1979

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KURT ROBERT DAHLBERG

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NL-339 (3/77)
AUTOACTIVATION OF SPORE GERMINATION IN THE
CELLULAR SLIME MOLD DICTYOSTELIUM DISCOIDEUM

by
Kurt Robert Dahlberg

A Dissertation submitted to the Faculty of Graduate Studies through the Department of Biology in Partial Fulfillment of the requirements for the Degree of Doctor of Philosophy at The University of Windsor

Windsor, Ontario, Canada
1979
ABSTRACT

AUTOACTIVATION OF SPORE GERMINATION IN THE
CELLULAR SLIME MOLD DICTYOSTELIUM DISCOIDEUM

by

Kurt Robert Dahlberg

Constitutively dormant Dictyostelium discoideum spores become capable of germinating in the absence of an exogenous activation treatment after 1-2 weeks of maturation in the intact sorocarp. Spontaneous mutants have been isolated which autoactivate without the need for spore aging. In addition, a variety of other slime mold species and strains also show spontaneous spore germination.

The process of autoactivation of D. discoideum spores is fundamentally different than that of exogenous spore activation by heat or chemicals. Spores at high densities autoactivate rapidly and to high percentages, while populations at low densities often fail to completely germinate. Spore germination in the absence of an activation treatment is mediated by soluble autoactivator factors released by spores during the swelling stage of germination. The autoactivator factors dramatically stimulate spore germination in all D. discoideum strains. Crude autoactivator preparations can be chromatographically fractionated into three active components. Two of these components are also present in dormant spores and
vegetative amoebae. The third is unique to the process of autoactivation. Attempts to identify the third component by spectroscopic and radioisotopic methods failed due to the minuscule concentrations of the substance. Autoactivator activity could not be mimicked by a variety of substances added to dormant spores. A number of bacteria release substances which mimic autoactivator activity.

Autoactivation of *D. discoideum* spore germination is sensitive to inhibitors of protein synthesis, and may also require RNA synthesis. The inhibition of spontaneous spore germination by some RNA and protein synthesis inhibitors is overridden by the autoactivator factors. Autoactivation is reversibly inhibited by high osmotic pressure and temperature extremes. Inhibitors of respiration such as cyanide and salicylhydroxamic acid delay, but do not prevent autoactivation and spore respiration. Addition of autoactivator substances stimulates the respiration and germination of spores treated with these compounds.

In contrast, *D. discoideum* spores activated by an exogenous treatment do not require protein or RNA synthesis to germinate, and spores do not interact during the process. Such spores do not release autoactivator substances, but rather release autoinhibitors. Mutant spores have altered requirements for exogenous spore activation and are insensitive to some deactivating
treatments.

Autoactivation occurs after spores have advanced from constitutive dormancy to "poised dormancy". Mutant spores are constantly in poised dormancy, while wild type spores require 1-2 weeks of aging to reach the state. Spore maturation may involve changes in spore permeability.

Three mechanisms of D. discoideum spore activation are now recognized: exogenous activation, response to substances released by bacteria, and autoactivation. Each of these mechanisms may occur in nature. Exogenous activation could result from heating of the soil by direct sunlight. Response to substances released by bacteria signals the presence of sufficient nutrients to support vegetative growth. Autoactivation may be a mechanism in which spores germinate in an environment depleted in nourishment, quickly aggregate to form a slug, and migrate in search of bacterial prey.
ACKNOWLEDGEMENTS

I would like to acknowledge with gratitude the assistance and support provided to me by a number of individuals: my friends, colleagues, and co-workers at The University of Windsor, faculty and staff within the Biology Department, members of my Doctoral Committee, and my family. Gratitude is also expressed to the Province of Ontario for the opportunity to study at The University of Windsor. Financial support was provided by various scholarships, bursaries, fellowships, and assistantships from The University of Windsor, the Province of Ontario, and the Mycological Society of America. Special thanks are expressed to Professor David A. Cotter, without whose direction and inspiration at both the undergraduate and graduate levels I would neither have begun nor completed my graduate education.
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INTRODUCTION

The cellular slime mold *Dictyostelium discoideum* is an organism which has attracted a considerable amount of attention from developmental, molecular, and cellular biologists. The organism, which has been variously categorized as a protozoan, a fungus within the class Mycetozoa (Olive, 1975), and "an organism of uncertain affinity" (Alexopoulos, 1962), possesses a number of features making it a model system for studies of eucaryotic development. The organism has simple growth requirements, is easily manipulated in the laboratory, and has a brief (about 4 d) life cycle. Of more importance, perhaps, is the fact that *D. discoideum* is a primitive eucaryote which is capable of complex cellular interactions, chemotaxis, and the development of cellular receptors and contacts. Because of its relative cellular simplicity and developmental patterns, *Dictyostelium discoideum* is considered by some to be the organism of choice for studies of differentiation of the lower eucaryotes.

Some aspects of the *D. discoideum* life cycle have been extensively studied. For example, aggregation of *D. discoideum* myxamoebae and subsequent sporulation have been investigated, and many of the molecular and biochemical events during these developmental stages have been elucidated (Loomis, 1975; Newell, 1978). Information
derived from such studies may be of value in furthering our understanding of cellular interactions in higher organisms.

One aspect of the *D. discoideum* life cycle that has not been so extensively studied is spore germination. The control of spore germination in *D. discoideum* is of interest for a variety of reasons. Perhaps the most compelling reason to study spore germination is that it represents a major control point in the life cycle of a spore forming organism. The process marks a transition point where most, if not all, of the organism's vegetative apparatus must be revived from a state of dormancy. The shift from dormancy to activity may be analogous to a variety of systems in higher organisms, such as the shifts of a cell from quiescence to active proliferation, or even the dramatic increases in cellular activity accompanying neoplasia. Knowledge gained from studies of simple organisms, such as *D. discoideum*, may be of value in understanding control mechanisms of organisms higher on the evolutionary scale.

The specific purpose of this research is to study the phenomenon of autoactivation, or spontaneous germination, of *D. discoideum* spores. The phenomenon is of interest because it represents an alternative method of spore germination to that heretofore described (see LITERATURE REVIEW or Cotter, 1975). The phenomenon may be observed as a result of spore aging or mutations,
suggesting that the mutations may affect the "temporal programming" of the organism. A number of distinct differences exist between constitutively dormant spores and those capable of autoactivating. Finally, the lack of severe treatments necessary to induce spontaneous spore germination suggests that it may be one method of D. discoideum spore germination in nature.
LITERATURE REVIEW

1. General Information of Dictyostelium discoideum

Dictyostelium discoideum was first discovered by Raper (1935) on decaying forest litter in North Carolina. It has since been isolated from a wide variety of climatic regions, and may be considered ubiquitous in distribution in many parts of the world.

In nature, D. discoideum obtains its nourishment by ingesting bacteria growing on the forest litter. In the laboratory, D. discoideum may be successfully grown with bacteria in two membered cultures, most frequently with Escherichia coli, Enterobacter aerogenes, or Klebsiella pneumoniae. Growth of D. discoideum is not limited to gram negative rod shaped bacteria, for it has also been grown on a variety of other bacteria (Raper, 1937; Depratère and Darmon, 1978). Axenic growth of the organism is also possible as a result of mutations (Loomis, 1975).

While Alexopoulos (1962) states that the cellular slime molds "undoubtedly have a role to play in the economy of nature", this role is probably minor in relation to the significant activities of other microorganisms. The primary importance to man of the cellular slime molds, most notably Dictyostelium discoideum, is their role as research tools. Their unique life cycle affords numerous opportunities to study cellular interactions at a simple level. The D. discoideum life cycle
is presented schematically in Fig. 1.

*Dictyostelium discoideum* myxamoebae grow by ingestion of bacteria and divide by binary fission. Upon exhaustion of the food supply, the myxamoebae aggregate via cAMP-oriented streaming to form a multicellular pseudoplasmidium (slug, grex). Amoebae within the pseudoplasmidium retain their individuality, but work in conjunction with one another to form a primitive "tissue". The pseudoplasmidium may migrate in search of additional nourishment, or may immediately enter the culmination stage of development. Pre-patterned cells within the pseudoplasmidium may either lay down cellulosic walls to form stalk cells or may lay down tripartite walls and enter dormancy to become spores. Spores and stalk cells collectively compose the sorocarp (sorus), or asexual sporangium.

It is not the intent of this dissertation to exhaustively review the *D. discoideum* life cycle. The remainder of this literature review and dissertation will specifically focus on *D. discoideum* spore dormancy and germination, with reference to autoactivation of spore germination. Additional information on the *D. discoideum* life cycle and details esoteric to this discussion may be found in the excellent reviews by Bonner (1967) and by Loomis (1975).
Figure 1. The life cycle of *Dictyostelium discoideum*

(Redrawn from Alexopoulos, 1962)
2. Activation and Germination of Constitutively Dormant
_Dictyostelium discoideum_ Spores

a. Maintenance of spore dormancy

Following the series of events of aggregation and sporulation, _D. discoideum_ asexual spores are held aloft within the sorocarp. These spores are constitutively dormant according to the definitions of Sussman and Halvorson (1966). Thus, the spores are in a reversible state of hypometabolism maintained by some innate property of the spores. Freshly formed spores are incapable of germinating in the absence of an activation treatment, as discussed below. In addition to the innate properties of the spores, dormancy is maintained by other factors within the sorocarp, including the presence of autoinhibitors and high osmotic pressure.

The first suggestion that _Dictyostelium_ spore germination was affected by self-inhibitors resulted from work by Russell and Bonner (1960), who demonstrated that germination of _D. mucoroides_ spores was inhibited at high spore densities. Snyder and Ceccarini (1966) later showed that such a germination inhibitor does, in fact, exist. The inhibitor is readily washed from spores with water and is resistant to boiling. The hierarchy of inhibitors from several acrasid species suggests that different slime molds show a differential susceptibility to the autoinhibitors, or possess different autoinhibitors.
Further investigation (Ceccarini and Cohen, 1967; Cohen and Ceccarini, 1967) showed that different species possess different autoinhibitors of varying efficacy, and which could affect other stages of the life cycle. Additionally, the *D. discoideum* germination inhibitor is dialyzable, with a molecular weight of about 200 daltons (Ceccarini and Cohen, 1967). The substance is effective at a concentration below that which would absorb ultraviolet light. Cohen and Ceccarini (1967) reiterated the concept of a species hierarchy of slime mold germination inhibitors, and demonstrated that the inhibitors could affect other stages of the life cycle, including aggregation and fruiting. In this way, the autoinhibitors could be partially responsible for the spacing of aggregation centers (Bonner and Hoffman, 1963).

Cotter and Raper (1968b) pointed out that while *D. discoideum* spore germination is affected by the autoinhibitor, other factors are also involved. For instance, spores induced to germinate by peptone do not germinate above $1 \times 10^6$ spores/ml even if washed or dialyzed free of the autoinhibitor prior to testing. The autoinhibitor has differential effects on spores treated with 1% peptone or heated at 45°C for 30 min: spores treated with peptone do not germinate in the presence of 1 mg/ml crude autoinhibitor preparation, while those activated by heat shock germinated to high percentages. Heat shocked spores required up to 20 mg/ml of the crude preparation
to be inhibited. However, at this high concentration, osmotic inhibition of spore germination probably results (Cotter, 1977).

The autoinhibitor does not irreversibly damage spores, but rather causes them to return to the dormant state. Spores activated with heat within the intact sorocarp or those activated with heat and incubated with natural concentrations of the autoinhibitor are deactivated during the preswelling period. A second activation treatment after washing the spores free of the autoinhibitor results in normal spore germination. Cotter and Raper (1968b) suggested that the autoinhibitor had physical properties similar to a sugar alcohol. In addition, both mannitol and sorbitol (20 mg/ml) resulted in a 65% inhibition of spore germination. Subsequent investigations (see below) demonstrated that the autoinhibitor was not a polyalcohol. The inhibition afforded by these sugar alcohols is probably due to osmotic effects (20 mg/ml mannitol or sorbitol = 0.11 M) which are now known to inhibit D. discoideum spore germination (Cotter, 1977).

The biological characteristics and identity of the slime mold autoinhibitors have been extensively studied. Katalus and Ceccarini (1975) investigated the autoinhibitor from D. purpureum, but could not estimate its molecular weight due to interactions of the molecule with Sephadex and Biogel gel filtration columns. The material
lost some activity after repeated freeze-thaw cycles, and absorbed UV light at around 200 nm. The inhibitor also causes a lengthening of the lag phases of *E. coli* and *Bacillus subtilis* growth curves. Presumably this allows myxamoebae to assume a dominant role in mixed cultures before inhibitory effects of the bacteria (such as lowered pH) become manifest. The autoinhibitor also inhibits *D. discoideum* aggregation (as previously shown by Cohen and Ceccarini, 1967), perhaps by interaction with cAMP. Addition of exogenous cAMP allows normal aggregation and sporulation.

The identity of the *D. discoideum* autoinhibitor has been the subject of some dispute. It was initially identified as 2-dimethylamino-6-oxypurine riboside (N,N-dimethylguanosine) and reported to yield 100% inhibitory activity at 50 ug/ml (Bacon et al., 1973). Further work suggested that the compound specifically inhibited protein synthesis, but had no effect on respiratory metabolism or RNA synthesis (Bacon and Sussman, 1973). However, subsequent investigations suggested that dimethylguanosine was not the authentic autoinhibitor of *D. discoideum* spore germination. Tanaka et al. (1974) could not demonstrate inhibition of *D. discoideum* spore germination with dimethylguanosine at up to 300 ug/ml. Additionally, under some chromatographic conditions dimethylguanosine and the autoinhibitor do not coelute. Thus it appears that dimethylguanosine
is not the authentic autoinhibitor (Obata et al., 1973; Tanaka et al., 1974).

These workers (Tanaka et al., 1974; Abe et al., 1976) identified the autoinhibitor as 3-(3-amino-3-carboxypropyl)-6-(3-methyl-2-butenylamino) purine, or "discadenine" (Fig. 2). This compound is reported to be 100% inhibitory to *D. discoideum* spore germination at 30 ng/ml (Tanakā et al., 1975) and has "significant" cytokinin activity (Tanaka et al., 1975; Nomura et al., 1977), as might be expected from its structure. The minuscule biological concentrations of this compound are perhaps best illustrated by noting that the isolation of 15 mg of pure discadenine required the use of 20,000 petri dishes (Abe et al., 1976).

Discadenine is synthesized from 5'AMP by the pathway shown in Fig. 2 (Taya et al., 1978a,b; Tanaka et al., 1978). Isopentenyladenine (i^6^Adé) is not produced by the degradation of tRNA, but rather is synthesized by the enzymatic addition of Δ_2_-isopentenylpyrophosphate to the 5'AMP, followed by the removal of ribose phosphate. Conversion of the i^6^Adé to discadenine occurs by the transfer of the 3-amino-3-carboxypropyl group from S-adenosylmethionine. This reaction is catalyzed by "discadenine synthetase", which has been isolated from cells in the late culmination stage of fruiting (Taya et al., 1978b).
Figure 2. Synthesis and structure of discadenine
(after Abe et al., 1976; Taya et al., 1978a,b; Tanaka et al., 1978)
The possible significance of this pathway of discadenine synthesis should not escape the careful reader. 5'AMP resulting from the degradation of cAMP during aggregation may be available for incorporation into discadenine. Thus, *D. discoideum* appears to demonstrate extreme thrift in its use of regulatory molecules. The same molecular skeletons responsible for control of aggregation may be used for the maintenance of dormancy.

Despite the meticulous work in elucidating the structure and synthesis of discadenine, little is presently known of its mode of action in inhibiting spore germination. It is not known if discadenine inhibits protein synthesis, as was the suggested mode of action for dimethylguanosine (Bacon and Sussman, 1973). It is likely that the autoinhibitor will be shown to affect a function required for spore germination, such as respiration. It is known, however, that residual *D. discoideum* autoinhibitor is released from spores during the swelling stage of spore germination (Dahlberg and Cotter, 1979). At this time its function is no longer required.

Whether the excreted autoinhibitor molecules are also recycled for other functions during vegetative growth remains to be determined.

In addition to the innate properties of the spores and autoinhibitors, spore dormancy is probably also maintained by high osmotic pressure within the sorocarps. The interstices between spores have been shown to contain
a variety of substances, including salts, proteins, pigments, carbohydrates (Loomis, 1975), and several enzymes, including acid phosphatase (Gezelius, 1972), β-glucosidase, and N-acetylglucosaminidase (Tisa and Cotter, personal communication). Cotter (1973b) has estimated that these components of the sorocarp collectively exert an osmotic pressure upon the spores of between four and five atmospheres, approximately equivalent to that exerted by 0.20 M sucrose. A variety of substances have been shown to osmotically prevent D. discoideum spore germination (Cotter, 1977). For instance, 0.20 M sucrose, and various other polyhydric compounds at 0.25 M prevent the germination of heat activated spores and cause them to return to dormancy within 6 h. Thus, conditions within the intact sorocarps and the sensitivity of the spores to high osmotic pressures suggest that the osmotic environment of the fruiting body contributes to the maintenance of D. discoideum spore dormancy.

b. Activation of Dictyostelium discoideum spores

Previously in this discussion, constitutive dormancy has been referred to. Constitutive dormancy is defined by Sussman and Halvorson (1966) as a reversible state of hypometabolism maintained by an innate property of the spore. This innate property may include a metabolic blockage or restriction on respiration, for example. One important feature of constitutive dormancy is that an
activation treatment is required to relieve the restrictions on vegetative growth and metabolism. Very often, the nature of the innate property of the constitutively dormant spore is best defined by the nature of the activation treatments necessary to break dormancy. Such is the case among *D. discoideum* spores.

"Activation" is here defined as any treatment which results in an increase in spore germination. *D. discoideum* spores may be activated by treatment with a racemic mixture of the hydrophobic amino acids tryptophan, phenylalanine, and methionine (Cotter and Raper, 1966), gamma irradiation (Hashimoto, 1971; Hashimoto and Yanagisawa, 1970; Khoury et al., 1970), heat shock (Cotter and Raper, 1966, 1968a), dimethylsulfoxide (Cotter et al., 1976), urea (Cotter and O'Connell, 1976), penetrating polyhydric compounds (Cotter, 1977), or a ninhydrin-negative "spore germination promoter" released by *Enterobacter aerogenes* (sic) cells (Hashimoto et al., 1976). All of these treatments inspire rapid and synchronous spore germination.

The observation that the common denominator for most of these treatments is protein denaturation led Cotter (1973a) to propose the "multistate model for spore activation". This model proposes that a mitochondrial regulatory protein restricts oxidative phosphorylation of ATP in the dormant spore. The activation treatments induce a conformational change in this regulatory protein,
resulting in an uncoupling of oxidative phosphorylation. A decay of the regulatory protein from its partially denatured state to a "relaxed" conformation results in a coupling of oxidative phosphorylation and allows ATP production. ATP formed in this way is then available for the myriad reactions of spore germination and vegetative growth.

It appears that the available evidence supports Cotter's (1973a) hypothesis. Thermodynamic considerations suggest that heat activation induces a partial helix-coil transition in a protein in the inner mitochondrial membrane. Suggestions that the mitochondria are involved in the activation process result from a theoretical analysis showing that the number of activation sites corresponds to the number of mitochondria in dormant spores. Additionally, mitochondria are the first organelles damaged by supraoptimal activation treatments (Cotter and George, 1975). Additional support for the model results from an analysis of treatments known to prevent spore activation or deactivate spores. Apparently, any treatment which blocks spores prior to the late post-activation lag phase (see below) results in a re-imposition of dormancy. For example, spores treated with 0.20 M sucrose, azide, cyanide, or pH or temperature extremes return to the dormant state within 5-6 h (Cotter et al., 1979). Such treatments apparently allow the regulatory protein to return to the "dormant config-
uration" by some "fail-safe" mechanism.

While Cotter's (1973a) activation model has gained wide acceptance and has been incorporated into a hypothesis on the entire *D. discoideum* spore germination sequence (Hohl, 1976), some workers have urged restraint in broadly accepting the model. Sussman (1976) argues that while Cotter's model is indeed plausible, protein denaturation is not the only possible result of the various activation treatments. Citing a considerable amount of data on *Neurospora* ascospore activation, as well as other spore systems, Sussman (1976) makes a cogent argument for the involvement of lipids in fungal spore activation. He cites numerous examples in which lipids are required for enzyme activity and shows, at least in the case of furfural activation of *Neurospora* ascospores, that the activating compound acts on membranes (cf. Eilers and Sussman, 1970 a,b). Thus, at least in some systems, activation treatments appear to be mediated by lipids or membranes. In *D. discoideum*, for example, activation results in a large difference in spore permeability (Hohl et al., 1978).

It is not possible at this time to accept or reject any of the hypotheses regarding activation of fungal spore germination. Firstly, fungi are notoriously diverse. The mechanism for activation of *D. discoideum* asexual spores may be totally different from that of *Neurospora*.
ascospores. Secondly, a cell or spore is not a packet of isolated components. Proteins and lipids are intimately associated and may transmit effects. Thus, while the primary effect of an activation treatment may be on a membrane, one would expect the effects of altered membrane fluidity to be manifest in the activity or location of membrane-associated enzymes. Finally, fungi are quite resourceful. It is possible, or likely, that there are multiple pathways leading from dormancy to vegetative growth. A fungal spore may be capable of germinating through several mechanisms depending on external conditions.

c. Germination of *Dictyostelium discoideum* spores

"Germination" is defined as "a process which leads to the first irreversible stage which is recognizably different from the dormant organism, as judged by physiological or morphological characteristics" (Sussman and Halvorson, 1966). In the case of *D. discoideum* spores, the first recognizably different stage is spore swelling. Swelling involves the formation of a lateral protuberance in the spore wall, and a distinct spore darkening when viewed under phase contrast microscopy (Cotter and Raper, 1966). Activated spores are committed to swell approximately 7 min before spore swelling
begins (Cotter, 1977). Thus, spore germination involves the processes that occur between 7 min before spore swelling and the actual swelling event: For simplicity in operational use, a swollen spore is considered to be germinated.

The time course of spore germination is presented in Fig. 3. Zero time is the end of the activation treatment, which in this case is heat activation at 45°C for 30 min. No observable changes immediately follow the activation treatment. This period is considered to be the "post-activation lag phase", and lasts approximately one hour. The swelling (germination) phase follows the lag phase, beginning at approximately one hour and being essentially completed by two hours after activation. During this time, spores lose their refractility under phase contrast microscopy and acquire a turgid appearance. Approximately 2.5 h after activation, a single myxamoeba begins to emerge from each swollen spore. This stage may be considered to be analogous to bacterial spore "outgrowth", but differs in that it requires no exogenous nutrients or other treatment (Cotter, 1975). By 5 h after activation, the entire sequence of events is completed, and vegetative amoebae are free to grow and divide.

Several metabolic events occur during the germination sequence. Spores begin to take up oxygen shortly after the end of the activation treatment (Cotter et al.,
Figure 3. The germination sequence of Dictyostelium discoideum spores heat activated at 45°C for 30 min. (redrawn from Cotter, 1975)
(○) percent spore swelling versus time in hours; (●) percent emergence of myxamoebae versus time in hours; morphological changes are shown schematically above the germination curves.
1976; Cotter, 1977; Cotter et al., 1979). Oxygen consumption continues throughout the entire process. Spores also begin to incorporate $^{14}$C-labelled amino acids into hot trichloroacetic acid (TCA) precipitable materials shortly after activation (Bacon and Sussman, 1973; Yagura and Iwabuchi, 1976; Giri and Ennis, 1977, 1978). This protein synthesis continues at a low rate until the beginning of spore swelling, whereupon the rate increases. RNA synthesis (as measured by incorporation of $^{14}$C uracil into cold TCA precipitable materials) is absent until the beginning of spore swelling, but begins about the same time that spores begin to swell (Yagura and Iwabuchi, 1976; Giri and Ennis, 1977, 1978). That early protein synthesis precedes the beginning of RNA synthesis suggests that "early" proteins are synthesized by pre-formed mRNA and ribosomes. The increased rate of protein synthesis following spore swelling may reflect synthesis on newly formed RNA templates. Neither rRNA nor general protein synthesis is required for heat induced spore swelling, since the event occurs normally in the presence of cycloheximide and actinomycin D (Cotter and Raper, 1970; Yagura and Iwabuchi, 1976). Cycloheximide completely inhibits the emergence of myxamoebae from swollen spores, suggesting that protein synthesis is required for this stage. Actinomycin D does not inhibit the emergence stage. However, Giri and Ennis (1977) have shown that other inhibitors of RNA synthesis, including daunomycin
and lomofungin, do inhibit emergence. These workers suggested that RNA synthesis is required for emergence, but that the spore is relatively impermeable to actinomycin D.

A variety of molecular events occur during the D. discoideum spore germination sequence. For instance, Giri and Ennis (1977) have shown that stage-specific proteins are synthesized at various times throughout the process. Similarly, quantitative and qualitative differences in RNA synthesis occur, including differences in the translational efficiency of poly A+ RNA (Giri and Ennis, 1978). DNA synthesis also begins two hours after spore activation (Yagura and Iwabuchi, 1976).

d. Spontaneous spore germination

Cotter and Raper (1968b) initially reasoned that Dictyostelium discoideum spores might be expected to communicate with each other during spore germination. Spores are in intimate contact with their environment, and it would not be surprising for them to detect the presence of other spores and respond accordingly. Such behavior would be consistent with their reputation as "social amoebae". However, under the experimental conditions employed by Cotter and Raper (1968b), each spore was independently activated, and germination was not affected by other spores in the suspension. Thus, there
was no evidence for autoactivation of *D. discoideum* spore germination.

In contrast, Kornfeld (1968) reported that *D. purpureum* spores germinate in a density dependent manner. Spore germination increases as spore density increases. Washing of spores results in a decrease in spore germination, suggesting the existence of an "endogenous germinant" of *D. purpureum* spore germination. Later work (Coco and Kornfeld, 1973) reiterated the concept that final percent germination of *D. purpureum* spores is dependent upon the number of times spores are washed and spore density. Readdition of the washings to spores results in a stimulation of spore germination. What is apparent from these reports is that an endogenous germinant is present in the sorocarp prior to the initiation of spore germination. In this way, *D. purpureum* spore germination may be similar to that found among many Myxomycetes (Wilson and Cadman, 1928; Smart, 1937; Dahlberg and Franke, 1977). It is not known if additional amounts of the endogenous germinant are released by germinating *D. purpureum* spores.

A similar phenomenon of "autoactivation" of *D. discoideum* spore germination has been discovered as a result of spontaneous mutations. This phenomenon is the subject of the present dissertation. Salient features from several published reports and oral presentations on
the subject of autoactivation of *D. discoideum* spore germination (Cotter and Dahlberg, 1977; Dahlberg and Cotter, 1977a,b, 1978a,b, 1979a,b) will be considered in detail herein.
MATERIALS AND METHODS

Culture conditions for *B. discoideum* and related species

A variety of species and strains of cellular slime molds were used in the course of this study. All were cultured in association with *Escherichia coli* B/r. With the exception of *Polysphondylium pallidum*, all species and strains were mixed with *E. coli* in a liquid medium containing per liter: 6.0 g Na₂HPO₄, 3.0 g KH₂PO₄, 1.0 g NH₄Cl, 0.13 g MgSO₄; 1–1.5 ml of this medium containing $10^3$–$10^4$ spores was aseptically transferred to petri dishes containing glucose-salts agar (containing the above components plus 4.0 g glucose and 20 g Difco agar per liter). The petri dishes were shaken to distribute the liquid and incubated at room temperatures in ambient light conditions. The shaking was repeated after 24–48 h. *Polysphondylium pallidum* was cultured in a similar manner except that spores were suspended in sterile distilled water with *E. coli* and plated on 0.1% L-P agar (1.0 g lactose, 1.0 g peptone, 20 g Difco agar, 1 l distilled water). Synchronous sporulation generally occurs within 3.5–4 d under these conditions.

When not in active culture, all species and strains are maintained at ca. 10 C on streaks of *E. coli* on glucose-salts or 0.1% L-P agar plates. Under these conditions, slime mold spores inoculated on one end of the streak germinate, and myxamoebae complete a vegetative life cycle with a minimum of migration. Following
sporulation, sorocarps topple over, and the spores repeat the process. Such stock cultures can generally be kept approximately 6 months before the slime molds reach the end of the streak. Spores of most slime mold species are also kept lyophilized.

Isolation of new strains, when performed, was by a modification of the methods of Cavender and Raper (1965). Soil samples were collected at Pt. Pelee National Park, Ontario, and at various locations adjacent to Massachusetts Routes 2 and 128, and U.S. Interstate 90 in Massachusetts and New York. Upon returning to the laboratory, 1.0 g. of each soil sample was suspended in 7.5 ml of sterile distilled water and vigorously shaken. After soil particles had sedimented, the suspension was diluted 1:10 in sterile distilled water, and mixed with a heavy inoculum of E. coli. Aliquots (0.5 ml) of each suspension were spread on 0.1% L-P agar plates and incubated under ambient laboratory conditions. At daily intervals, the plates were examined for clear plaque-like areas in the E. coli lawn, or for sorocarps. Spores from upright sorocarps were transferred with a fine wire needle to streaks of E. coli on glucose-salts or 0.1% L-P agar plates. Under these conditions, the slime molds generally migrate free of bacterial and fungal contaminants to yield pure cultures. Slime molds were identified according to the characteristics discussed by Bonner (1967).
Dictyostelium discoideum and other slime molds were cloned, when necessary, by a similar plating technique. Spores were suspended in sterile phosphate buffer (10 mM, pH 6.5), salts medium, or distilled water, and concentrations were determined with a hemacytometer. Serial dilutions of the spore suspensions were performed to yield 5-10 spores/ml. A heavy inoculum of E. coli was added to each suspension, and 0.5 ml aliquots were spread on glucose-salts or 0.1% L-P agar plates. Plates were inspected daily for plaque-like clearing in the E. coli lawn. Amoebae from such clearing were transferred to glucose-salts agar plates and allowed to grow and sporulate. Resulting spores were tested for phenotype by usual procedures.

Optimal conditions for spore germination

Dictyostelium discoideum spores were generally harvested from agar plates by holding a moistened glass microscope slide several millimeters above the agar surface and gently rotating the petri dish. Spores from the sorocarps adhering to the slide were rinsed off into 5-10 ml of distilled water in a 50 ml beaker, and the suspension was poured into 15 ml conical glass centrifuge tubes. The preparation was centrifuged at high speed in an IEC Clinical Centrifuge to pellet the spores, and the supernatant was poured off and saved as "crude auto-inhibitor preparation". Spores were resuspended in 5 ml
of 10 mM phosphate buffer, pH 6.5 and recentrifuged. This washing procedure was repeated twice.

If an activation procedure was to be performed, spores were suspended in 5 ml of phosphate buffer in a conical glass centrifuge tube. The sides of the tube were carefully wiped to remove any spores that might not be exposed to the thermal treatment. The entire tube was exposed to the activating regimen: 45°C for 30 min in a Braun Thermomix II circulating water bath or 37-41°C for 30-60 min in a Julabo P5 water bath. Following, or in the absence of an activating treatment, spore concentrations were determined with a hemacytometer, and the spore densities were adjusted with phosphate buffer. Spores were generally incubated at between 1 x 10^6 and 1 x 10^7/ml at 23.5°C with stirring supplied by 10 mm magnetic stirring bars. Volumes were generally kept below 4 ml in 1 x 10 cm glass tubes. Percent spore germination was monitored periodically by placing a small volume of the spore suspension on a glass microscope slide and examining it with a Zeiss phase contrast microscope at 650 power magnification. A minimum of 200 objects were examined and scored as "dormant spores" (phase-bright, regularly-shaped spores), "swollen spores" (phase dark spores with distended spore cases), or "emerged myxamoebae" (amoebae not enclosed in spore cases).

In experiments to determine the times of autoactivator and autoinhibitor release, spores were suspended at
$1 \times 10^7$/ml in 25 ml phosphate buffer in ehrlemeyer flasks with vigorous stirring. Use of such larger volumes often delayed spore germination, presumably due to lowered oxygen tensions and despite vigorous stirring. At intervals, aliquots of the spore suspensions were removed, filtered through 0.45 um Millipore filters, and frozen pending detection of autoactivators and autoinhibitors. Time of loss of cycloheximide sensitivity was determined by removing 0.5 ml aliquots of the spore suspensions at intervals, and adding 0.1 ml of cycloheximide (Sigma) at 1.67 mg/ml. Percent spore swelling in each aliquot was scored after the untreated population had reached maximal spore germination.

In experiments in which spores were germinated *en masse* for generation of autoactivator factors, spores were washed from the petri dishes into a 250 ml beaker with distilled water. The resulting suspension, containing spores, stalk cells, bacteria, and autoinhibitors, was adjusted to approximately $1 \times 10^7$ spores/ml by the "Iball" technique and made 10 mM with respect to phosphate by the addition of 100 mM phosphate buffer, pH 6.5. Spore germination was monitored microscopically, and upon the completion of germination, the preparation was filtered through cheesecloth and centrifuged at 10,000 x g in a Sorvall RC2-B centrifuge. The supernatant was poured into Nalgene bottles and frozen. Such preparations were normally lyophilized to dryness and extracted twice.
with 80% ethanol. Following centrifugation to pellet the precipitated proteins and cellular components, the ethanol was removed by evaporation in vacuo at 45°C, and the preparations were lyophilized to dryness.

Detection of autoactivators and autoinhibitors

Detection of autoactivator substances was by mixing a volume (at least 0.1 ml) of putative autoactivator solution with an equal volume of 1-5 d old strain NC4 spores, strain SG1 spores, or strain SG2 spores inhibited from autoactivating by cycloheximide. Test spore suspensions were at 2 x 10^6 spores/ml and 200 μg/ml cycloheximide so that mixing with the test solutions brought the final spore concentration to 1 x 10^6/ml and cycloheximide to 100 μg/ml. Under these conditions, endogenous autoactivation is at a minimum, and the presence of exogenous autoactivator substances results in a stimulation of spore germination. Detection of the presence of autoinhibitor substances was by a similar method, but conditions were adjusted to give maximum autoactivation. The presence of autoinhibitors results in a decrease in spore germination.

Chromatographic techniques

All biological materials (i.e., materials from D. discoideum or bacteria) to be subjected to chromatographic separation were first extracted twice with 80% ethanol. Ethanol soluble materials were reduced to a small volume
by evaporation at 45 °C and were lyophilized to dryness. Routinely, 100 mg of this material was suspended in 1-2 ml of distilled water, applied to a 3 x 55 cm Biogel\textsuperscript{©} P2 column, and eluted with distilled water at flow rates of ca. 40 ml/h (20 cm head pressure). Little or no change in the appearance of the major peaks of activity was detected when the column was eluted with 20 mM phosphate buffer, pH 6.5. Fractions consisting of 200 drops (8 ml) were mechanically collected until two times the internal volume of the column had eluted. Each fraction was tested for absorbance at 260 and 280 nm in a Beckman DB Spectrophotometer, and tested for autoactivator (or autoinhibitor) activity as described above. Purified substances to be tested for column elution characteristics were chromatographed in a similar manner without prior extraction in ethanol.

Selected fractions from the Biogel P2 column were often pooled, lyophilized to dryness, applied to a 1 x 18 cm Sephadex\textsuperscript{®} LH-20 column, and eluted with distilled water. Fractions (1-2 ml) were mechanically collected and tested for ultraviolet absorbance and autoactivator activity. Since active substances generally showed high biological activities with little or no ultraviolet absorbance, no further chromatographic purification was deemed necessary.
Radioisotopic methods

On several occasions in this study, attempts were made to incorporate radioactive precursors into autoactivator substances. Such attempts were made by adding the radioactively labelled compounds (usually 5 uCi) directly to petri dishes in which D. discoideum strain SG1 amoebae were growing in two membered culture with E. coli. In several instances the E. coli was inhibited by prior addition of streptomycin sulfate (to 100 ug/ml), while in others the bacteria were allowed to grow normally. Following aggregation and sporulation of the amoebae, spores were harvested into sterile phosphate buffer to approximately 1 x 10^7 spores/ml and allowed to autoactivate. The resulting supernatants were tested for autoactivator activity, lyophilized to dryness, mixed with 75 mg of an 80% ethanol extract of unlabelled SG1 autoactivator preparation, and applied to a 3 x 55 cm Biogel P2 column as above. Each fraction was tested for UV absorbance and autoactivator activity. Selected fractions were tested for the presence of radiolabel: 0.1 ml samples were added to 10 ml of a scintillation cocktail (16.5 g PPO, 0.36 g POPOP, 1 l Triton X 100, 2 l toluene) and counted in either a Nuclear-Chicago Mark II or a Beckman LS3150P liquid scintillation counter with appropriate preset channels and quench correction techniques. Labelling of autoactivator factors was also attempted by harvesting unlabelled D. discoideum
strain SG1 spores and adding labelled compounds (5 uCi/ml) to the suspension. Similar chromatographic and isotopic techniques were employed.

**Respiration experiments**

Oxygen uptake by germinating spores was determined with a Clark type oxygen monitor (YSI Model 53, Yellow Springs Instrument Co., Yellow Springs, OH). Spores subjected to various treatments were incubated at 23.5°C in the YSI Model 5301 standard bath assembly coupled to a Haake model HE constant temperature circulator. Spores were suspended in 5 ml of phosphate buffer to 3-10 x 10⁶/ml. Oxygen consumption was recorded as a function of air saturation (6.139 ul O₂/ml at 23.5°C) and was recorded at 30 min intervals. If air saturation fell below 55-60% during the course of an experiment, the samples were reaerated by bubbling air through the suspension. Spores were kept in suspension by stirring with 10 mm magnetic stirring bars. All results were corrected to ul oxygen consumed per hr per 1 x 10⁷ spores.
RESULTS

1. Isolation of *Dictyostelium discoideum* Autoactivator (Spontaneous Germinator) Mutants

   Freshly formed wild type (i.e., strains NC4 or V12) *Dictyostelium discoideum* spores are constitutively dormant. Thus, these spores do not germinate in the absence of an exogenously applied activation treatment (Cotter, 1975; Fig. 4). In laboratory cultures, *D. discoideum* spores are normally sown on glucose-salts agar plates in association with *Eschericia coli* B/r. The glucose-salts medium contains no components capable of activating the spores. However, *E. coli* growing on the glucose-salts medium may release materials capable of activating the spores, including the amino acids tryptophan, phenylalanine, and methionine (Cotter and Raper, 1966), or other substances (see below). One must assume, however, that there is a period during which the bacteria are growing before spore activating components are present in sufficient concentrations to induce spore germination. Any spontaneous mutations which decrease the length of time spores remain ungerminated under such conditions would have a selective advantage in these

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1 Note that the terms "spontaneous germination" and "autoactivation" will be used interchangably. Spontaneous germination refers to the ability of spores to germinate in the absence of an activation treatment. Autoactivation will be defined in the DISCUSSION section of this dissertation.
Figure 4. Dictyostelium discoideum spore germination. Percent spore germination is plotted vs time in hours. (▲) 2 d old strain NC4 spores, no activation treatment; (●) 2 d strain NC4 spores activated at 45°C for 30 min; (●) 2 d strain SG1 spores, no activation treatment; (□) 2 d strain SG2 spores, no activation treatment; (△) 14 d strain NC4 spores, no activation treatment.
cultures. Thus, over several years of serial transfers of *D. discoideum* strain NC4 (haploid) in the laboratory of Professor D. A. Cotter, it is not surprising that a sub-population of spores was detected which did not require an exogenously applied activation treatment to germinate. Cultures containing this subpopulation were designated SG10 (10% Spontaneous Germination).

Mutant spores from SG10 cultures were concentrated by allowing large numbers of washed spores to germinate in small droplets on non-nutrient agar. The majority of young wild type spores do not germinate under these conditions, but mutant spores do so rapidly. Amoebae within the droplet quickly aggregate to form small fruiting bodies due to the lack of nutrients required for vegetative growth. Spores from these diminutive sori were cloned, and spores from individual clones were tested for the ability to autoactivate. After cloning a second time, a homogeneous population of spores capable of spontaneously germinating was obtained. This population was designated SG1. The primary characteristic of *D. discoideum* strain SG1, its ability to demonstrate spore germination in the absence of an exogenous activation treatment, is shown in Fig. 4.

Mutant strain SG2 arose spontaneously in stock cultures of *D. discoideum* strain V12 which was maintained on streaks of *E. coli* on glucose-salts agar at ca. 10°C (see MATERIALS AND METHODS). Over several six month
periods between transfers of stock cultures, the entire population became capable of spontaneously germinating without the need for cloning. The phenotype was first detected when it was observed that strain V12 spores had germinated within the intact sorocarps in the humid stock cultures. Investigation of the strain revealed the presence of a homogeneous population capable of spontaneous spore germination. The population was designated SG2; its characteristic of spore germination in the absence of an activation treatment is shown in Fig. 4.

2. Age Induced Autoactivation of Wild Type *Dictyostelium discoideum* Spores.

During the course of studies of the *D. discoideum* spontaneous germination phenomenon, wild type strain NC4 spores were often used as controls. The purpose of such controls was to ensure that various manipulations of the spores were not in themselves activation treatments. If wild type spores undergoing the various treatments did not germinate, the treatments were considered non-activating.

During one experimental series on the effects of age on the ability of mutant spores to autoactivate, it was noted that strain NC4 control spores also germinated to a high percentage. Since the spores received no treatment except aging in the sorocarp, the effect of maturation on strain NC4 spores was further investigated.
The data of Fig. 4 show an example of the effect of age on autoactivation of strain NC4 spores. Freshly formed (i.e., 2 d after fruiting) spores are constitutively dormant, and thus do not germinate without an activation treatment. Spores aged in the sorus at 23°C at ca. 50% relative humidity for 14 d germinate synchronously and to high percentages when suspended in phosphate buffer. The aged wild type spores require a longer lag time prior to the beginning of spore swelling than autoactivating mutant spores, but otherwise the spontaneous germination is normal. Results plotted in Fig. 5 demonstrate that wild type spores become capable of autoactivating after 6-7 d in the sorus, reach a peak of almost 90% germination at 10 d, and then rapidly lose their germinating ability. A comparison of the percent germination at 6 and 8 h after suspension of the spores in phosphate buffer suggests that one effect of age on the ability of spores to autoactivate is to alter the lag times. Thus, spores aged 7 d show about 1% germination at 6 h and 17% at 8 h, while those aged 10 d show 17% germination at 6 h and 86% at 8 h. The entire curve is shifted to shorter lag times, but the synchrony of spore germination is retained despite aging. Note that spores aged 14 d (Fig. 4) still germinate to at least 95%, but have a lag period in excess of 9 h.

The percent germination at 10 h is not shown in Fig. 5 since myxamoebae in suspension tend to aggregate,
Figure 5. Age induced autoactivation of wild type (strain NC4) Dictyostelium discoideum spores. Percent spore germination at 4 h (Δ), 6 h (○), and 8 h (□) after suspension in phosphate buffer is plotted vs spore age in days.
making accurate counts at this time difficult. Spores which have lost the ability to spontaneously germinate with advanced age retain their viability since they are capable of germinating in response to a heat shock (data not shown). Experiments were not conducted past 20 d after fruiting since sorocarps dry to such an extent that harvesting of spores is difficult.

*D. discoideum* strain NC4 is normally considered "wild type". However, this strain has been in laboratory culture for many years. Since laboratory culture conditions are known to exert selective pressures on *D. discoideum* (note the isolation of the SG mutants), the possibility was considered that the effect of age on strain NC4 spores was an artifact of long term laboratory selection. In order to determine whether the aging phenomenon was natural (i.e., a characteristic of true "wild type" spores), or an artifact, similar tests were conducted on a fresh *D. discoideum* isolate. *D. discoideum* strain JC1 was a gift of Dr. James Cavender (Ohio Univ., Athens, OH), and was freshly isolated from the soil (August, 1977). Immediately upon receipt of the culture, spores were lyophilized to preserve the "wild type" phenotype. Spores were revived from lyophilization and cultured in such a manner that the working material represented products of only the third sporulation in laboratory culture. Data in Fig. 6 demonstrate that strain JC1 spores also autoactivate as a result of spore
Figure 6: Age induced autoactivation of wild type (strain JC1) Dictyostelium discoideum spores. Percent spore germination is plotted vs. time in hours after suspension in phosphate buffer; (○) 2 d old spores; (□) 9 d old spores; (□) 16 d old spores.
maturation. While the kinetics of age induced germination
differ between strains JC1 and NC4, the observation of the
autoactivation phenomenon suggests that it is not an
artifact of laboratory selection.

It should be noted that age induced autoactivation
of wild type D. discoideum spores is somewhat variable.
The periods of time before the onset and peak of auto-
activating ability are not very reproducible. Minor
variations in laboratory temperature and humidity probably
account for most of this variation.

Investigations of age induced spore autoactivation
were not exhaustively pursued due to this variability
and the inconvenience of the requirements for aging and
long lag times. Autoactivation of wild type spores was
investigated only when necessary to confirm characteris-
tics of mutant spore autoactivation. As will become
apparent below, the mutations affecting strains SG1 and
SG2 probably induce a "premature maturation" phenomenon.
Strains SG1 and SG2 were investigated as a paradigm
for autoactivation in general.

3. Autoactivation of Spore Germination Among Other
Acrasids

Spores of other strains of D. discoideum also
demonstrate autoactivation to a limited extent. For
instance, D. discoideum strains B, AX3, and MC91 spon-
taneously germinate to approximately 25% within 5 h after
suspension in phosphate buffer. No effort was made to extensively study autoactivation in these strains, however, for the following reason: these genetically marked strains were isolated after mutagenesis with nitrosoguanidine (Ennis and Sussman, 1975; Loomis, 1975), and appear to have multiple genetic defects. For instance, spores of strain B are oval rather than the normal capsule shape of strain NC4. Additionally, strain B spores germinate somewhat asynchronously after heat activation at 45 °C for 30 min (Ennis and Sussman, 1975). D. discoideum strains AX3 (axenic growth) and HC91 (brown pigmented sori) are similarly disturbed. Because of the mutagenesis in their histories, these strains are considered to be defective, and any data obtained from them must be considered suspect. Therefore, these strains were studied only for the purpose of comparison with data obtained from the spontaneous germinator mutants.

Other slime mold species demonstrate autoactivation of spore germination. Spores of two strains of Dictyostelium purpureum spontaneously germinated up to about 50% within 5 h. Cloning of these populations would probably yield a homogeneous population similar to that reported by Coco and Kornfeld (1978). Dictyostelium mucoroides spores may also autoactivate. Of ten strains tested, four demonstrated spontaneous spore germination. Of these, three autoactivated to high percentages. D. mucoroides var. Stoloniferum spores have previously
been shown to germinate immediately after sorocarps topple over (Cavender and Raper, 1968). Thus, spontaneous germination in this variety is not surprising. D. mucoroides strains MA1 and NY2 do not appear to be members of the Stoloniferum variety, however, since spores do not germinate on the spent agar surface when sorocarps collapse. The difference in the ability of spores to autoactivate could reflect their status in the "D. mucoroides complex" (Cavender and Raper, 1968). Spores from Polysphondylium pallidum and Polysphondylium violaceum do not appear to be capable of autoactivation.

Autoactivation was not extensively studied in species other than D. discoideum. Very little is known of spore germination in D. purpureum and D. mucoroides compared to D. discoideum. Therefore, emphasis was placed on furthering our knowledge of the one species rather than extending our studies to others. Autoactivation of spore germination in D. mucoroides and D. purpureum was examined only for the purpose of comparison with the process in D. discoideum.

4. General Aspects of *Dictyostelium discoideum*

Autoactivation

a. Comparison of growth and development of wild type strain NC4 and mutant strains SG1 and SG2

Mutant strains SG1 and SG2 are similar or identical to wild type strain NC4 (haploid) in regard to growth
and sporulation. The fruiting bodies of strains SG1 and SG2 are equal to or slightly larger in size than those of strain NC4. No abnormalities are noticeable in the morphology of fresh sorocarps from the mutant strains. Strain SG2 is occasionally observed to form "secondary sori" in which spores within the sorus germinate and immediately reform fruiting bodies. Such sori extend from within the primary sorocarps. The phenomenon is especially evident in very humid cultures. The humid conditions allow sorocarps to take up considerable amounts of moisture as evidenced by their turgid appearance. This dilutes the autoinhibitor and reduces the osmotic pressure, allowing spores to germinate in situ. When sorocarps from strain SG2 are incubated at ambient laboratory conditions at ca. 96% relative humidity, approximately 50% of the spores germinate within 5 d. They do not germinate in situ at ambient laboratory humidities. Strain SG1 spores do not appear to germinate in situ under similar conditions, and rarely form secondary sori.

Mutant strains complete their life cycle somewhat more rapidly than strain NC4. At 23 C, strains SG1 and SG2 sporulate approximately 3-3.5 d after inoculation onto glucose-salts agar plates. Strain NC4 requires approximately 4 d to complete its life cycle. Presumably, this difference is due to the capacity of the mutant spores to germinate earlier than those of the wild type.
Thus, these strains begin their vegetative phase earlier rather than have accelerated growth rates. Spores from mutant strains are virtually indistinguishable from those of strain NC4 (haploid). The germination process of mutant strains SG1 and SG2 is identical to that of strain NC4 after exogenous activation. Strain SG2 is, of course, the opposite mating type from strains SG1 and NC4, reflecting its origin from *D. discoideum* strain V12.

b. Density dependence of autoactivation

In previous representations of spontaneous germination (Fig. 4-6) spore densities have been adjusted to $1 \times 10^7$ spores/ml. Under these conditions, spores capable of autoactivating do so synchronously and to high percentages. However, there is a marked positive concentration dependence of autoactivation. Spores suspended in phosphate buffer at $1 \times 10^6$/ml germinate asynchronously and to lower percentages. The results presented in Fig. 7 show that SG1 spores at $1 \times 10^6$/ml germinate to only about 30% within 4 h, while those at $1 \times 10^7$/ml show essentially complete germination at this time. Similarly, SG2 spores germinate very poorly at $1 \times 10^6$/ml, while those at $1 \times 10^7$/ml germinate rapidly and synchronously (Fig. 7). Aged strain NC4 spores also demonstrate that autoactivation is density dependent (data not shown).

While density dependence of autoactivation can be demonstrated in a variety of spore preparations, it is
Figure 7. Density dependence of autoactivation.
Panel A: strain SG1 spores; Panel B: strain SG2 spores; (△) spores at 1 x 10^7/ml; (▲) spores at 1 x 10^6/ml.
variable. At some times spores at $1 \times 10^6$/ml germinate poorly, while at other times they germinate to high percentages, with a rate and final percentage approaching that of spores at $1 \times 10^7$/ml (Fig. 8). The data in Fig. 7 and 8 represent the two extremes of density dependence. Subjective observations suggest that the variability in density dependence may be due to the effects of humidity. Spores at $1 \times 10^6$/ml generally germinate well in the warm summer months, but do so poorly during the winter months. While ambient laboratory temperatures are relatively uniform throughout the year, the humidity in the laboratory is quite low in winter months and higher in the summer. This difference is especially apparent in preparing cultures of *D. discoideum*. In the summer months, 1.0 ml of a medium containing spores and *E. coli* is added to each petri dish. After approximately 3 d, the agar surface is dry enough to allow aggregation and culmination of amoebae without interference by liquid. In winter months, up to 2.0 ml of the liquid must be added to each petri dish to obtain a similar result due to the lowered humidity. Additional variations in humidity could result from differences in the amount of agar in each petri dish, location of the dishes within a stack, and the temperature of the agar when poured, for example. Thus, the variations in density dependence of autoactivation could result from a number of factors affecting the humidity within the petri dish. The
Figure 8. Reduced density dependence of autoactivation. Panel A: strain SG1 spores; Panel B: strain SG2 spores; (Δ) spores at 1 x 10⁷/ml; (▲) spores at 1 x 10⁵/ml.
possible effect of humidity on autoactivation will be further discussed below.

c. Mediation by autoactivator substances

The observation of a positive density dependence (higher levels of germination with higher spore densities) of autoactivation suggests the presence of substances stimulatory to spore germination similar to that found in D. purpureum (Coco and Kornfeld, 1978). This effect contrasts with the negative density dependence of auto-inhibition of D. discoideum spore germination, whereby higher spore densities result in decreased germination (Russell and Borner, 1960). Solutions in which D. discoideum spores have previously (spontaneously germinated are stimulatory to subsequent germination due to the presence of "autoactivator factors". When spores are added to filtered supernatants in which spores have previously spontaneously germinated at 1 x 10⁷/ml, germination proceeds rapidly and to high percentages.

The information presented in Fig. 9 shows the effect of various "activated supernatants" on SG1 spores at 1 x 10⁶/ml. Supernatants were derived from SG1 and SG2 autoactivation. In both cases, the supernatants stimulate spore germination to an extent greater than if spores were suspended at 1 x 10⁷/ml. Activated supernatants also stimulate strain SG2 spores in a similar manner (Fig. 9). Supernatants in which aged strain NC4 spores
Figure 9. Effect of activated supernatants on strain SG1 and SG2 spore germination. All data are plotted as percent germination vs. time in hours after harvesting spores. Panel A: strain SG1 spores; Panel B: strain SG2 spores; (Δ) spores at 1 x 10⁷/ml; (○) spores at 1 x 10⁶/ml in a solution in which SG1 spores have previously autoactivated; (□) spores at 1 x 10⁶/ml in a solution in which SG2 spores have previously autoactivated.
have autoactivated are also highly stimulatory to subsequent spore germination (data not shown).

Fresh wild type strain NC4 spores are also markedly stimulated by the activated supernatants. While these spores will normally not germinate in the absence of an activation treatment, addition of SG1 or SG2 autoactivator preparations results in rapid, synchronous spore germination (Fig. 10). The rate and final percent germination compare favorably to that obtained when spores are heat activated at 45°C for 30 min. Again there is some variability in the phenomenon. At times strain NC4 spores germinate rapidly in response to the autoactivators. At other times, however, NC4 spores germinate asynchronously (Fig. 10), but still well above control levels. Whether this variability is due to differences in the efficacy of the activated supernatants or in the responses of the spores is unknown, but the former possibility is suspected.

Fresh wild type spores show temporal variations in response to the autoactivator preparations. Spores treated with the autoactivators immediately after suspension in phosphate buffer respond well (albeit variably) to the germinant. However, if spores are incubated for 1 h prior to the addition of the autoactivators, the response is diminished or totally abolished (Fig. 10). This finding suggests that there is a "competence period" during which the substances operate. The period is about
Figure 10. Effect of activated supernatants on strain NC4 spore germination. Percent spore germination vs time in hours. (Δ) 2 d old NC4 spores at 1 x 10^7/ml; (▲) same, heat activated at 45°C for 30 min. (□) same, plus activated supernatant from SG1 spores; (○) same, plus activated supernatant from SG1 spores from a different lot added at t = 0 h; (●) same, plus activated supernatant from SG1 spores from a different lot added at t = 1 h; (●) same, plus activated supernatant from SG2 spores.
1 h in duration, after which spores become refractory to the signal (Fig. 11). The spores are still viable, since they respond to heat activation (data not shown). Mutant strains SG1 and SG2, in contrast, have no such competence period. Activated supernatants added at any time after suspending the spores in phosphate buffer result in a stimulation of spore germination. Data on the response of SG2 spores to SG1 autoactivator preparation added at various times is shown in Fig. 11.

The effect of strain NC4 spore age on the response to the autoactivator signal is complex. If the activator substances are added to variously aged strain NC4 spores immediately after suspension in phosphate buffer, all spores respond well (Table 1). Aged (5, 7, and 9 d) spores may respond slightly better than fresh (3 d) spores, but the differences are minor. However, variously aged spores show altered responses 1 h after suspension of the spores. Those aged 9 d germinate well, while fresh spores show a greatly reduced response to the delayed autoactivator signal (Table 1). Thus, one effect of age on spores may be to eliminate the competence period. This finding is consistent with the observation that advancing spore age induces the spontaneous germinator phenotype. Aged NC4 spores become more like mutant spores in that they become capable of responding to the autoactivator signal at any time.
Figure 11. Competence period in response to SG1 auto-activator preparations. Data represent a plot of percent germination at 5 h vs time of addition of SG1 autoactivator preparation. Spores in both cases were treated with cycloheximide (100 ug/ml final concentration) to inhibit endogenous spore activation. (▵) response of NC4 spores; (○) response of SG2 spores.
<table>
<thead>
<tr>
<th>Spore age (d)</th>
<th>% autoactivation at 5 h</th>
<th>% germination&lt;sup&gt;a&lt;/sup&gt; at 5 h, &quot;AA&quot; added t = 0 h</th>
<th>% germination at 5 h, &quot;AA&quot; added t = 1 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.0</td>
<td>97.0</td>
<td>27.0</td>
</tr>
<tr>
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<td>0.0</td>
<td>97.0</td>
<td>58.0</td>
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<tr>
<td>7</td>
<td>0.0</td>
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<td>68.0</td>
</tr>
<tr>
<td>9</td>
<td>0.0</td>
<td>99.0</td>
<td>84.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> "AA" refers to addition of autoactivator preparation resulting from strain SG1 spontaneous spore germination at 1 x 10<sup>7</sup> spores/ml.
Spores show a graded response to the addition of autoactivator factors. A volume of the activated supernatant is normally added to an equal volume of spores in phosphate buffer. When the activated supernatant is derived from mutant spores which have spontaneously germinated at $1 \times 10^7$ spores/ml, the resulting 50% autoactivator solution is sufficient to stimulate the entire spore population (see, for example, Fig. 9 and 10). Further dilution of the activated supernatants results in diminished spore germination. Fig. 12 shows an example of a dilution series on the SG2 activated supernatant. In this case, the autoactivator preparation is tested against strain SG2 spores inhibited from spontaneously germinating by cycloheximide (100 μg/ml). This drug inhibits autoactivation, but its effect may be overridden by the addition of autoactivator factors (see below). (The purpose of inhibiting autoactivation in this case is to prevent autocatalysis of spore germination). As can be seen from Fig. 12, dilution of the supernatant reduces the stimulation of spore germination.

The plot of percent spore germination (relief from cycloheximide inhibition) versus the percentage of the solution as activated supernatant results in a hyperbolic curve (Fig. 12). That this is hyperbolic is supported by the linearity of a double reciprocal plot of the data points (data not shown). This saturation-type plot is
Figure 12. Dilution series on SG2 activated supernatant. SG2 spores at $2 \times 10^6$/ml plus 200 ug/ml cycloheximide were mixed with an equal volume of various dilutions of SG2 activated supernatant. (●) represents percent spore germination at 5 h after harvesting vs. percentage of the suspension as SG2 activated supernatant.
not unlike that shown by many enzymes. These data may suggest that multiple molecules of the autoactivator substances must interact with each spore to induce germination. Were only a single molecule required to activate each spore one would expect a more linear relationship between autoactivator concentration and spore germination. A similar hyperbolic relationship exists between the concentration of the autoactivators from strain SG1 and percent spore germination (data not shown).

a. Release of autoactivator substances during spontaneous spore germination

Autoactivator substances are not present in the spore suspension prior to germination. It is only after spores have begun to germinate that supernatants become stimulatory to subsequent spore germination. Data in Fig. 13 demonstrate that autoactivator substances are released during the swelling stage of germination of both SG1 and SG2 spores. The ability of supernatants to stimulate spore germination closely parallels the onset of spore swelling. Whether these substances are specifically excreted at this time or are simply components of the "osmotically active material" released during spore swelling (Hohl et al., 1978) is not known.

Autoactivator substances are not released under conditions in which spontaneous spore germination is inhibited. Thus, spores prevented from autoactivating by
Figure 13. Release of autoactivator factors during spore swelling. Panel A: SG1 spores; Panel B: SG2 sporas; (Δ) percent spore swelling at $1 \times 10^7$/ml vs. time in hours after harvesting; (▲) percent emergence of myxamoebae; (○) and (□) ability of supernatants to stimulate subsequent spore germination: percent germination of SG2 spores (plus cycloheximide) is plotted vs time of sampling. Percent germination was scored at 5 h after addition of spores to the aliquots.
various metabolic inhibitors or inhibitory conditions (see below) do not release the stimulatory compounds.

Autoactivators are not released by spores activated to germinate by the application of a heat shock. Regardless of the strain, no autoactivators are present in the supernatants after spores have germinated in response to heat activation.

e. Effects of inhibitors and inhibitory conditions on autoactivation

Autoactivation of D. discoideum spores may be inhibited by a variety of drugs and treatments. Complete inhibition of autoactivation is considered to be the prevention of spores from spontaneously swelling and releasing autoactivator factors. A number of other drugs and treatments which are known to allow spontaneous spore swelling and release of autoactivators but inhibit the emergence of myxamoebae will not be considered here.

Caution must be exercised in interpreting the results of inhibitor studies in D. discoideum spore autoactivation. One cannot always be sure that a specific drug is entering the spores, or that it has specific effects. As will be seen below, such considerations are important in interpreting these experimental results.

Cycloheximide

One drug of particular interest to a study of D. discoideum spore germination is cycloheximide, an inhibi-
itor of peptide elongation (Davis et al., 1973). Several previous studies have reported that cycloheximide does not block the swelling stage of spores activated to germinate by a heat shock or dimethylsulfoxide (DMSO) treatment (Cotter and Raper, 1966, 1970; Bacon and Sussman, 1973; Yagura and Iwabuchi, 1976; Giri and Ennis, 1977). This inhibitor does, however, prevent the emergence of myxamoebae. Several workers have used this evidence to suggest that protein synthesis is not required for heat induced spore swelling (Cotter and Raper, 1966, 1970; Yagura and Iwabuchi, 1976). However, Bacon and Sussman (1973) inferred that protein synthesis is required for spore swelling but that dormant spores are impermeable to the drug.

Autoactivation of *D. discoideum* spores is sensitive to cycloheximide at 100 µg/ml. Spores fail to spontaneously swell in the presence of the drug (Table 2 and Fig. 14). Thus, spores capable of autoactivation are permeable to cycloheximide. More tellingly, SG spores activated by heat shock in the presence of cycloheximide swell normally (Fig. 14). Therefore, protein synthesis is not required for swelling of heat activated spores, since the event still occurs under conditions where cycloheximide can enter spores and inhibit protein synthesis by greater than 90% (Cotter et al., 1979).
Table 2. Effect of various inhibitors and inhibitory conditions on autoactivation

<table>
<thead>
<tr>
<th>Spores</th>
<th>Drug or treatment</th>
<th>concentration (μg/ml)</th>
<th>+ drug</th>
<th>+AA</th>
<th>145°C, 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG1</td>
<td>control</td>
<td>---</td>
<td>100.0^a</td>
<td>96.0</td>
<td>95.0</td>
</tr>
<tr>
<td>SG1</td>
<td>cycloheximide</td>
<td>100.</td>
<td>9.0^b</td>
<td>99.0^b</td>
<td>99.0^b</td>
</tr>
<tr>
<td>SG1</td>
<td>Edeine</td>
<td>200</td>
<td>18.0^b</td>
<td>94.5^b</td>
<td>98.5^b</td>
</tr>
<tr>
<td>SG1</td>
<td>Thiolutin</td>
<td>100^c</td>
<td>2.5^b</td>
<td>81.5^b</td>
<td>93.5^b</td>
</tr>
<tr>
<td>SG1</td>
<td>Daunomycin</td>
<td>250</td>
<td>0.0</td>
<td>1.0^b</td>
<td>89.0^b</td>
</tr>
<tr>
<td>SG1</td>
<td>4NQO</td>
<td>---</td>
<td>8.0^b</td>
<td>87.0^b</td>
<td>36.0^b</td>
</tr>
<tr>
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<td>62.5^b</td>
<td>99.0^b</td>
<td>99.0^b</td>
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<td>98.0</td>
<td>96.0</td>
<td>94.0</td>
</tr>
<tr>
<td>SG2</td>
<td>cycloheximide</td>
<td>100</td>
<td>0.0</td>
<td>99.0^b</td>
<td>98.0^b</td>
</tr>
<tr>
<td>SG2</td>
<td>Edeine</td>
<td>200</td>
<td>9.5^b</td>
<td>98.5^b</td>
<td>97.5^b</td>
</tr>
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<td>Thiolutin</td>
<td>100^c</td>
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<td>69.5^b</td>
<td>94.5^b</td>
</tr>
<tr>
<td>SG2</td>
<td>Daunomycin</td>
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<td>3.0^b</td>
<td>4.0^b</td>
</tr>
<tr>
<td>SG2</td>
<td>4NQO</td>
<td>---</td>
<td>3.5^b</td>
<td>96.0^b</td>
<td>58.0^b</td>
</tr>
<tr>
<td>SG2</td>
<td>UV</td>
<td>---</td>
<td>47.0^b</td>
<td>99.5^b</td>
<td>99.0^b</td>
</tr>
</tbody>
</table>

^a All values represent percent spore germination 5 h after harvesting of spores.

^b Emergence of myxamoebae inhibited.

^c Saturated solution.

^d In all cases, spores were at a density of 1 x 10^7/ml.
Figure 14. Effect of cycloheximide on SG2 spore germination. All points represent percent spore germination vs time in hours after spore harvesting. Panel A: (△) SG2 spores at 1 x 10^7/ml; (▲) SG2 spores at 1 x 10^7/ml in 100 μg/ml cycloheximide; (□) SG2 spores at 1 x 10^7/ml in 100 μg/ml cycloheximide plus SG1 autoactivator solution; Panel B: (○) SG2 spores heat activated at 45°C for 30 min; (●) SG2 spores heat activated at 45°C for 30 min in 100 μg/ml cycloheximide.
The effect of cycloheximide on autoactivation is another phenomenon subject to some variability. While SG2 spores are consistently sensitive to cycloheximide, SG1 spores range in sensitivity from 0 to 100%. The sensitivity of strain SG1 spores to cycloheximide generally correlates with the concentration dependence of autoactivation, and may therefore be attributable to humidity differences. Accordingly, this topic will be discussed below in that context.

Cycloheximide inhibition of autoactivation is overcome by the addition of autoactivator substances (Table 2 and Fig. 14). Whereas the drug alone completely inhibits SG2 spore swelling, cycloheximide plus crude autoactivator preparations allows a high percentage of spore germination. While there is often an alteration in the rate of autoactivation and a portion of the spores remain ungerminated, the autoactivator preparation dramatically stimulates spore germination. Similar results are obtained with SG1 spores treated with cycloheximide (Table 2). Thus, spontaneous spore swelling (germination) apparently requires protein synthesis, but this requirement can be obviated by the addition of autoactivator factors. However, emergence of myxamoebae is still prevented by cycloheximide.

Both SG1 and SG2 spores remain sensitive to cycloheximide until approximately 0.5 h before the onset of spore swelling (Fig. 15). If the drug is added after this time,
Figure 15. Time of loss of sensitivity to cycloheximide. Panel A: SG1 spores; Panel B: SG2 spores. (△) percent spore germination at 1 x 10^7/ml vs time in hours after harvesting; (○) percent spore germination at 5 h vs time of addition of cycloheximide to 100 μg/ml.
spores swell normally. Thus, a critical synthetic event occurs about 0.5 h before spore swelling; after this synthetic event, spores are committed to swell. The synthetic event does not correspond to the release of the autoactivator factors, since this occurs concomitantly with spore swelling, not before (see Fig. 13).

**Edeine**

Another inhibitor of macromolecular synthesis which inhibits spontaneous spore germination is edeine at 200 ug/ml (Table 2). Edeine is an inhibitor of the initiation stage of protein synthesis which acts by interfering with the binding of methionyl-tRNA<sub>f</sub> to the 40S ribosomal subunit (Odom et al., 1978). The effect of this drug supports the contention that protein synthesis is required for autoactivation. Addition of autoactivator substances overrides the inhibition of spore swelling caused by edeine. Application of a heat shock also allows spores to swell. Thus, the effect of edeine on autoactivation appears to be identical to that of cycloheximide.

**Thiolutin**

Thiolutin (3-4 ug/ml) has been shown to inhibit RNA synthesis by affecting DNA-dependent RNA polymerase activity (Tipper, 1973). This drug also inhibits autoactivation, and may be overridden by addition of autoactivator substances (Table 2). Its effect may also be overridden by heat shock. This observation suggests that
RNA synthesis is also required for autoactivation. However, it should be pointed out that thiolutin may have multiple effects on the spores. For instance, some data suggest that the drug may also inhibit protein synthesis (D. J. Tipper, personal communication to D. A. Cotter and L. S. Tisa). Thiolutin may also affect respiratory metabolism, since treatment of vegetative amoebae with the drug results in an 80% decrease in ATP levels (F. J. Garnish, L.S. Tisa, and D. A. Cotter, personal communication). Whatever the cellular effect, it is obvious that thiolutin readily enters the spores and inhibits autoactivation.

The observation that thiolutin inhibition of germination is overridden by an exogenous activation treatment conflicts with that reported by Giri and Ennis (1977). These workers showed a failure of DMSO activated spores to swell in the presence of 100 ug/ml thiolutin, and used this evidence to suggest that RNA synthesis is required for spore "germination" (these workers did not state whether they considered spore swelling or emergence of myxamoebae to be germination). It is possible that these conflicting results may be due to Giri and Ennis' (1977) use of 1% DMSO to increase the solubility of thiolutin. The drug used in these experiments was at 100 ug/ml, but the solutions were saturated. Therefore, the effective concentration of the drug was reduced.
The difference in results is not due to the use of heat activation rather than DMSO activation, since thiolutin in 1% DMSO also inhibits the swelling stage of heat activated D. discoideum spores (L. S. Tisa and D. A. Cotter, personal communication). Since satisfactory inhibition of autoactivation was obtained without the use of 1% DMSO, no attempt was made to increase the solubility of the drug.

4-Nitroquinoline-1-oxide

The drug 4-nitroquinoline-1-oxide (4NQO) is a potent carcinogen and mutagen (Tada and Tada, 1976). Its primary cellular effect is mediated through a highly carcinogenic intermediate, 4-hydroxyaminoquinoline-1-oxide, which binds to purines (especially guanine), but does not significantly affect pyrimidines (Tada and Tada, 1976). The drug inhibits RNA synthesis by reducing the template ability of DNA (Tada et al., 1967), and by inhibiting DNA-dependent RNA polymerase activity (Paul et al., 1967). 4NQO results in lowered levels of protein, RNA, and DNA in HeLa cells (Amsterdam et al., 1967). The drug apparently results in the synthesis of RNA fragments, as there is an increase in the levels of soluble, non-amino acid accepting RNA (Amsterdam et al., 1967; Paul et al., 1967). The effect of 4NQO has been likened to ultraviolet irradiation in that the 4NQO-purine complex is inhibitory to RNA transcription, and the excision repair of the lesions results in high rates of mutation.
(Ikenaga et al., 1975).

4NQO is inhibitory to autoactivation of mutant strains SG1 and SG2 (Table 2). In the presence of saturated concentrations of the drug, germination is inhibited by greater than 90%. The effect of 4NQO is overridden by the addition of SG1 autoactivator preparation, but is only partially overridden by the application of a heat shock. Emergence of myxamoebae is prevented by the presence of 4NQO.

While this data might suggest that RNA synthesis is required for autoactivation, the results are again equivocal. 4NQO binds to purine residues, but apparently does not distinguish between RNA and DNA (Tada and Tada, 1967). Thus, the drug could affect the integrity of the RNA template and reduce or prevent protein synthesis. Several groups have reported reductions in total cellular protein after treatment with 4NQO (Taya et al., 1967; Namba et al., 1977). The observation that heat activation does not completely overcome 4NQO inhibition of germination suggests that the drug may not be specifically inhibiting RNA synthesis, since such synthesis is apparently not required for spore swelling (Cotter and Raper, 1966; Yagura and Iwabuchi, 1976). If 4NQO were affecting only RNA synthesis, one would expect spore swelling despite the presence of the drug.
Daunomycin

Daunomycin is an inhibitor of RNA synthesis which acts in a manner similar to actinomycin D, ethidium bromide, and related drugs (Ward et al., 1965; Kersten, 1971). That is, the drug intercalates DNA molecules and disrupts the template. Its effect is to prevent the DNA-dependent RNA polymerase from transcribing the DNA.

Daunomycin is inhibitory to autoactivation of SG1 and SG2 spores (Table 2). However, its effects cannot be overcome by the addition of SG1 autoactivator preparation, and SG2 spores are not relieved from daunomycin inhibition by heat shock. SG1 spores swell to a high percentage after heat shock (Table 2). The lack of relief by autoactivator substances and heat shock (SG2) suggests that daunomycin may be cytotoxic to functions other than RNA synthesis; thus, these data do not allow one to speculate about the requirement for RNA synthesis during autoactivation.

Ultraviolet irradiation

From the data above, it is possible to suggest that protein synthesis is required for autoactivation of mutant strains SG1 and SG2. A similar requirement for RNA synthesis has not been unequivocally demonstrated due to the possibility of their being secondary effects from the drugs. One further treatment used in an attempt to determine the requirement for RNA synthesis
during autoactivation was the use of ultraviolet (UV) irradiation. The major cellular effect of UV is the induction of pyrimidine dimers in nucleic acids (Beukers and Berends, 1961; Setlow, 1966), rendering DNA unsuitable as a template for RNA synthesis. Thus, UV treatment may be considered an RNA synthesis inhibitor.

Heat activated *D. discoideum* spores swell normally despite the UV treatment (Table 2 and Fig. 16), but myxamoebae fail to emerge. A portion of the mutant spores spontaneously swell despite the UV treatment, but the kinetics of autoactivation are different than those of non-irradiated spores (Fig. 16). Unirradiated spores germinate rapidly with sigmoid kinetics. UV treated spores begin to swell normally, but soon show a distinct alteration in germination kinetics. Final percentages of germination are often reduced (Table 2). Even massive doses (15 min, 20 cm) of UV radiation do not appear to increase the inhibition of spore germination. Autoactivator substances added to the UV irradiated spores overrides the effect, and spores germinate normally (Fig. 16).

Supernatants derived from UV irradiated spores are only minimally stimulatory to subsequent spore germination (data not shown). In contrast, supernatants from the non-irradiated spores are highly stimulatory. This observation may suggest that irradiation of spores inter-
Figure 16. Effect of ultraviolet irradiation on auto-activation. All data points represent percent spore germination vs time in hours after harvesting of spores or end of activation treatment. (△) SG1 spores at 1 x 10⁷/ml; (▲) SG spores at 1 x 10⁷/ml, UV irradiated for 2 min at 20 cm lamp height; (⊙) SG1 spores, UV irradiated and heat shocked at 45°C for 30 min; (●) SG1 spores, UV irradiated and treated with SG1 autoactivator preparation.
fere with the synthesis or release of autoactivator factors. However, low concentrations of the factors synthesized or already present despite the UV irradiation might be sufficient to induce at least partial spore germination. The response of spores to autoactivator factors is not affected by UV; rather, the autocatalysis of germination appears to be affected.

The observation that UV irradiation affects autoactivation and release of autoactivator factors suggests that RNA synthesis is required for the process. Ostensibly, DNA within the spore is damaged to the extent that it can no longer serve as a template for RNA synthesis. One must, however, consider this data to be equivocal. While the primary photoproducts of UV irradiation are thymine dimers (Beukers and Berends, 1961; Setlow, 1966), one must also consider that other pyrimidine dimers, strand breaks, and damage to bases also occurs. These other lesions are not necessarily specific to DNA, but rather can also occur in RNA. Thus, while the data appear to show that RNA synthesis is involved in autoactivation, no concrete evidence exists. In summary, the process of autoactivation of D. discoideum spore germination appears to require protein synthesis, and may or may not also require RNA synthesis.

Deactivating conditions

Various other treatments inhibit autoactivation of
mutant spores. Many of these treatments induce "deactivating conditions" that render even heat activated spores incapable of germination (Cotter et al., 1979). Thus, it is not surprising that some of these treatments interfere with autoactivation (the effects of these treatments on heat activated mutant spores will be considered below).

Mutant spores treated with high osmotic pressure induced by 0.25 M sucrose fail to germinate. Addition of autoactivator substances does not override this inhibition. Spores washed from the sucrose solution or sucrose plus autoactivator solution and resuspended in phosphate buffer germinate normally and to high percentages (data not shown).

Mutant spores treated with the temperature extremes of 0 C and 37 C fail to autoactivate. Upon release from the 37 C treatment, SG spores germinate to high percentages (37 C treatment is in itself an activation treatment—see below). Upon release from 0 C treatment after 24 h, SG spores germinate to high percentages but require a somewhat longer lag period. Autoactivator substances added to spores at either temperature extreme fail to induce spore germination, but spores germinate rapidly upon return to the permissive temperature of 23.5 C (data not shown). Mutant spores treated at 50 C for 30 min fail to autoactivate even after 5 h at 23.5 C
regardless of whether autoactivator substances are added to the suspension (data not shown). This observation is consistent with earlier data (Cotter and Raper, 1968a) which showed that 50°C for 30 min is lethal to the majority of the spore population.

Spores are also temporarily inhibited from autoactivating by cyanide. This drug is an inhibitor of the mitochondrial electron transport system. In the presence of 2 x 10^{-3} M cyanide, SG1 spores autoactivate to only 1.5% after 5 h (Table 3). However, after 18 h spores have germinated to high percentages. Autoactivation is not completely inhibited by cyanide, but rather is delayed. Germination in the presence of cyanide appears to be normal, since viable-appearing myxamoebae emerge from swollen spores. Autoactivator substances added to cyanide treated strain SG1 spores stimulate spore germination. Addition of autoactivator factors results in 48% germination after 5 h (Table 3). Heat activation of cyanide treated SG1 spores appears to result in a slight stimulation of spore germination. However, this germination is probably due to autoactivation rather than response to the heat treatment, since cyanide causes deactivation of exogenously activated wild type spores (Cotter et al., 1979 and Table 5).

Oxygen uptake data are consistent with the above observations. Spores treated with cyanide show an
Table 3. Effects of cyanide and SHAM on *D. discoideum* strain SG1 spore germination

<table>
<thead>
<tr>
<th>Treatment</th>
<th>oxygen uptake</th>
<th>% germination at 5 h</th>
<th>% germination at 18 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>17.90</td>
<td>98.5</td>
<td>---</td>
</tr>
<tr>
<td>CN</td>
<td>8.05</td>
<td>1.5</td>
<td>90.0^c</td>
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<tr>
<td>SHAM</td>
<td>18.84</td>
<td>10.5^d</td>
<td>34.0^d</td>
</tr>
<tr>
<td>CN + SHAM</td>
<td>3.45</td>
<td>0.0</td>
<td>32.0^d</td>
</tr>
<tr>
<td>AA</td>
<td>59.67</td>
<td>99.0</td>
<td>---</td>
</tr>
<tr>
<td>AA + CN</td>
<td>14.10</td>
<td>48.0</td>
<td>80.0^c</td>
</tr>
<tr>
<td>AA + SHAM</td>
<td>30.77</td>
<td>79.0^d</td>
<td>79.0^d</td>
</tr>
<tr>
<td>AA + CN + SHAM</td>
<td>3.60</td>
<td>0.0</td>
<td>82.0^d</td>
</tr>
<tr>
<td>HS</td>
<td>---</td>
<td>97.5</td>
<td>---</td>
</tr>
<tr>
<td>HS + CN</td>
<td>---</td>
<td>8.5</td>
<td>91.5</td>
</tr>
<tr>
<td>HS + SHAM</td>
<td>---</td>
<td>89.0^d</td>
<td>94.0^d</td>
</tr>
<tr>
<td>HS + CN + SHAM</td>
<td>---</td>
<td>0.0</td>
<td>83.0</td>
</tr>
</tbody>
</table>

^a Abbreviations: CN = treatment with 2 x 10^{-3} M cyanide; SHAM = treatment with 5 x 10^{-3} M salicylhydroxamic acid; AA = addition of SG1 autoactivator preparation; HS = heat activation at 45° C for 30 min.

^b Oxygen uptake is expressed as ul O₂/h/1 x 10⁷ spores.

^c Estimated germination due to clumping of myxamoebae.

^d Emergence of myxamoebae inhibited.
approximately 50% reduction in oxygen consumption. Treatment of spores with autoactivator factors markedly stimulates oxygen uptake in untreated spores. Addition of the factors to spores treated with cyanide results in an increase in oxygen uptake to levels approaching that of the controls (Table 3).

Salicylhydroxamic acid (SHAM) is also inhibitory to strain SG1 autoactivation. SHAM is an inhibitor of the cyanide insensitive alternate oxidase pathway. Treatment of spores with SHAM differs from cyanide in that amoebae do not emerge from swollen spores, suggesting that the drug is cytotoxic. SHAM does not affect oxygen uptake by autoactivating spores (Table 3).

Addition of autoactivator factors to SG1 spores treated with SHAM stimulates germination. However, amoebae still fail to emerge from swollen spores. SHAM inhibits the autoactivator-induced increase in oxygen consumption by about 50%. Thus, it is possible that at least part of the increase in oxygen uptake by spores treated with the autoactivator factors is attributable to SHAM sensitive respiration. SHAM alone does not inhibit the swelling of heat activated SG1 spores, but appears to delay it somewhat. This observation is consistent with that reported by Cotter et al., (1979). SHAM does not induce spore deactivation and reduces oxygen uptake by only 18%.
Treatment of SG1 spores with both cyanide and SHAM also results in an inhibition of autoactivation and oxygen consumption (Table 3). The oxygen uptake under these conditions is probably attributable to use by the Clark type oxygen electrodes. Although no spore germination is detected after 5 h, spores swell to some extent within 18 h. This germination after 18 h may be due to loss of the potassium cyanide as cyanic acid vapors.

Spores of strain SG2 respond in a similar manner to treatment with cyanide and/or SHAM (Table 4). While there are several differences in the extent of responses to inhibitory conditions, the results are consistent. That is, cyanide and SHAM both delay autoactivation, but this delay is partially overcome by the addition of autoactivator substances or heat activation.

*D. discoideum* strain NC4 spores show no germination after 5 h regardless of whether they are treated with cyanide and/or SHAM (Table 5). This is consistent with their being constitutively dormant. After 18 h some autoactivation of untreated spores has occurred, and this autoactivation is sensitive to cyanide and SHAM. Strain NC4 spores treated with autoactivator factors respond to treatments in a manner similar to the mutant spores. Wild type spores activated by the application of a heat shock respond as reported by Cotter et al., (1979). That is, spores are deactivated by cyanide, but
Table 4. Effects of cyanide and SHAM on D. discoideum strain SG2 spore germination

<table>
<thead>
<tr>
<th>Treatment\textsuperscript{a}</th>
<th>% germination at 5 h</th>
<th>% germination at 18 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>96.0</td>
<td>--</td>
</tr>
<tr>
<td>CN</td>
<td>0.0</td>
<td>60.5\textsuperscript{c}</td>
</tr>
<tr>
<td>SHAM</td>
<td>2.0\textsuperscript{b}</td>
<td>26.0\textsuperscript{b}</td>
</tr>
<tr>
<td>CN + SHAM</td>
<td>0.0</td>
<td>1.5\textsuperscript{b}</td>
</tr>
<tr>
<td>AA</td>
<td>99.5</td>
<td>--</td>
</tr>
<tr>
<td>AA + CN</td>
<td>14.5</td>
<td>55.0\textsuperscript{c}</td>
</tr>
<tr>
<td>AA + SHAM\textsuperscript{b}</td>
<td>94.0\textsuperscript{b}</td>
<td>--</td>
</tr>
<tr>
<td>AA + CN + SHAM</td>
<td>0.0</td>
<td>24.5\textsuperscript{b}</td>
</tr>
<tr>
<td>HS</td>
<td>99.0</td>
<td>--</td>
</tr>
<tr>
<td>HS + CN</td>
<td>14.0</td>
<td>62.0\textsuperscript{c}</td>
</tr>
<tr>
<td>HS + SHAM</td>
<td>85.0\textsuperscript{b}</td>
<td>--</td>
</tr>
<tr>
<td>HS + CN + SHAM</td>
<td>0.0</td>
<td>12.0\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Abbreviations: CN = treatment with 2 x 10\textsuperscript{-3} M cyanide; SHAM = treatment with 5 x 10\textsuperscript{-3} M salicylhydroxamic acid; AA = addition of SG1 autoactivator preparation; HS = heat activation at 45°C for 30 min.

\textsuperscript{b}Emergence of myxamoebae inhibited.

\textsuperscript{c}Estimated germination due to clumping of myxamoebae.
Table 5. Effects of cyanide and SHAM on D. discoideum strain NC4 spore germination

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>oxygen uptake</th>
<th>% germination at 5 h</th>
<th>% germination at 18 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>---</td>
<td>0.0</td>
<td>64.0c,d</td>
</tr>
<tr>
<td>CN</td>
<td>6.59</td>
<td>0.0</td>
<td>2.0</td>
</tr>
<tr>
<td>SHAM</td>
<td>---</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CN + SHAM</td>
<td>1.63</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>AA</td>
<td>---</td>
<td>92.0</td>
<td>---</td>
</tr>
<tr>
<td>AA + CN</td>
<td>14.24</td>
<td>1.0</td>
<td>43.0d</td>
</tr>
<tr>
<td>AA + SHAM</td>
<td>---</td>
<td>45.0e</td>
<td>52.0e</td>
</tr>
<tr>
<td>AA + CN + SHAM</td>
<td>2.12</td>
<td>0.0</td>
<td>2.0e</td>
</tr>
<tr>
<td>HS</td>
<td>---</td>
<td>96.0</td>
<td>---</td>
</tr>
<tr>
<td>HS + CN</td>
<td>---</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>HS + SHAM</td>
<td>---</td>
<td>69.0e</td>
<td>---</td>
</tr>
<tr>
<td>HS + CN + SHAM</td>
<td>---</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

aAbbreviations: CN = treatment with $2 \times 10^{-3}$ M cyanide; SHAM = treatment with $5 \times 10^{-3}$ M salicylhydroxamic acid; AA = addition of SG2 autoactivator preparation; HS = heat activation at 45°C for 30 min.

boxygen uptake is expressed as ul O₂/h/1 x 10⁷ spores, and was measured during the first 5 h only.

cspores were 5 d old and showed some age induced autoactivation between 5 and 18 h.

destimated due to clumping of myxamoebae.

eemergence of myxamoebae inhibited.
SHAM alone merely slows germination.

Several bits of information may be gleaned from the data in Tables 3-5. Spores are capable of consuming oxygen and autoactivating despite treatment with 2 x $10^{-3}$ M cyanide. This germination is extremely slow and asynchronous. The process appears to occur normally and viable amoebae emerge from the swollen spores. The spores are apparently obtaining the energy necessary for spore germination from a source other than oxidative phosphorylation by the usual electron transport system. Treatment of the spores with 5 x $10^{-3}$ M SHAM largely abolishes the ability of spores to consume oxygen in the presence of cyanide. It therefore appears that the SHAM sensitive pathway produces the energy necessary for spore germination. SHAM itself is inhibitory to autoactivation, although it does not seem to affect oxygen consumption in the absence of cyanide. The failure of myxamoebae to emerge from SHAM treated spores suggests that the compound is cytotoxic to vegetative cells. Spores which germinate are killed, but SHAM only delays spore germination.

Treatment of cyanide treated spores with autoactivator factors results in a stimulation of germination and an increase in oxygen consumption. The stimulation in oxygen consumption is not observed when spores are also treated with SHAM. Thus, some oxygen consumed results
from a stimulation of the SHAM sensitive pathway by the autoactivator substances. In the absence of cyanide and presence of SHAM, autoactivators stimulate cyanide sensitive respiration. Thus, the autoactivator factors are capable of stimulating both cyanide sensitive and SHAM sensitive respiratory pathways. In the presence of both cyanide and SHAM, no increase in oxygen consumption is observed when spores are treated with the autoactivators.

4. Exogenous Activation of Mutant and Wild Type

Dictyostelium discoideum Spores

a. Lowered activation requirements

Dictyostelium discoideum spores may be activated to germinate by the application of a heat shock. Optimal thermal activation occurs after treatment at 45°C for 30 min (Cotter and Raper, 1966, 1968; Cotter, 1975). Such a treatment results in rapid and synchronous germination of the constitutively dormant spores. Apparently all D. discoideum strains are activated well with this

1 This dissertation deals largely with the phenomenon of autoactivation of D. discoideum spore germination rather than activation in general. The data in this section are presented only in the interest of characterizing the phenotypes of the mutant strains, not characterizing exogenous activation. Therefore, some details are not exhaustively pursued.
Table 6. Activation conditions for mutant and wild type Dictyostelium discoideum spores

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SG1</th>
<th>Strain SG2</th>
<th>NC4</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 °C, 30 min</td>
<td>99.5</td>
<td>99.0</td>
<td>99.5</td>
</tr>
<tr>
<td>37 °C, 60 min</td>
<td>63.0</td>
<td>13.5</td>
<td>0.0</td>
</tr>
<tr>
<td>37 °C, 30 min</td>
<td>53.5</td>
<td>4.5</td>
<td>0.0</td>
</tr>
<tr>
<td>40 °C, 60 min</td>
<td>---</td>
<td>80.5</td>
<td>0.0</td>
</tr>
<tr>
<td>40 °C, 30 min</td>
<td>---</td>
<td>53.0</td>
<td>0.0</td>
</tr>
<tr>
<td>no treatment</td>
<td>9.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*aAll spores were treated with the activation regimen in the presence of 100 μg/ml cycloheximide to inhibit autoactivation. Values represent percent spore germination after 5 h.*
Figure 17. Lowered activation requirements of strain SG2 spores. Panel A: (□) SG2 spores treated at 45°C for 30 min; (▲) SG2 spores treated at 40°C for 60 min; (△) SG2 spores treated at 40°C for 30 min; (○) NC4 spores treated at 40°C for 60 min; all data points represent percent germination vs time in hours after the end of the activation treatment. Panel B: (○) percent spore germination at 5 h vs time at 40°C. All spores were treated with 100 µg/ml cycloheximide.
37°C for 30 min in Table 6).

Spore activation at lower temperatures was studied in the presence of 100 μg/ml cycloheximide to inhibit autoactivation of spore germination. Mutant spores activated at 37°C for 1 h in the absence of cycloheximide germinate to high percentages and release autoactivator factors. Such germination is largely attributable to autoactivation rather than exogenous activation.

It should be noted that mutant spores activated at the lower temperature regimens have a very short lag time prior to the onset of spore swelling (Fig. 17). This observation is consistent with earlier reports that the length of the lag phase is proportional to the severity of the activation treatment (Cotter and George, 1975; Cotter et al., 1976). For instance, wild type spores maintained at 45°C for 60 min have a longer lag period than those heated for 30 min. Similarly, spores activated by "milder" treatments such as 20% DMSO for 60 min have shorter lag times (Cotter et al., 1976). Therefore, the shortened lag times for mutant spores activated at lower temperatures is not surprising.

Evidence that this is exogenous activation of spores rather than a stimulation of autoactivation results from several observations. Firstly, mutant spores activated at the lower temperatures germinate independently of spore density. Thus, spores at $1 \times 10^6$/ml and $1 \times 10^7$/ml
germinate with similar kinetics. Secondly, activation at the lower temperatures occurs despite the presence of 100 μg/ml cycloheximide. It may be recalled that autoactivation is sensitive to cycloheximide while exogenous activation is not affected by the drug. Thirdly, autoactivator factors are not released from mutant spores activated at the lower temperatures. Spores activated to germinate at 45°C have never been observed to release autoactivator factors, while no instance has ever been noted in which spontaneously germinating spores have failed to release the factors. Finally, the percent spore germination in the presence of cycloheximide is proportional to the duration of the activation treatment (Fig. 17). Therefore, spore germination after treatment at the lower temperatures results from exogenous activation rather than from a stimulation of autoactivation. In general, it can be concluded that mutant spores have lowered activation requirements.

b. Sensitivity to deactivating conditions

Wild type D. discoideum strain NC4 spores which have been exogenously activated at 45°C for 30 min or DMSO treatment may be deactivated by a variety of treatments. Such treatments include temperature extremes, pH extremes, high osmotic pressure, low oxygen tension, and autoinhibitors (Cotter and Raper, 1968b; Cotter et al.
1976; Cotter, 1977; Cotter et al., 1979). Spores generally return to the dormant state within 5-6 h after the application of the deactivating treatment (Cotter et al., 1979).

Mutant spores appear to be insensitive to some of these deactivating treatments. For instance, whereas wild type spores are completely deactivated by treatment with 0.25 M sucrose, SG1 spores germinate well despite the presence of sucrose (Fig. 18). The mutant spores are insensitive to the high osmotic pressure induced by the sucrose and germinate at a rate only slightly lower than spores in the absence of sucrose. Note that this germination is not due to autoactivation, since spores fail to spontaneously germinate in the presence of sucrose.

Mutant spores are also relatively insensitive to deactivating conditions within the intact sporocarps. Spores can be activated in situ by placing petri dishes containing upright fruiting bodies in a humidified oven at 45 °C for 30 min (Cotter and Raper, 1968b). The extent of spore activation and the rate of deactivation are determined by removing spores from the fruiting bodies at various times and incubating the washed spores in phosphate buffer. When spores are immediately removed from freshly heated plates, the average germination of mutant spores is 99%, while wild type spores germinate to 86.5%. Activated wild type spores return
Figure 18. Insensitivity of D. discoideum strain SG1 spores to deactivating conditions. All data are plotted as percent spore germination vs time in hours after the end of the activation or deactivation treatment. Panel A: (○) strain NC4 spores activated at 45°C for 30 min; (□) same, in the presence of 0.25 M sucrose; (●) SG1 spores activated at 45°C for 30 min; (■) same, in the presence of 0.25 M sucrose; Panel B: (○) strain NC4 spores activated in situ and removed from fruiting bodies after 5 h; (●) SG1 spores, same treatment.
to the dormant state if not washed free of autoinhibitors and osmotically active materials within the sorocarps (Cotter and Raper, 1968b). Thus, no germination of wild type strain NC4 spores is detected under such conditions (Fig. 18). In contrast, SG1 spores germinate to approximately 20% within the sorocarps when activated in situ (Fig. 18). When SG1 spores are washed free of autoinhibitors and osmotically active materials after 6h, an additional 40% of the spores germinate. Thus, mutant spores are relatively insensitive to the deactivating conditions of high osmotic pressure and the presence of autoinhibitors. Strain SG2 spores also germinate within the intact sorocarps if incubated for 5 d at a high relative humidity, as discussed above.

Data on the effects of cyanide and SHAM on exogenously activated and spontaneously germinating spores has been presented above (Tables 3-5). Note that mutant spores still germinate, although at greatly diminished rates, in the presence of cyanide, while wild type spores are completely deactivated.

In general, mutant spores are relatively insensitive to deactivating conditions imposed by osmotic pressure, autoinhibitors, and respiratory poisons. With spores germinating in the presence of cyanide, it appears that while the exogenous activation mechanism is deactivated, spores still autoactivate despite the inhibition
of respiration. In the case of osmotic pressure, spores simply fail to deactivate. The effect of heat activation in situ is probably more complex. Some spores germinate within the sorocarps, probably in response to the heat treatments, while the spores germinating after being washed free of the autoinhibitors and osmotically active compounds probably do so by the autoactivation mechanism.

c. Release of autoinhibitors

*Dictyostelium discoideum* spores activated to germinate by the application of a heat shock do not release autoactivator substances, as noted above. Instead, supernatants in which exogenously activated spores have germinated are inhibitory to subsequent spore germination. Inhibitory materials are released by both mutant and wild type spores activated by a heat shock (Fig. 19). Inhibitory material is released rather asynchronously throughout the later portions of spore swelling (Fig. 20) rather than during early swelling. Autoactivator substances are released from spontaneously germinating spores during early swelling (Fig. 13). Note that spores which have not been heat activated also release inhibitory substances, but at a reduced rate (Fig. 20).

The inhibitory material released from heat activated *D. discoideum* spores may be discadenine, the autoinhibitor of spore germination (Abe et al., 1976). This material
Figure 19. Release of inhibitor from spores activated by heat shock. All data points represent percent spore germination vs time in hours after harvesting. (△) SG1 spores at 1 x 10^6/ml; (○) SG1 spores in an activated supernatant derived from SG1 spores; (■) SG1 spores in a solution in which NC4 spores have germinated after heat shock at 45°C for 30 min; (●) SG1 spores in a solution in which SG1 spores have germinated after heat shock at 45°C for 30 min.
Figure 20: Time of release of inhibitors from heat activated NC4 spores. (△) percent spore swelling vs time in hours after end of treatment at 45°C for 30 min; (▲) percent emergence of myxamoeba; (○) percent germination at 5 h of SG1 spores incubated in aliquots of the spore suspension vs time of sampling; (●) percent germination of SG1 spores incubated in aliquots of a spore suspension that was not subjected to heat activation vs time of sampling.
elutes after the internal volumes of Biogel and Sephadex gel filtration columns (data not shown). The inhibition observed could not be due to osmotic effects, since osmotically active materials are released into the supernatant during the early swelling stage of spore germination (Hohl et al., 1978), not late swelling.

Some inhibitory material is also released by spontaneously germinating spores. The inhibitor released is not detected in crude autoactivator preparations, presumably because the autoactivator factors exert a more powerful effect. This inhibitory material elutes from a Biogel P2 column at the same position as the inhibitory substance released from heat activated spores (data not shown).

5. Characterization of *Dictyostelium discoideum* Autoactivator Factors
   a. General information

Autoactivator substances resulting from the spontaneous germination of *D. discoideum* spores are unstable in crude form. That is, crude activated supernatants rapidly lose their ability to stimulate spore germination upon incubation at room temperature. The autoactivators are relatively stable when frozen, but lose activity upon repeated freezing and thawing. Boiling of the crude autoactivator preparations yields a 40% loss in
activity after 10 min, but flash evaporation at 35°C results in an almost complete loss of activity. Lyophilized preparations are comparatively stable if the preparations remain dry. Ethanol extraction of the crude lyophilized preparations also results in an increased stability of the materials. Thus, it appears that an enzyme is present in the crude preparations which results in an inactivation of the autoactivator factors. Denaturation of the enzyme with heat or ethanol results in increased stability of the factors, while freezing and lyophilization reduce the enzyme activity. Conditions allowing enzyme activity (room temperature or 35°C) result in a rapid loss of activity.

Autoactivator factors are soluble in 80% ethanol, suggesting that the substances are small non-polymeric molecules. Extraction in 80% ethanol is normally incorporated as a preliminary purification procedure to rid the preparations of large proteins and other macromolecules. Autoactivator substances appear to be non-dialyzable. Attempts to dialyze crude preparations yielded no activity outside the dialysis bags and no apparent increase in activity inside the bags. Since further work demonstrated that the factors show affinity for a number of substances, it is believed that the factors bind to the dialysis membranes. Thus, no estimate of molecular weight is available from dialysis experiments,
and the procedure is unsuitable as a purification step.

b. Chromatography of autoactivator substances
Autoactivator factors are readily chromatographed by standard laboratory techniques. Routinely, activated supernatants are lyophilized to dryness to yield a yellow-brown powder. Crude autoactivator factors are extracted twice with 80% ethanol. Ethanol is evaporated away at 45°C and the preparation is lyophilized to dryness. One gram of crude autoactivator preparation yields approximately 75 mg of ethanol soluble material. Ethanol soluble material is taken up in distilled water to 100 mg/ml, and 1 ml is applied to a 3 x 55 cm Biogel P2 column. The column is eluted with distilled water at a flow rate of ca. 40 ml/h with a head pressure of 20 cm. Fractions (200 drops = 8 ml) are mechanically collected until twice the internal volume (Vi) has eluted (ca. 50 fractions). Each fraction is tested for absorbance at 260 and 280 nm, and tested for autoactivator activity (see MATERIALS AND METHODS). A typical elution profile of the SG1 autoactivator preparation is presented in Fig. 21.

SG1 autoactivator preparations are fractionated into three major peaks of activity. Peak A corresponds to a peak of UV absorbance and elutes at the void volume (Vo) of the column. Material eluting in peak A is highly stimulatory to strain SG2 spores inhibited from germin-
Figure 21. Fractionation of SG1 autoactivator preparation
100 mg of 80% ethanol soluble material from
a crude SG1 autoactivator preparation were
fractionated through a 3 x 55 cm Biogel P2
column as described in MATERIALS AND METHODS
and the text. Top panel shows absorbance of
each fraction at 260 and 280 nm; lower panel,
upper tracing shows response of SG2 spores
inhibiting from autoactivating by 100 ug/ml
cycloheximide to each fraction; lower panel;
lower tracing shows response of strain NC4
spores to each fraction.
ating by cycloheximide, but shows minimal stimulation of strain NC4 spores. The ability of peak A material to stimulate strain NC4 spores is somewhat variable. Peak B elutes at or slightly after the internal volume of the column. It is associated with minimal UV absorbance. The peak C material elutes well after the internal volume of the column and appears to be associated with a peak of UV absorbance at 260 nm, with little at 280 nm. Both peaks B and C are highly stimulatory to cycloheximide treated SG2 spores, and moderately stimulatory to NC4 spores.

Peak B and C materials appear to interact with the Biogel P2 column. Thus, these materials elute from the column at a position after small molecules such as salts. Therefore, no estimate of molecular weight is available from this procedure. A similar interaction of the auto-activator substances occurs when they are fractionated on Sephadex columns. This characteristic of late elution due to interactions with the column is not uncommon when fractionating aromatic or heterocyclic compounds on Biogel or Sephadex columns under conditions of low ionic strength (Anonymous, 1971; Anonymous, 1975). For instance under conditions normally employed for chromatography, adenine, thymine, and various plant hormones (kinetins, auxins, etc) also elute after the internal volume (data not shown). The characteristic interaction with the
column is apparently due to the presence of a few carboxyl groups on the acrylamide (Biogel) or dextran (Sephadex) polymers (Anonymous, 1971; Anonymous, 1975). Compounds with a positive charge may interact with these carboxyl residues and be retarded on the columns. Both Sephadex and Biogel product instructions suggest that these ionic interactions may be avoided by eluting the columns with buffers with an ionic strength in excess of 20 mM. However, little or no difference in elution profiles of the autoactivator peaks was observed when the column was eluted with 20 mM phosphate buffer, pH 6.5. While it may be possible to prevent this retardation by further adjusting the ionic strength or pH of the eluent, this was not attempted. *D. discoideum* spore germination is sensitive to high osmotic pressure and alterations in pH (Cotter et al., 1979). Any attempt to detect the presence of the autoactivators by bioassay may be befuddled by such suboptimal conditions. Additionally, the late elution characteristic proves useful in purifying the factors, so elution with distilled water was normally employed.

Peak C autoactivator substance was chosen for further investigation for several reasons. Firstly, it is more accessible in purified form; that is, it is free from many contaminants within the internal volume of the column. Secondly, it appears to be purine-like in that
it elutes from the column near adenine and related purine bases. It may be recalled that discadenine, the autoinhibitor of *D. discoideum* spore germination, is a substituted adenine molecule (Abe et al., 1976). Thirdly, of the three peaks of autoactivator activity, peak C alone is unique to the activated supernatants. That is, peak C material is absent from dormant spores and vegetative amoebae (see below).

Peak C material from several fractionation runs was pooled and refractionated through the Biogel P2 column. This material represented approximately 20 $A_{260}$ units. The material was then concentrated to 1 ml, applied to a Sephadex LH-20 column (1 x 20 cm), and eluted with distilled water. Normally, 50 1.5 ml fractions were collected. Distilled water was chosen as the eluent since results were satisfactory, and water was more convenient than use of 80% or absolute ethanol or absolute methanol. A typical Sephadex LH-20 elution profile of SG1 autoactivator peak C is presented in Fig. 22. Note that the peak of activity is totally separated from the $A_{260}$ absorbing material, and elutes after the internal volume of the column. The peak of activity is not associated with any ultraviolet absorbance, and could not be shown to absorb UV even after active fractions were pooled and lyophilized to a small volume. When the tube containing the active material was lyophilized to dryness
Figure 22. Fractionation of SG1 autoactivator peak C material on Sephadex LH-20 (1. x 20 cm). Top panel shows absorbance of each fraction at 260 and 280 nm; lower panel shows percent germination response of strain NC4 spores to each fraction.
and visually inspected, no residue was observed.

It is possible to estimate the effective concentration of autoactivator peak C material if one accepts several assumptions. While these assumptions may not be valid, it allows at least an approximate estimation of concentrations. One may assume that the molecule is not unlike discadenine, with a molecular weight of about 300 daltons and a molar absorptivity coefficient at 260 nm of $17.5 \times 10^3$. In such a case, 1.0 $A_{260}$ units equals $5.7 \times 10^{-5}$ M. Absorption at 260 nm of less than 0.01 units would suggest that the effective concentration of the substances is less than $5-6 \times 10^{-7}$ M. This concentration takes into account 100% activity in purified form, but does not attempt to estimate the initial effective concentration in the activated supernatant. More than 2 l of activated supernatant was initially used for this fractionation run (several hundred petri dishes containing ca $5 \times 10^7$ spores/petri dish). It is apparent that the autoactivator peak C material is effective at minuscule concentrations.

Autoactivator factors derived from spontaneous germination of aged, wild type, strain NC4 spores appears to be identical to that from strain SG1. Fractionation of 80% ethanol extracts of the NC4 autoactivator preparation shows three peaks of activity eluting from the Biogel P2 column at the same positions as those from strain SG1 (Fig. 23). No differences between the preparations are
Figure 23. Fractionation of strain NC4\textsuperscript{/} autoactivator preparation. 100 mg of 80\% ethanol soluble material from a crude NC4 autoactivator preparation was fractionated through a 3 x 55 cm Biogel P2 column. Top panel shows absorbance of each fraction at 260 and 280 nm; lower panel, upper tracing shows response of SG2 spores inhibited from autoactivating by 100 \textmu g/ml cycloheximide to each fraction; lower panel, lower tracing shows response of strain NC4 spores to each fraction.
notable. Since strain SG1 is derived from strain NC4, and aging appears to confer the mutant phenotype on NC4 spores, it has been assumed that the substances are identical. Combining of autoactivator peak C materials from SG1 and aged NC4 preparations and refractionating on Biogel P2 or Sephadex LH-20 yields single peaks of activity (data not shown).

The autoactivator preparations from spontaneous germination of strain SG2 is similar to that from strain SG1. Peak A and B materials elute at the same positions, but autoactivator peak C activity is absent (Fig. 24). The $A_{260}$ material coeluting with SG1 autoactivator peak C is present in the SG2 preparation but does not inspire spore germination. It is likely that the lack of peak C material in SG2 autoactivator preparations represents strain specific differences. It should be recalled that strain SG2 is derived from D. discoideum strain V12, which is the opposite mating type from strains NC4 and SG1. No attempt was made to exhaustively analyze the SG2 autoactivator preparation.

c. Presence or absence of the autoactivator substances in dormant spores and amoebae

Attempts were made to determine if autoactivator factors are present at any other point in the D. discoideum life cycle. Therefore, dormant spores and vegetative amoebae were examined for the presence of autoactivators.
Figure 24. Fractionation of strain SG2 autoactivator preparation. 100 mg of 80% ethanol soluble material from a crude SG2 autoactivator preparation was fractionated through a 3 x 55 cm Biogel P2 column. Top panel shows absorbance of each fraction at 260 and 280 nm; lower panel shows percent germination response of strain NC4 spores to each fraction.
Myxamoebae from strains SG1, SG2, and NC4 were grown in association with *E. coli* B/r on glucose-salts agar plates. Amoebae were washed from the plates during logarithmic growth with phosphate buffer, washed three times, and resuspended in fresh phosphate buffer. Amoebae were either disrupted immediately by vortexing in the presence of glass beads (Van Etten and Freer, 1978) or incubated at 23.5°C overnight. Following incubation, amoebae were pelleted, and the supernatant was used for further investigation. In each case, suspensions were lyophilized to dryness, resuspended in distilled water, and fractionated on a 3 x 55 cm Biogel P2 column.

Spores were tested for the presence of autoactivators by a similar procedure. Fresh SG1, SG2, and NC4, and aged NC4 spores were harvested from sori with glass slides. Spores were pelleted, and the supernatants were removed and saved as "autoinhibitor preparations" (contents of the sori, including autoinhibitors, exclusive of spores). Spores were washed in phosphate buffer as usual, and disrupted with glass beads. These preparations or "spore extracts" were centrifuged to remove debris. Spore extracts and autoinhibitor preparations were lyophilized to dryness and fractionated on a Biogel P2 column. All fractions were tested for UV absorbance and autoactivator activity as usual. Results from the above-described investigations are summarized in Table 7.
Table 7. Presence or absence of autoactivator factors in vegetative amoebae, dormant spores, and sori of strains SG1, SG2, and fresh and aged strain NC4

<table>
<thead>
<tr>
<th>Preparation a</th>
<th>Autoactivator factor b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>SG1 autoactivator preparation</td>
<td>+</td>
</tr>
<tr>
<td>SG2 autoactivator preparation</td>
<td>+</td>
</tr>
<tr>
<td>NC4 autoactivator preparation</td>
<td>+</td>
</tr>
<tr>
<td>SG1 amoebae</td>
<td>+</td>
</tr>
<tr>
<td>SG2 amoebae</td>
<td>+</td>
</tr>
<tr>
<td>NC4 amoebae</td>
<td>+</td>
</tr>
<tr>
<td>SG1 autoinhibitor preparation</td>
<td>+</td>
</tr>
<tr>
<td>SG2 autoinhibitor preparation</td>
<td>+</td>
</tr>
<tr>
<td>fresh NC4 autoinhibitor preparation</td>
<td>+</td>
</tr>
<tr>
<td>aged NC4 autoinhibitor preparation</td>
<td>+</td>
</tr>
<tr>
<td>SG1 spore extract</td>
<td>-</td>
</tr>
<tr>
<td>SG2 spore extract</td>
<td>-</td>
</tr>
<tr>
<td>fresh NC4 spore extract</td>
<td>+</td>
</tr>
<tr>
<td>aged NC4 spore extract</td>
<td>-</td>
</tr>
</tbody>
</table>

a see text for details.

b "+" indicates the presence of that component in the preparation, "-" indicates the absence of that component.
What is clearly apparent from Table 7 is that autoactivator factors A and B are commonly associated with life cycle stages other than spore germination. In contrast, autoactivator factor C is present only after spore germination and is absent or undetectable in vegetative amoebae and dormant spores. These data suggest that of all the factors, it is factor C which is uniquely associated with spore germination.

The situation with factors A and B is more complex than their simple presence during all stages of the life cycle. These factors are apparently absent from dormant spores of strains SG1, SG2, and aged NC4. They are present in the sorocarps of strains SG1, SG2, and aged NC4, however. The lack of factors A and B in dormant spores which are capable of autoactivating may be explained by differences in spore permeability. As will be discussed in the DISCUSSION section of this dissertation, one hypothesis regarding the mutant phenotype suggests that mutant spores are more permeable than wild type spores. It is conceivable then that autoactivator factors are present in dormant spores with the autoactivator phenotype, but that they readily leak out of the spores during washing.

The one inescapable conclusion from these results is that autoactivator factors A and B are common cellular components: Factor A, which elutes at the void
volume of the Biogel P2 column, may be a peptide that is soluble in 80% ethanol but is above the 1800 dalton exclusion limit of the column. Factor B may be another small peptide which interacts with the column by virtue of an aromatic amino acid residue. One may recall that several amino acids are stimulatory to *D. discoideum* spore germination under other conditions (Cotter and Raper, 1966). Inclusion of tryptophan, phenylalanine, and/or methionine into a small peptide may alter the characteristics of stimulation of spore germination.

d. Radiolabelling experiments

Various attempts have been made to identify the autoactivator factors, particularly factor C. This component is of interest, as noted above, because it is unique to the activated supernatants. Initial attempts to identify factor C spectroscopically proved to be impractical due to the minuscule concentrations of this material. The amount of factor C from several hundred petri dishes was undetectable by ultraviolet spectroscopy. A similar problem had been encountered by groups attempting to identify the *D. discoideum* spore germination inhibitor. The first reproducibly successful identification of this molecule required the use of 20,000 petri dishes to yield 15 mg of the pure substance (Abe et al., 1976). A similar attempt at large scale culture was not attempted for practical and economic reasons. Thus,
other avenues of approach to the problem were needed.

One method of approaching the problem was the use of radioisotopes. Incorporation of a radiolabel into a compound is of value for at least two major reasons. Firstly, it allows one to conveniently monitor the presence of the compound under various chromatographic conditions. Detection of an isotope by liquid scintillation is less subject to extremes of pH and osmotic pressure than by bioassay. Secondly, the nature of the precursors incorporated into the molecule provides some insight into the nature of the molecule. For these reasons, attempts were made to incorporate a variety of \(^{14}\text{C}\)-containing substances into autoactivator factor C. The procedures used are outlined in the MATERIALS AND METHODS section of this dissertation.

Attempts were made to incorporate the following radioactive precursors into SG1 autoactivator factor C: \([8-^{14}\text{C}]\) 5'AMP, \([^{14}\text{C}]\) glycine, \([^{14}\text{C}-\text{U}]\) algal protein hydrolysate, \([^{14}\text{C}-\text{U}]\) glucose, and \([^{14}\text{O}]\) \(\text{NaHCO}_3\). At no time has incorporation of any radioactive precursor into SG1 autoactivator C been detected. Radiolabelled 5'AMP, algal protein hydrolysate, glycine, and glucose were incorporated into various components of the spores, but never into factor C. Sodium bicarbonate was incorporated into spores poorly. To prevent the bicarbonate from being released as \(\text{CO}_2\), it was necessary to increase the pH of the glucose-salts agar above the normal 6.5.
At pH 7.5 the sorocarps were only sparsely produced; those which did form were obviously aberrant. At pH 7.2 sorocarps appeared to be normal, but very little $^{14}$C was incorporated into spore material. For this experiment, petri dishes were incubated in a moist chamber fitted with a CO$_2$ trap. During the growth and sporulation of the myxamoebae, much of the CO$_2$ released was collected in this trap. Final calculations revealed that greater than 90% of the label detected was released as $^{14}$CO$_2$. Thus, bicarbonate is a poor choice as a precursor for the radiolabelling of D. discoideum cellular components.

Two possible explanations could account for the failure to detect incorporation of radiolabel into autoactivator factor C. The first, that factor C is inorganic, is implausible. One would not expect an inorganic molecule to exhibit the lability observed for the autoactivator preparations. The second possibility, that autoactivator factor C is present in undetectably low amounts, is more reasonable. The possibility exists that so little material is present that radioactive incorporation is below background levels. It should also be borne in mind that the period of radiolabelling is one in which differentiation (sporulation) is occurring. Thus, many stalk cell and spore components are being synthesized. Much of the radiolabel added to the cells.
undoubtedly is incorporated into other such components.

e. Attempts to mimic autoactivator activity with exogenously applied compounds

During the course of this investigation, a variety of compounds were tested against *D. discoideum* spores in an attempt to mimic the activity of the authentic autoactivator factors. The reasoning behind the addition of each of these compounds ranged from a careful consideration of the biological functions of the compounds to a fervent desire to achieve results through pure luck. For instance, a variety of plant hormones were tested for activity since they have important roles in regulating dormancy, germination, and general metabolic activity in higher plants. Additionally, the cytokinins have been implicated in the regulation of *D. discoideum* spore dormancy in that dicadenine has cytokinin activity (Tanaka et al., 1975; Nomura et al., 1977). Some other compounds were tested without regard for their possible biological or molecular significance.

Table 8 lists a number of compounds which were tested for autoactivator activity but which failed to elicit a germination response. Undoubtedly many more compounds could be added to this list but are omitted. It should be sufficient to comment that a large number of substances were tested, but none mimicked the effect of substances released by spontaneously germinating *D.
Table 8. A list of substances which do not mimic the activity of autoactivator factors

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Diguanosine tetraphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casamino acids</td>
<td>Zeatin</td>
</tr>
<tr>
<td>Nutrient broth</td>
<td>6-furfurylaminopurine</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>6-benzylpurine</td>
</tr>
<tr>
<td>Biotin</td>
<td>Indole-3-acetic acid</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>Gibberellic acid (GA3)</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>Folic acid</td>
<td>Isopentenyl adenine</td>
</tr>
<tr>
<td>Inositol</td>
<td>Isopentenyl adenosine</td>
</tr>
<tr>
<td>Niacin</td>
<td>Adenine</td>
</tr>
<tr>
<td>P-aminobenzoic acid</td>
<td>Thymine</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>Guanine</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>Uracil</td>
</tr>
<tr>
<td>Thiamine</td>
<td>Pronase</td>
</tr>
<tr>
<td>Boric acid</td>
<td>Cellulolysin</td>
</tr>
<tr>
<td>NAD+</td>
<td>Macerase</td>
</tr>
<tr>
<td>NADH+H+</td>
<td>Cellulase II</td>
</tr>
<tr>
<td>Succinate</td>
<td>Phosphate</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>Molybdenum</td>
</tr>
<tr>
<td>Maleate</td>
<td>Zinc</td>
</tr>
<tr>
<td>cAMP</td>
<td>Sulfate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Manganese</td>
</tr>
</tbody>
</table>
It should be borne in mind that many of the compounds listed in Table 8 can stimulate D. discoideum spore germination under specifically defined conditions. For instance, amino acids, purines, and pyrimidines can activate strain NC4 spores (Cotter and Raper, 1968c). However, activation by these compounds is at rather high concentrations (10 mM each, tryptophan, phenylalanine, and methionine, or 5 mg/ml adenine or thymine). Additionally, such activation requires that spores be maintained at very low densities (fewer than $1 \times 10^6$ /ml) on a solid agar substrate. Response to autoactivator factors, in contrast, occurs independently of spore density and proceeds quite well in liquid suspensions.

Many of the compounds listed in Table 8 also result in a stimulation of autoactivation. For instance, addition of amino acids results in an acceleration in the rate of spontaneous germination. However, these compounds are not themselves autoactivator factors since they fail to override the inhibition of autoactivation caused by cycloheximide, and fail to activate strain NC4 spores under the conditions tested. Thus, while a number of substances affect the rate of spontaneous germination, none are authentic autoactivator factors.
f. Autoactivators from other sources

Compounds stimulatory to *D. discoideum* spore germination have been detected from sources other than autoactivating spores. For instance, Hashimoto et al. (1976) reported that *Aerobacter aerogenes* (sic *K. pneumoniae*) cells release a ninhydrin-negative "spore germination promoter" (SGP) during logarithmic growth. *D. discoideum* spores incubated in the presence of the SGP in a proteose peptone medium germinate synchronously and to high percentages. The SGP in the absence of peptone, however, does not inspire spore germination. Hashimoto et al. (1976) also examined *E. coli* cells for the production of a similar SGP, but could not detect any activity. This group speculated that long term coculturing of their *D. discoideum* strain NC4 resulted in an insensitivity of the spores to respond to an *E. coli* SGP. Such *E. aerogenes*-grown strains also grow poorly on *E. coli*.

In contrast to that reported by Hashimoto et al. (1976), results from this laboratory indicate that a number of bacterial species produce substances which stimulate *D. discoideum* spore germination. For instance, *E. coli* B/r produces several substances which stimulate *D. discoideum* spores (Fig. 25). One of these substances elutes from a Biogel P2 column at the same position as autoactivator factor A, while several substances elute in the same area as factor C. None of these substances
Figure 25. Substances stimulatory to *D. discoideum* spore germination released by *E. coli*. 
*E. coli* cells were grown in 500 ml of a glucose salts minimal medium for 72 h at 30 C. Cells were removed by centrifugation, and the supernatant was treated as a super-
from *D. discoideum* spore germination. Upper tracing shows absorbances at 260 and 280 nm of each fraction; lower tracing shows percent germination response of strain NC4 spores to each fraction.
is identical to peak C material since they do not coelute with factor C on a Sephadex LH-20 column (data not shown).

The substances released by logarithmically growing *E. coli* cells are probably not the same SGP substances reported by Hashimoto et al. (1976). These activator factors do not require the presence of peptone to inspire spore germination, as does the SGP. The possibility exists that the SGP substances merely potentiate the characteristics of peptone or amino acid induced spore activation rather than be activating substances themselves. The alternative is that the substances detected by Hashimoto et al. (1976) are similar to the factors noted above, but display a much reduced potency in activating *D. discoideum* spores. The substances isolated from *E. coli* may themselves be spore activators not unlike the *D. discoideum* autoactivator factors.

A number of bacteria produce activating factors of one kind or another. For instance, *Enterobacter aerogenes*, *Myxococcus xanthus*, *Micrococcus luteus*, and *Pseudomonas aeruginosa* also release spore activating substances (Fig. 26). *E. coli*, *E. aerogenes*, and *M. xanthus* cells produce a number of substances which are similar to autoactivator factor C in that they elute from a Biogel P2 column after the internal volume under the conditions used. In contrast, *M. luteus* produces
Figure 26. Release by bacteria of substances capable of activating *D. discoideum* spores. Panel A: substances released by *Pseudomonas aeruginosa* growing in nutrient broth; Panel B: substances released by *Micrococcus luteus* growing in glucose-salts broth fortified with peptone; Panel C: substances released by *Enterobacter aerogenes* growing in glucose-salts broth; Panel D: substances released by *Myxococcus xanthus* growing on CT medium. None of the media themselves contain components capable of activating *D. discoideum* spores.
only a single substance corresponding to factor A. The production of autoactivator factor C-like materials may correspond to the ability of *D. discoideum* amoebae to grow well upon the bacteria. Depratière and Darmon (1978) have shown that *D. discoideum* myxamoebae grow less well upon *Sarcina lutea* (*Micrococcus luteus*) than on other bacteria such as *E. coli* and *E. aerogenes*. Similarly, *Pseudomonas aeruginosa* produces no type C material, and also does not support the growth of *D. discoideum* amoebae (Fig. 26).

In general then, a variety of bacteria produce substances which are capable of stimulating *D. discoideum* spore germination. While it is unlikely that these substances are identical to the *D. discoideum* autoactivator factors, they may be structural analogs or similar compounds. The presence of these factors also suggests a mechanism of *D. discoideum* spore germination in nature. This subject will be further discussed below.
DISCUSSION

It should be pointed out at the immediate outset of this discussion that the phenomenon of autoactivation of *Dictyostelium discoideum* spore germination is not a bizarre consequence of mutagenesis. It is a comparatively "natural" process that was first noticed as a result of spontaneous mutations. While the spontaneous germinator mutants are phenotypically altered, the mutations affect only the timing of spore maturation. Autoactivator mutants do nothing which the wild type strains are incapable of; they simply do it without the need for a long (1-2 week) maturation period. This is in sharp contrast with earlier attempts to induce *D. discoideum* spore mutants with mutagens such as nitrosoguanidine (Ennis and Sussman, 1975). Such mutants are often grossly defective, and may even fail to germinate. The fact that the SC mutants arose spontaneously in response to mild selective pressures suggests that they are not grossly distorted, and probably do not possess multiple genetic defects.

Since autoactivation of *D. discoideum* spore germination is a natural process, that is, wild type spores are capable of germinating in this manner, this discussion can be subdivided into two main categories. The first will consider the general characteristics of autoactivation. The mechanisms of autoactivation will be considered
in this category insofar as can be concluded from this preliminary analysis of the phenomenon. The second section of this discussion will consider the process of *D. discoideum* spore germination in nature. Sufficient evidence has accumulated that educated speculations can be made about how spores behave in their natural habitats.

1. Characteristics of Autoactivation

   Autoactivation of *D. discoideum* spore germination, whether in mutant or aged wild type spores, is a process that requires no exogenous trigger. Spores suspended in phosphate buffer at pH 6.5 or even in distilled water with the pH maintained near neutrality, germinate rapidly, synchronously, and to high percentages. The morphological changes undergone by autoactivating spores are identical to those observed in spores activated by a heat shock at 45°C for 30 min. Both autoactivating and exogenously activated spores initially enter a lag period during which no morphological changes are evident. Following the lag period, spores swell and become non-refractile under phase contrast microscopy. After a suitable period, a single myxamoeba emerges from each swollen spore. Even the time course of the series of events is similar. Spores from mutant strains SG1 and SG2 begin to germinate (swell) at approximately the same time as heat activated SG1, SG2, or NC4 spores. Aged wild type spores differ in that longer lag periods are
required prior to the beginning of spontaneous spore swelling. Thus, from all outward appearances, the characteristics of spore germination following autoactivation and exogenous activation are identical. However, it is apparent that the processes leading to spore germination are not identical, and may not, in fact, even be similar.

Autoactivation proceeds in the absence of an exogenous activation treatment. The rates and final percentages of spore germination are dependent on spore density within the suspensions. Spores at a density of $1 \times 10^7$/ml germinate synchronously and to high percentages, while spores at $1 \times 10^6$/ml often germinate poorly. This positive density dependence suggests the existence of germination stimulants. Autoactivator factors are detectable in suspensions in which spores have spontaneously germinated at $1 \times 10^7$/ml. These factors markedly stimulate spore germination in all $D. discoideum$ strains tested, and even show some stimulatory activity to spores from other $Dictyostelium$ species. These autoactivator substances are released during the early swelling stage of spontaneous spore germination.

$D. discoideum$ spontaneous spore germination appears to occur by autocatalysis. A few spores in the population autoactivate and release the stimulatory factors. These factors in suspension may then in turn stimulate other spores in the suspension. Thus, only a fraction of the
population may initiate the autoactivation process, but other spores respond and relay the signal. The entire population is activated upon the instigation of a fraction of the group. The long lag period prior to the beginning of autoactivation of aged wild type spores may indicate that several hours of permissive conditions are required before the first spores in the population are capable of autoactivating. Once this fraction begins to germinate, the remainder of the population follows suit.

This characteristic response to a stimulus followed by a signal relay is not unprecedented in *D. discoideum*. A similar system is in operation in organizing the aggregation of myxamoebae. Initially, a few myxamoebae under starvation conditions begin to pulse cAMP (Loomis, 1975). Other amoebae in the vicinity respond to the signal, and shortly begin to generate a signal of their own. While the aggregation phenomenon is complex, it does illustrate that a similar autocatalytic relay system does exist in other stages of the *D. discoideum* life cycle.

Essentially all freshly harvested *D. discoideum* spores respond to the autoactivator signal. Mutant spores and wild type spores freshly harvested from the fruiting bodies are dramatically stimulated by the activated supernatants. Mutant spores and aged wild type spores retain the ability to respond to the autoactivator
factors even after they have remained in suspension for several hours. Unaged strain NC4 spores, however, lose the ability to respond to the factors after about one hour after removal from the sorus, indicating the existence of a competence period. The major point to be made from the finding that aged wild type spores have lost the competence period is that aging of spores confers the mutant phenotype on wild type spores. Aged strain NC4 spores behave in a manner identical in many ways to strain SG1 spores, including similar drug sensitivities and release of apparently identical autoactivator factors.

It is therefore concluded that mutant strain SG1 autoactivation is the same process that occurs in aged strain NC4 spores, differing only in the length of the lag period. Thus, the mutations inducing the SG phenotype may affect the "temporal programming" of D. discoideum. Similar mutations have been detected in other stages of the D. discoideum life cycle (Sonneborn et al., 1963).

In contrast to the interactions between autoactivating spores, exogenously activated spores are independently activated (Cotter and Raper, 1968b) and germinate equally well at $1 \times 10^6$/ml and $1 \times 10^7$/ml. The only density effect on exogenously activated spores is that caused by low oxygen tension at very high spore densities (Cotter and Raper, 1968b).

A number of other characteristics additionally
distinguish autoactivation from exogenous activation. Spontaneously germinating spores apparently require protein synthesis, since germination is inhibited by cycloheximide and edeine. In contrast, spores activated by a heat shock or DMSO treatment swell normally in the presence of these drugs (Cotter and Raper, 1970; Bacon and Sussman, 1973; Yagura and Iwabuchi, 1976; Giri and Ennis, 1977). RNA synthesis is apparently not required for exogenous spore activation (swelling) (Cotter and Raper, 1970; Yagura and Iwabuchi, 1976), though others have argued otherwise (Giri and Ennis, 1977). The requirement for RNA synthesis for autoactivation is similarly inconclusive. A number of drugs and treatments which are known to inhibit RNA synthesis also inhibit autoactivation; however, in most cases it is not possible to conclude that the treatments are specific for RNA synthesis inhibition. For instance, thiolutin, daunomycin, 4NQO, and UV irradiation are inhibitory to autoactivation. These treatments may also disturb the integrity of the RNAs already present in the spore, however, and therefore effectively block protein synthesis. Detailed studies of RNA synthesis will be required to conclusively demonstrate whether the process is required for autoactivation of *D. discoideum* spore germination.

One notable result of studies of the effects of
various RNA and protein synthesis inhibitors is the observation that in many cases, exogenous activation treatments or addition of autoactivator factors override the inhibition. The effect of heat activation on inhibited spores is not surprising, since protein and RNA syntheses are apparently not required for exogenously activated spore swelling. The overriding of inhibition afforded by the autoactivator factors suggests that protein and perhaps RNA syntheses are required for the synthesis or release of the factors. In the case of cycloheximide inhibition of autoactivation, addition of the autoactivator factors markedly stimulates spore swelling. The need for protein synthesis required for the synthesis of the factors is obviated by the addition of the factors. Cycloheximide added to spore suspensions up to 30 min before swelling prevents germination. One may conclude from this datum that a protein is synthesized at this time which is responsible for the autoactivation process. Two observations suggest that the loss of cycloheximide sensitivity is not due to the synthesis of the autoactivators themselves. Firstly, autoactivator substances are apparently non-proteinaceous. Secondly, they are released during early spore swelling, not 30 min before.

Autoactivation of D. discoideum spore germination is sensitive to a number of treatments. For instance,
high osmotic pressure induced by 0.25 M sucrose, and
temperature extremes of 0 and 37°C prevent spontaneous
spore germination. Spores germinate following their
return to permissive conditions. Spores treated at 0°C
for 24 h germinate slowly upon being returned to 23.5°C,
while those at 37°C for 60 min. show an increased rate of
germination. Treatment at 37°C exogenously activates
mutant spores (see below). That these treatments are
inhibitory to autoactivation is not surprising, since
they also induce deactivation of exogenously activated
wild type spores (Cotter et al., 1979).

Autoactivation of D. discoideum spore germination
occurs slowly despite the presence of \(2 \times 10^{-3}\) M
cyanide. While the process is dramatically slowed, it
still occurs to relatively high percentages within
about 18 h. Viable myxamoebae emerge from swollen spores.
Spores apparently derive energy from some source other
than oxidative phosphorylation by the usual electron
transport system. Additional evidence for this results
from the observation that spores consume some oxygen
despite the cyanide. Addition of autoactivator factors
stimulates spore germination and increases oxygen
consumption by the spores.

The effect of salicylhydroxamic acid (SHAM) on
spores treated with cyanide suggests that spores derive
some energy as a result of the SHAM sensitive alternate
oxidase system. Treatment of spores with SHAM plus cyanide almost completely prevents oxygen uptake, and reduces the percentage of spores germinating. Addition of autoactivator factors does not stimulate oxygen consumption under these conditions. SHAM alone delays spontaneous spore swelling, which is consistent with earlier observations (Cotter et al., 1979). The drug is apparently toxic, since myxamoebae fail to emerge from swollen spores in the presence of SHAM.

Autoactivator substances generally stimulate oxygen uptake by autoactivating spores. The stimulation is observed in all cases except when spores are treated with cyanide plus SHAM. While an attractive explanation for this stimulus would be to suggest that the autoactivator factors directly stimulate spore respiration, such a suggestion is premature. The possibility exists that autoactivators stimulate some aspect of spore metabolism, and the resulting metabolic fluxes result in an apparent increase in respiration. The nature and function of the SHAM sensitive alternate oxidase pathway is not well understood, so it is dangerous to speculate on its role in autoactivation. However, increased oxygen consumption could perhaps result from a stimulation of glycolysis and the TCA cycle. The resulting increase in $\text{NADH}^+$ levels may increase the flux through the electron transport or alternate oxidase systems to regenerate
NAD$^+$ and cause an increase in oxygen consumption. However, further investigations are required to understand the nature of the autoactivator-induced changes.

Spores from mutant strains SG1 and SG2 also differ from wild type strain NC4 spores in that their requirements for exogenous activation are reduced. Wild type spores are activated to germinate at 45°C for 30 min, but are only minimally activated at lower temperatures. This observation confirms earlier work (Cotter and Raper, 1968c). Mutant spores, in contrast, are partially activated after a treatment at 37°C for 60 min. Better activation occurs at slightly higher temperatures (39-40°C), at which activation of wild type spores is minimal. No autoactivator substances are released when mutant spores are activated at these lower temperature regimens. Wild type and mutant spores activated by thermal treatment release a substance not unlike discadenine (Abe et al., 1976) during the late swelling stage. This compound is no longer necessary for the maintenance of dormancy at this time, and is apparently excreted.

An additional difference between SG and wild type spores is that heat activated mutant spores are insensitive to some deactivating conditions. For instance, spores germinate despite the high osmotic pressure induced by 0.25 M sucrose. Spores may not be able to
detect the high osmotic pressure as a result of differences in spore permeability, as discussed below. In contrast, germination of wild type spores is inhibited by 0.25 M sucrose, and spores return to the dormant state (Cotter et al., 1979). Other treatments known to deactivate wild type spores, including 0 °C and treatment with cyanide apparently also deactivate the exogenously activated mutant spores. However, SG spores still autoactivate slowly in the presence of cyanide, and those relieved for 0 °C treatment after 24 h are still capable of autoactivation.

One conclusion that becomes increasingly apparent throughout the course of this discussion is that autoactivation and exogenous activation of D. discoideum spores are fundamentally different processes. Whereas autoactivation apparently requires macromolecular synthesis, exogenous activation of spores does not. While spontaneous germination is mediated by autoactivator substances, heat induced spores germinate independently of one another. Young wild type spores require harsh treatments to break dormancy, while mutant spores may be activated by milder treatments or can autoactivate in the absence of an activation treatment. Since autoactivation and exogenous activation are so different, one must consider what alterations are brought about by both aging and mutations, and how these alterations
result in the spontaneous germinator phenotype. Before considering these topics, however, it is necessary to define both autoactivation and exogenous activation:

**Exogenous activation of D. discoideum spores** is a process whereby spores are relieved from a state of constitutive dormancy by comparatively severe, externally applied treatments. Breakage of dormancy does not require macromolecular synthesis, and may involve a relaxation of restraints on oxidative phosphorylation.

This definition reiterates that *D. discoideum* spores are constitutively dormant according to the definitions of Sussman and Halvorson (1966). The restraint on oxidative phosphorylation by the electron transport system is likely to be the innate property of the spore which is ultimately responsible for spore dormancy. Evidence for this restriction of oxidative phosphorylation is based on the multi-state model for spore activation (Cotter, 1973) and related work. In contrast:

**Autoactivation of D. discoideum spore germination** is a process by which spores are relieved from a state of poised dormancy (cf.) by mechanisms inherent to the spore. The process requires macromolecular synthesis and is mediated by autoactivator factors released from spores as they germinate. The autoactivator factors stimulate additional spores to germinate, resulting in an autocatalysis of spore germination.

Poised dormancy is a reversible state of hypometabolism distinct from exogenous dormancy. It is special case of constitutive dormancy in that it is maintained by an innate property of the spore, but is reversed by a mechanism inherent to the spore.

Autoactivation occurs among spores in a state of
of hypometabolism here termed "poised dormancy" for want of a better name. Spores are not in a state of exogenous, or environmental, dormancy. Exogenous dormancy is maintained only by the lack of a critical component of the environment, such as water or nutrients (Sussman and Halvorson, 1966). When spores are provided with the necessary component, they germinate without the need for an activation treatment. Poised dormancy is a special state of constitutive dormancy which is distinguished by the fact that while an activation treatment is necessary to break dormancy, the treatment is provided by the spores themselves. Spores possess an inherent mechanism of activating themselves (autoactivation) when environmental conditions become favorable. Thus, they are in a state of dormancy which is poised to be broken under appropriate circumstances.

Spores of mutant strains SG1 and SG2 are continuously in a state of poised dormancy. Thus, they are capable of autoactivating at any time they are exposed to permissive conditions. In contrast, wild type spores are initially constitutively dormant, but gradually enter poised dormancy after aging. The alterations induced by spore aging are apparently the same, as those induced by mutations. The question arises, of course, of what these alterations are.

One possible alteration resulting from spore aging
and mutations is of spore permeability. Some evidence exists which suggests that mutant spores have altered permeability. For instance, mutant spores activated by heat shock are not inhibited by 0.25 M sucrose. They may simply fail to detect the osmotic pressure because sucrose penetrates the spores. Additionally, some auto-activator factors seem to readily leak from mutant and aged wild type spores, while they are retained by fresh wild type spores.

Differences in spore permeability could result from mutations. Single gene alterations are known to be capable of causing alterations in membrane integrity and composition. The genetic defects can simultaneously affect plasma and mitochondrial membranes (Rank et al., 1977). Spore permeability could also be affected by maturation. Membrane permeability differences have been implicated in aging of rat brain and liver cells (Zs.-Nagy, 1978). Additionally, a variety of enzymes are present in the sorocarp in the interstices between spores (Gezelius, 1972; Tisa and Cotter, personal communication). It is conceivable that enzymes present could affect the spore coat or plasma membrane and alter permeability. Maximal enzyme activity would be expected when the sorocarps are fully hydrated, such as occurs when they are incubated at a high relative humidity. In contrast, lower humidities could result in lowered
enzyme activities (Giese, 1973). Thus, these considerations could in part explain the variability observed in age induced autoactivation of strain NC4 spores, and variations in the density dependence of mutant spore autoactivation. Humid conditions such as experienced in summer months would promote rapid maturation of wild type spores, whereas drier conditions would retard spore aging. One could not discount the possibility that metabolism within the spores also contributes to alterations in spore permeability. Many dormant spores are not ametabolic, but rather are hypometabolic. For instance, dormant Rhizopus stolonifer spores incorporate $^{14}\text{C}$ into various spore components when incubated in an atmosphere of $^{14}\text{CO}_2$ for long periods (Van Etten, personal communication). Thus, slow metabolic processes could contribute to spore maturation, or entrance into poised dormancy.

The manner in which altered spore permeability could induce the spontaneous germination phenotype is unknown. Permeability differences have been implicated in the germination of several types of seeds and spores (Sussman, 1976). Furfural activation of Neurospora ascospores is mediated through membrane effects (Eilers et al., 1970a,b). Heat activation of D. discoideum results in changes in spore permeability and the distribution of membrane particles, and also causes a large
efflux of osmotically active materials from spores (Hohl et al., 1978). Thus, some association has been shown between spore permeability and release from dormancy. Exactly how these phenomena are associated is unknown, and the subject requires additional investigation.

An additional question to be considered is the nature and mode of action of the autoactivator substances. These subjects also require additional investigation, and only preliminary observations can be considered here. A distinct problem encountered in studies of the autoactivator factors is that they are present and function at minuscule concentrations. Factor C was chosen for further studies primarily because it is unique to spore germination. Factors A and B may be common cellular components, as noted previously. All attempts to identify autoactivator factor C were unsuccessful. Sufficient material was not available for spectroscopic analysis, no radioisotopic precursors could be incorporated into the factors, and its activity could not be mimicked by exogenously applied compounds. Several bacterial species release compounds which mimic the activity of autoactivator factor C. Some of these compounds may be structural analogs. Therefore, future efforts to identify the stimulatory substances may be devoted to investigations of the bacterial factors, since the bacteria can be grown with less effort and expense than D. discoideum.
Autoactivator factor C may be a small aromatic compound. Its elution characteristics on Biogel and Sephadex gel filtration columns are similar to those of purines and pyrimidines. Additionally, *D. discoideum* is notorious for its use of purines to regulate various portions of its life cycle. For instance, cAMP organizes the aggregation of myxamoebae (Loomis, 1975; Newell, 1978), while discadenine is involved in the maintenance of spore dormancy (Abe et al., 1976). Beyond the speculation of its being a purine and noting its activity at very low concentrations, it is impossible to add any information about autoactivator factor C.

The mode of action of the autoactivator substances in inducing spore germination is also unknown. It can be stated that the factors result in an increase in respiration as measured by oxygen consumption. Additionally, the hyperbolic curve of autoactivator dilution versus percent spore germination suggests that multiple molecules of the substance are required to activate each spore. However, no further evidence is available on how the substances work. A full understanding of how the factors stimulate spore germination may not be available until the substances are identified.
2. Germination of *Dictyostelium discoideum* Spores in Nature

Although this investigation has been primarily laboratory oriented, efforts have been made to relate the results obtained to events in nature. While *D. discoideum* is a "laboratory animal" and is often considered only in that context, it also commonly resides in the soil and leaf litter of deciduous, subtropical forests (Raper, 1935). Studies of what an organism does in the laboratory are merely academic if the organism does not also perform the feat in nature. Therefore, the following discussions will attempt to relate the laboratory results to what may actually occur in nature.

Three distinct stimuli exist which could presumably trigger *D. discoideum* spore germination in nature: exogenous activation, response to substances released by bacteria, and autoactivation. Each of these activation treatments will be considered separately.

Exogenous activation of *D. discoideum* spores is one possible mechanism of spore germination in nature. Since slugs are positively phototactic (Loomis, 1975), sorocarps would tend to be present on the surface of the soil or leaf litter. The fragile sorocarps may be knocked over by wind, rain, or disturbances by insects or other denizens of the leaf litter. Once spores are released from the sorus, autoinhibitors and high osmotic
pressures are diluted by ground moisture. One can envision a variety of conditions in which spores may be activated. For instance, spores may be activated by exposure to high temperatures caused by direct sunlight. Temperatures in the soil and its microenvironment may rise to as high as 50°C when exposed to full midday sun, and dark soils may reach 70°C (Brook, 1970). Thus, thermal activation of spores is possible. Spores reaching a temperature of 45°C for 20-30 min germinate with a lag period of about 1 h when cooled to 23°C (Cotter and Raper, 1966). Even spores maintained at this higher temperature for longer periods germinate, but require a longer lag period (Cotter and George, 1975). One can envision that such thermal activation would occur most frequently in the late spring, when temperatures are warm and the deciduous forests have not yet developed a full leaf cover.

A number of other triggers for exogenous spore activation have been studied under laboratory conditions. Treatment with 20% DMSO or 8 M urea causes activation of D. discoideum spores (Cotter and O'Connell, 1976; Cotter, 1977). It seems unlikely, however, that spores would be exposed to these treatments in nature. These severe treatments apparently mimic the effects of thermal treatments in nature. Another harsh condition which might exogenously activate D. discoideum spores in nature
but which has not been studied is ingestion by insects, worms, or other animals. The low pH or abrasion within the intestinal tracts of such creatures may aid in relieving dormancy. Spores of the Myxomycete, Didymium sp., isolated from feces of the mite Tyrophagus putrescentiae have been shown to germinate normally (Keller and Smith, 1978), so ingestion is not necessarily synonymous with digestion.

A second possible trigger of D. discoideum spore germination is one that is intuitively obvious: spores germinate in response to a signal that sufficient nourishment is available to support vegetative growth. Thus, spores germinate in response to the presence of suitable bacterial prey. This phenomenon has been observed countless times in two membered cultures of D. discoideum with an appropriate bacterium. One would expect a similar phenomenon to occur in the soil. Spores respond to substances released by the common soil bacteria Enterobacter aerogenes, Pseudomonas aeruginosa, Myxococcus xanthus, and Micrococcus luteus, as well as Escherichia coli. Dictyostelium discoideum has apparently evolved the ability to detect and respond to metabolites or waste products released by bacteria. The organism can apparently also lose the ability to respond to some bacteria, since Hashimoto et al. (1976) reported that their D. discoideum strain NC4 fails to respond to
E. coli supernatants after long term coculture with E. aerogenes.

The most likely time of year for D. discoideum spores to respond to the presence of bacteria is in early summer to early fall. During these times the mild climatic conditions would favor rapid bacterial growth. The time required for D. discoideum aggregation and sporulation might allow repletion of bacterial populations in the soil. Exogenous activation of spores at these times is probably minimal due to blockage of sunlight by the leaf cover.

Autoactivation, the subject of this dissertation, is the third possible mechanism of D. discoideum spore germination in nature. Spores in the sorocarps or soil or leaf litter which are not exogenously activated by heat (sunlight) or bacterial products may autoactivate. One may consider it an act of desperation: spores which have remained dormant for 1-2 weeks without an activation treatment are obviously not in an environment conducive to growth. Sufficient moisture and mild temperatures are required for autoactivation so that the process will not occur in hostile environments. Thus, spores germinate en masse to reenter the vegetative state. The resulting myxamoebae may immediately aggregate to form a slug, which can then migrate in search of nourishment.

Several characteristics of autoactivation may be of
survival value to *D. discoideum* in nature. The density dependence of the process would insure that sufficient numbers of myxamoebae are present to form a slug. Also note that the rate of migration of the pseudoplasmodium is related to its size: larger slugs migrate faster and farther than smaller ones (Bonner et al., 1953). Slugs may migrate for days, covering tens of centimeters (Loomis, 1975). The 1-2 week lag period prior to auto-activation of wild type spores would insure that the germination process is not premature. The lack of nourishment in the environment is not transient, but rather is long term. Thus, there is survival value in migrating away from a depleted area, perhaps to another area where nourishment is available. At the very least the process may allow *D. discoideum* to resporulate in a position more favorable for future spore activation.

It must occur that at certain times of the year, spores present in the soil or leaf litter are not exposed to any of the above activation treatments. Perhaps sunlight is waning due to the approach of the autumnal equinox, or is shielded due to leaf litter or overhead leaf cover. Under these circumstances, spores would not be exogenously activated. Similarly, reduction of bacterial growth rates due to autumnal cooling would reduce spore activation by bacterial substances. Auto-activation would be delayed by slower maturation rates.
due to cooler temperatures. Under such conditions, spores would remain dormant in the soil and would overwinter. Töbler and Hohl (1977) have reported that 60-85% of the cellular slime mold clone forming units in the soil are attributable to spores, even in the spring and summer when myxamoebae are not killed by freezing. Thus, it is likely that slime molds overwinter as spores. Germination may occur in the spring by any of the mechanisms outlined above.
LITERATURE CITED


Setlow, R. B. 1966. Cyclobutane-type dimers in poly-

Smart, R. F. 1937. Influence of certain external
factors on spore germination in the Myxomycetes.

Snyder, H. M., III and C. Ceccarini. 1966. Interspec-
ific spore germination inhibitor in the cellular

A mutation affecting both rate and pattern of
morphogenesis in Dictyostelium discoideum.
Devel. Biol. 7:79-93.

Sussman, A. S. 1976. Activators of funal spor e germa-
(D. J. Weber and W. M. Hess, Eds.). John Wiley &

Sussman, A. S., and H. O. Halvorson. 1966. Spores:
Their Dormancy and Germination, Harper and Row,

Tada, M., and M. Tada. 1976. Main binding sites of the
carcinogen, 4-nitroquinoline-1-oxide in nucleic

of a carcinogen, 4-hydroxyaminoquinoline-1-oxide,
29:469-477.

Tanaka, Y., K. Yanagisawa, Y. Hashimoto, and M. Yamaguchi.
1974. True spore germination inhibitor of a
cellular slime mold, Dictyostelium discoideum.

Partial structure of a spore germination inhibitor
from a cellular slime mold Dictyostelium discoideum.

Tanaka, Y., H. Abe, M. Uchiyama, Y. Taya, and S.
Nishimura. 1978. Isopentyladenine from Dictyo-
stelium discoideum. Phytochemistry 17:543-544.

Taya, Y., Y. Tanaka, and S. Nishimura. 1978a. 5'AMP
is the direct precursor of cytokinin in Dictyos-


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