined in *D. discoldeum* may have some significance in spore germination.

Cathepsin B-like protease may have another function. The cell wall layers of *D. discoldeum* are made up of polysaccharides and glycoproteins. With this being the case a group of enzymes, and not an individual enzyme, may be responsible for the breakage of the cell wall layers during
germination. The enzymes required to degrade the cell wall may include the following: (i) cellulase, which has been shown by Rosness (1968) and Jones et al. (1979) to be a preformed enzyme in the dormant spores. This enzyme is released into the extracellular medium during spore germination; (ii) protease(s) which are required to degrade the polypeptide linkages in the cell wall layers (Hemmes et al., 1972); and (iii) glycosidases which may facilitate the breakage of carbohydrate linkages in the cell wall layers. Chan and Cotter (1982) and Hemmes et al. (1972) have shown that Pronase used in sequence with cellulase will release amoebae from cycloheximide-treated spores. Thus, the endogenous and/or exogenous cathepsin B-like protease may have a role in this group of enzymes, specifically the breakage of the cell wall polypeptide linkages. In the related species, Polysphondyllum pallidum, O'Day (1976) also examined two acid proteases with similar pH values to those found in D. discoideum. During microcyst germination the pH 6.0 protease increased 3- to 4-fold and was excreted. O'Day suggested a role for the extracellular acid protease in cell wall removal during germination. Chemical analysis of the microcyst wall has revealed it to be made up of lipid, cellulose, glucose polymers, and large amounts of protein (Toama and Raper, 1967).

Analysis of protease activity in the spontaneous
germination mutants SG1 and SG2 may give some indication of
the involvement of cathepsin B-like protease activity in the
degradation of the spore wall. In 2-day old SG1 and SG2
mutants cathepsin B-like protease activity was extremely low
(Figs. 21, 22). After 7 days of aging there was a
significant burst of activity which later decreased but
still remained high when compared to 2-day old fruiting
bodies. This increase in cathepsin B-like protease activity
in aging spores may reflect a need for the spontaneous
germination mutants to produce cell wall degrading enzymes.

The final proteolytic enzyme examined was leucine
aminopeptidase. If proteins are degraded to small peptides,
then enzymes such as aminopeptidases should play a role in
their conversion to "free" amino acids. Firtel and
Brackenbury (1972) found evidence for this during
multicellular development when protein was used as the
primary energy source. Indeed, aging spores showed little
change in activity after 13 days which probably is
indicative of the enzymes involvement during multicellular
development (Fig. 24). Dormant spores contained a high
level of leucine aminopeptidase activity (Table V) but the
enzyme did not change significantly at any time during
germination (Fig. 19). This evidence again suggests that
protein is not involved in energy production. Aminopeptidase
activity may remain throughout germination to degrade
proteins in order to fuel the period of protein synthesis
during germination.

A preliminary investigation of various metabolic
inhibitors was undertaken in an attempt to better understand
energy-related metabolism. In order for trehalose to serve
as an endogenous energy source the glycolytic pathway must
be employed. Hence, arsenate and fluoride were used since
both had been reported to effect different areas of
glycolysis.

It initially appeared that both inhibitors failed to
enter the spore and only affected germination when the cell
became more permeable. When each of the inhibitors were
added after activation only the emergence of myxamoebae was
delayed (Figs. 26, 28, 30, 31). When activated in the
presence of either inhibitor and then immediately washed
out, normal germination proceeded (Figs. 27, 32).

The amount of endogenous lipid present in the spore may
itself be sufficient to fuel the entire germination process
but at a slower rate. The slower rate may in part be due to
the loss of potential ATP from trehalose hydrolysis during
the period of germination when ATP levels had decreased
ten-fold. In the presence of arsenate oxygen utilization
had increased 12% by the end of germination (Fig. 25). This
increase began during the post-activation lag period.
Contrary to earlier results this suggested that arsenate was
able to enter the cell early in the germination process and may effect glycolysis as outlined by Lehninger (1975). In the presence of arsenate the R. Q. values indicated a shift in metabolism to lipid oxidation (Fig. 29). This occurred in a period when under normal germination conditions trehalose was also hydrolyzed. The delay in emergence of myxamoebae, as seen with arsenate, may reflect a lag period due to the shift in metabolism.

Iodoacetamide and iodoacetic acid can inhibit a variety of enzymes (Stryer, 1981). This includes cathepsin B-like protease at a concentration of 1 mM (Fong and Rutherford, 1978). It was hoped that both these inhibitors might only have effected the emergence of myxamoebae. This then, may have shed some light as to the possible involvement of cathepsin B-like protease in cell wall degradation leading to emergence. It is apparent that these inhibitors were too general in their action(s) and probably had a multiplicity of effects which are uncertain at this time. In the presence of 1 mM iodoacetamide the emergence of myxamoebae was completely inhibited under all the germination conditions examined (Figs. 33, 34, 35). Spore swelling was also inhibited except in one case. When spores were heat-shocked in phosphate buffer and resuspended in 1 mM iodoacetamide, spore swelling never exceeded 50% (Fig. 33). The effects of iodoacetic acid were similar under these same
experimental conditions (Fig. 36). Therefore, the results using these two inhibitors are inconclusive.
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