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AUTOACTIVATION OF SPORE GERMINATION IN THE

CELLULAR SLIME MOLD <u>DICTYOSTELIUM</u> <u>DISCOIDEUM</u>

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 complete-
ly 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 chromatographi-
cally 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 com-
ponent 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 auto-
activator 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

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INTRODUCTION

The cellular slime mold <u>Dictyostelium discoideum</u> 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 eucary-
otic development. The organism has simple growth
requirements, is easily manipulated in the laboratory,
and has a brief (about 4 d) life cycle. Of more import-
ance, perhaps, is the fact that <u>D. discoideum</u> is a
primitive eucaryote which is capable of complex cellu-
lar interactions, chemotaxis, and the development of
cellular receptors and contacts. Because of its relative
cellular simplicity and developmental patterns,
<u>Dictyostelium discoideum</u> is considered by some to be the
organism of choice for studies of differentiation of
the lower eucaryotes.

Some aspects of the <u>D. discoideum</u> life cycle have
been extensively studied. For example, aggregation of
<u>D. discoideum</u> myxamoebae and subsequent sporulation have
been investigated, and many of the molecular and biochem-
ical events during these developmental stages have been
elucidated (Loomis, 1975; Newell, 1978). Information

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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 germina-tion, 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; Depraitère 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 "undoubtably have a role to play in the economy of nature", this role is probably minor in relation to the significant activities of other micro-organisms. 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 inter-actions at a simple level. The *D. discoideum* life cycle

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is presented schematically in Fig. 1.

Dictyostelium discoideum myxamoebae grow by ingestion
of bacteria and divide by binary fission. Upon exhaus-
tion of the food supply, the myxamoebae aggregate via
cAMP-oriented streaming to form a multicellular pseudo-
plasmodium (slug, grex). Amoebae within the pseudoplas-
modium retain their individuality, but work in conjunction
with one another to form a primitive "tissue". The
pseudoplasmodium may migrate in search of additional
nourishment, or may immediately enter the culmination
stage of development. Pre-patterned cells within the
pseudoplasmodium 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 exhaus-
tively review the D. discoideum life cycle. The remainder
of this literature review and dissertation will specifi-
cally focus on D. discoideum spore dormancy and germina-
tion, with reference to autoactivation of spore germina-
tion. 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).
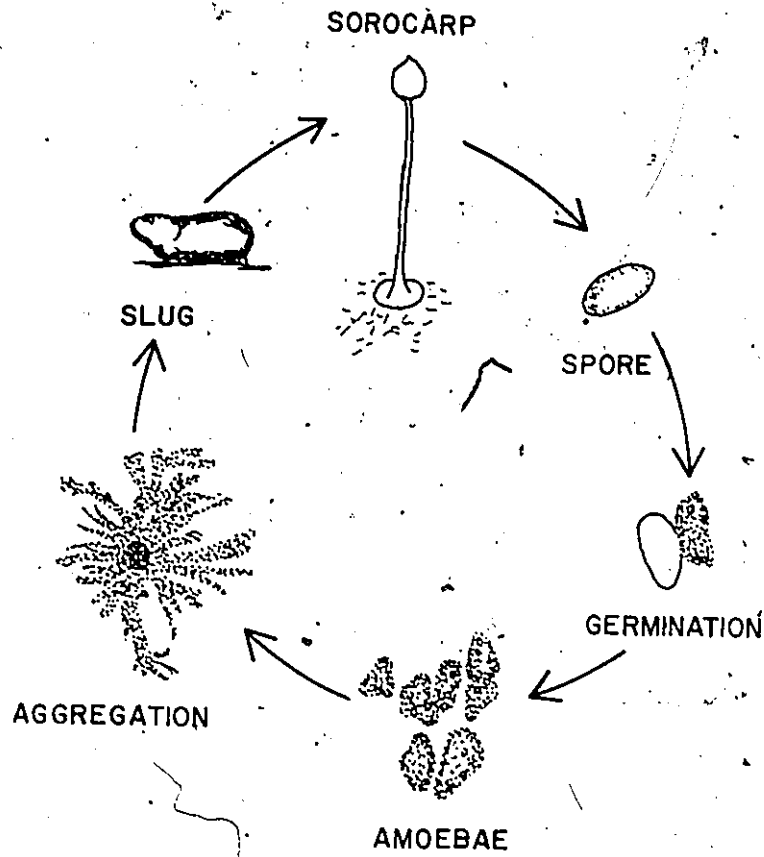
Figure 1. The life cycle of <u>Dictyostelium</u> <u>discoideum</u>
(Redrawn from Alexopoulos, 1962)

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 auto-inhibitors 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 ultra-violet 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 auto-inhibitor, other factors are also involved. For instance, spores induced to germinate by peptone do not germinate above 1×10^6 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. Katilus 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 culture 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 (Tanaka 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^6Ade) 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^6Ade 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).

5' AMP

isopentenyl pyrophosphate → → pyrophosphate

i^6 AMP

→ ribose phosphate

i^6 ADE

S-adenosyl-methionine → → 5' methylmercap-toadenosine

HN—CH$_2$—CH=C CH$_3$
                      CH$_3$

CH$_2$—CH$_2$—C—C
                   H   O
                   OH
              NH$_2$
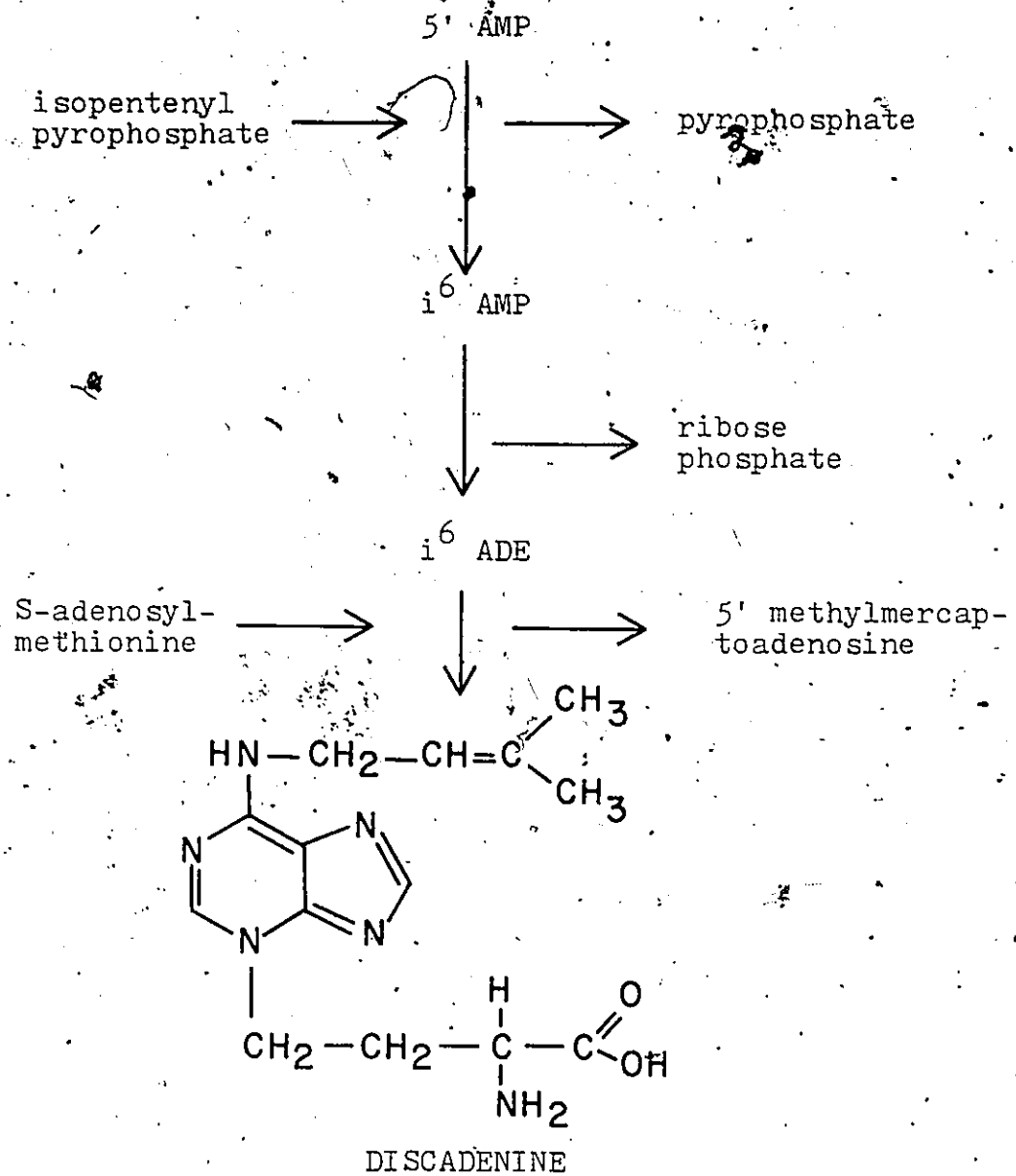
DISCADENINE

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 disc-
adenine 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 present-
ly 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 respir-
ation. 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, pig-
ments, carbohydrates (Loomis, 1975), and several enzymes,
including acid phosphatase (Gezelius, 1972), β -gluco-
sidase, 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 osmptically 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 vegeta-
tive growth.

It appears that the available evidence supports
Cotter's (1973a) hypothesis. Thermodynamic considera-
tions suggest that heat activation induces a partial
helix-coil transition in a protein in the inner mitochon-
drial membrane. Suggestions that the mitochondria are
involved in the activation process result from a theoret-
ical analysis showing that the number of activation sites
corresponds to the number of mitochondria in dormant
spores. Additionally, mitochondria are the first organ-
elles 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. Apparent-
ly, 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 tempera-
ture 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 funal 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 Dictystelium 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.

T   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.,
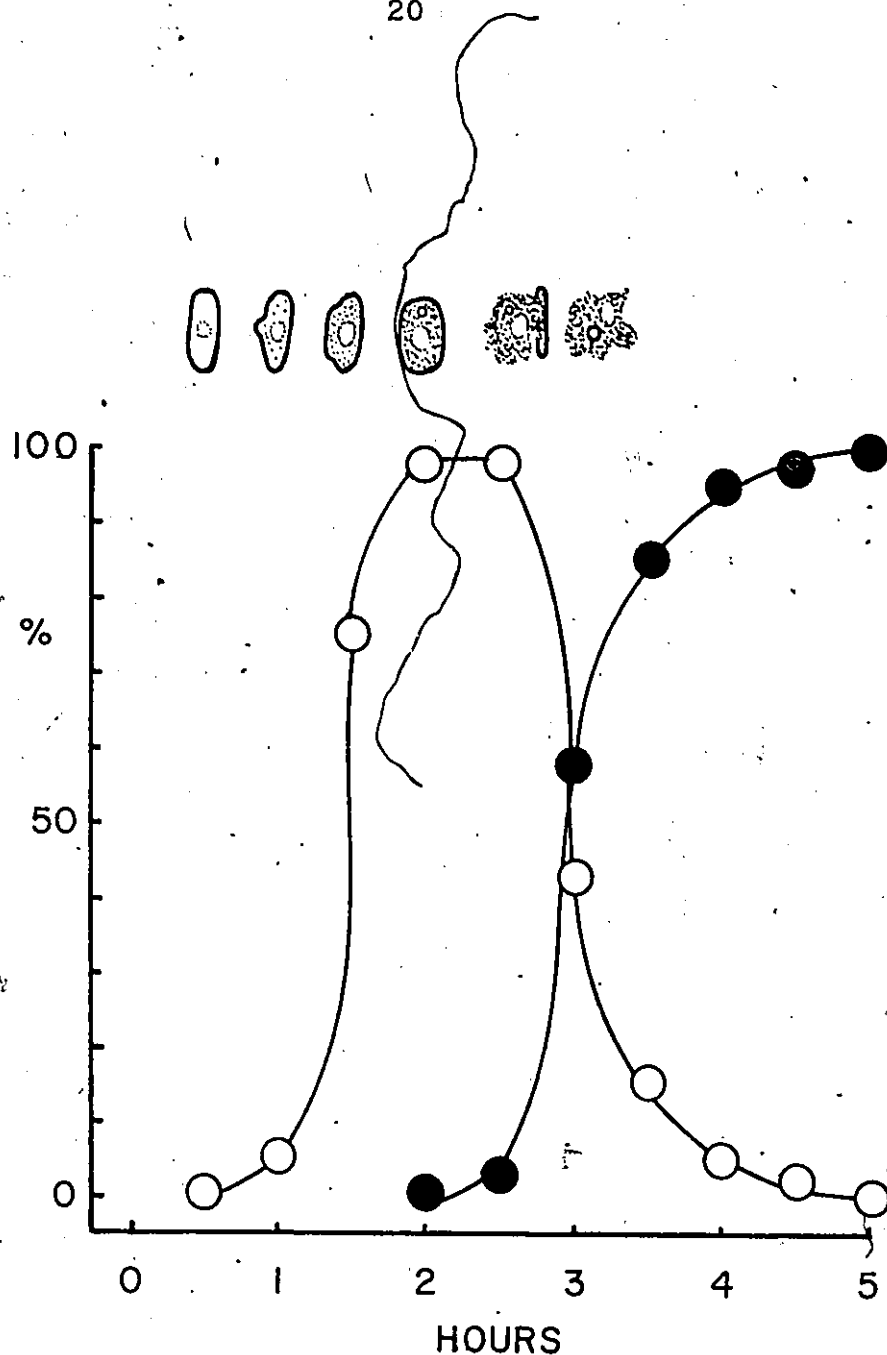
Figure 3. The germination sequence of <u>Dictyostelium</u>
<u>discoideum</u> spores heat activated at 45 C for
30 min. (redrawn from Cotter, 1975)
(O) percent spore swelling versus time in
hours; (●) percent emergence of myxamoebae
versus time in hours; morphological changes
are shown schematically above 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 cyclo-
heximide 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 actino-mycin 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, 1978) 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 <u>D. discoideum</u> 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 D. 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 Na_2HPO_4, 3.0 g KH_2PO_4, 1.0 g NH_4Cl, 0.13 g $MgSO_4$; 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 1×10^6 and 1×10^7/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

1×10^7/ml in 25 ml phosphate buffer in ehrlenmeyer 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 1×10^7 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 2×10^6 spores/ml and 200 ug/ml cycloheximide so that mixing with the test solutions brought the final spore concentration to 1×10^6/ml and cycloheximide to 100 ug/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 ®
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 phos-
phate 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 autoactiva-
tor (or autoinhibitor) activity as described above.
Purified substances to be tested for column elution char-
acteristics 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 ® 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 auto-activator 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 auto-activator activity, lyophilized to dryness, mixed with 75 mg of an 80% ethanol extract of unlabelled SG1 auto-activator 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 10^6/ml.
Oxygen consumption was recorded as a function of air
saturation (6.139 ul O_2/ml at 23.5) 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 h per 1 x 10^7 spores.

RESULTS

1. Isolation of <u>Dictyostelium discoideum</u> Autoactivator (Spontaneous Germinator) Mutants [1]

Freshly formed wild type (i.e., strains NC4 or V12) <u>Dictyostelium discoideum</u> 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, <u>D. discoideum</u> spores are normally sown on glucose-salts agar plates in association with <u>Eschericia coli</u> B/r. The glucose-salts medium contains no components capable of activating the spores. However, <u>E. coli</u> growing on the glucose-salts medium may release materials capable of activating the spores, including the amino acids trypto-phan, 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 difined in the DISCUSSION section of this disser-tation.
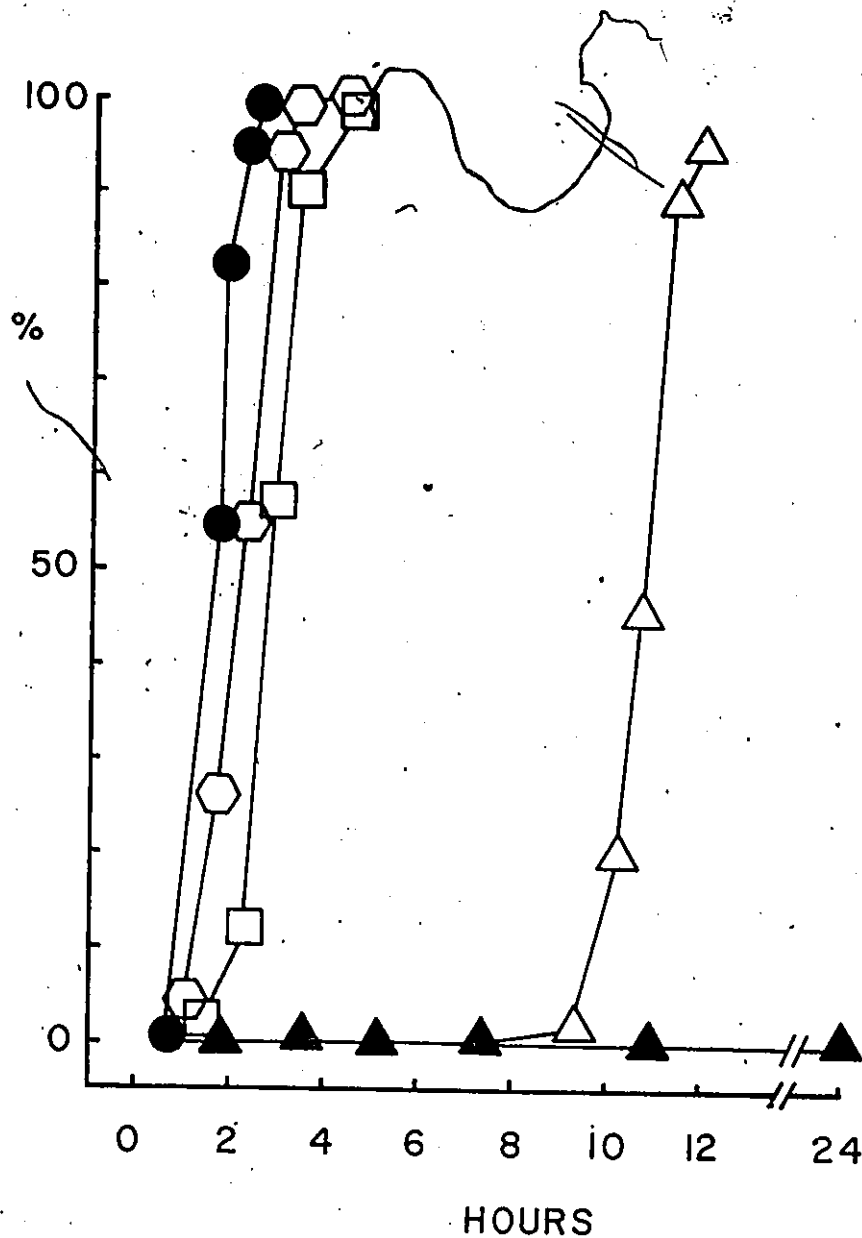
Figure 4. Dictyostelium discoideum spore germination. Percent spore germination is plotted vs time in hours. (▲) 2 d old strain NC4 spores, no activation treatment; (⬤) 2 d strain NC4 spores activated at 45 C for 30 min; (⬡) 2 d strain SG1 spores, no activation treatment; (□) 2 d strain SG2 spores, no 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 subpopulation 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,
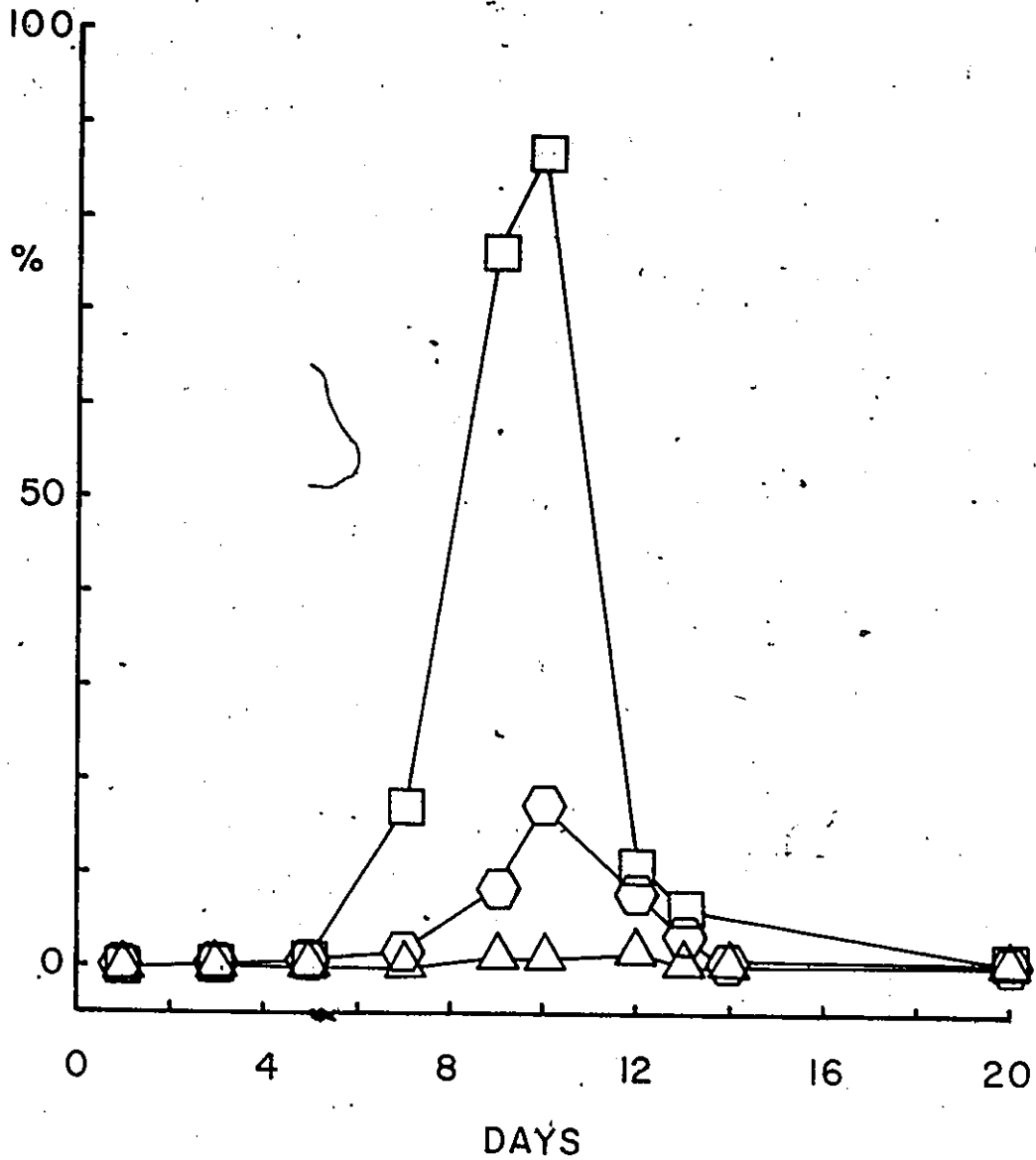
Figure 5. Age induced autoactivation of wild type
(strain NC4) <u>Dictyostelium</u> <u>discoideum</u> spores.
Percent spore germination at 4 h (△),
6 h (○), and 8 h (□) after suspension in
phosphate buffer is plotted vs spore age
in days.

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 condi-
tions 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
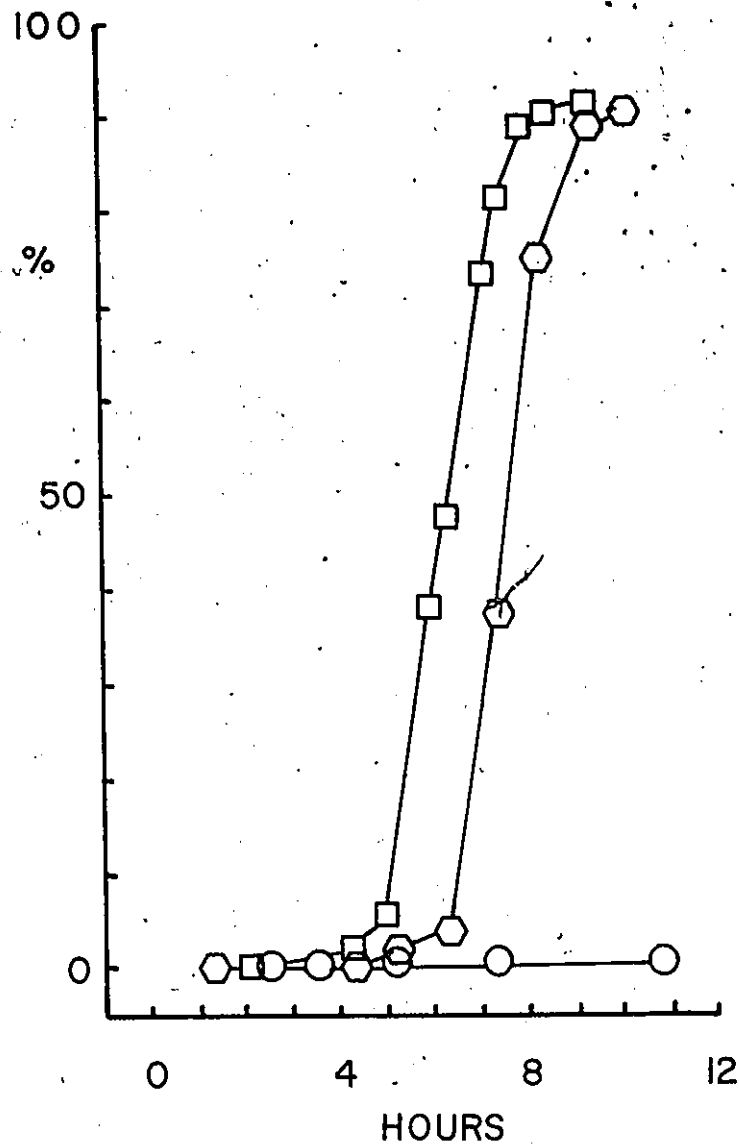
Figure 6. Age induced autoactivation of wild type
(strain JC1) Dictyostelium discoideum spores.
Percent spore germination is plotted vs time
in hours after suspension in phosphate buffer;
(○) 2 d old spores; (⬡) 9 d old spores;
(□) 16 d old spores.

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 characteristics 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 HC91 spontaneously 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 nitroso-guanidine (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. mucor-
oides_ 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
vioaceum_ 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. Autoactiva-
tion 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 1×10^7 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 1×10^6/ml germinate asynchronously and to lower percentages. The results presented in Fig. 7 show that SG1 spores at 1×10^6/ml germinate to only about 30% within 4 h, while those at 1×10^7/ml show essentially complete germination at this time. Similarly, SG2 spores germinate very poorly at 1×10^6/ml, while those at 1×10^7/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
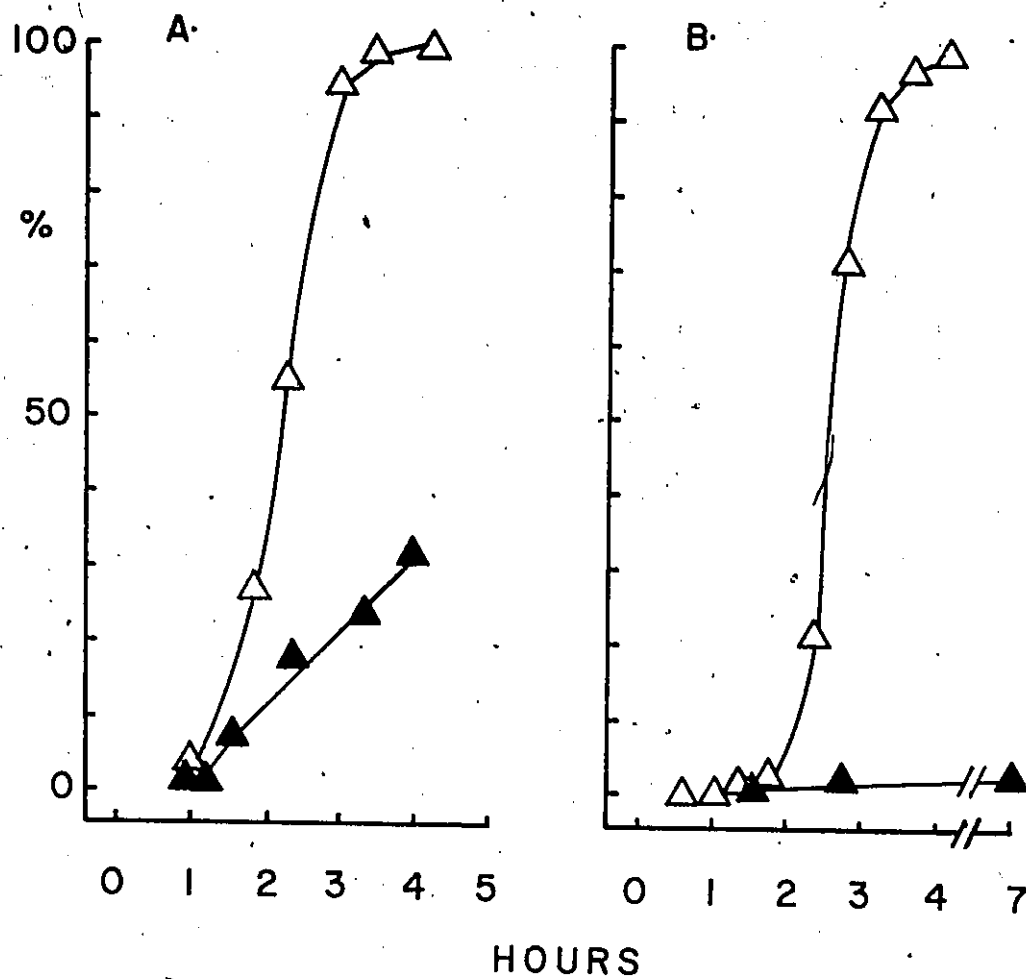
Figure 7.   Density dependence of autoactivation.
Panel A: strain SG1 spores;   Panel B: strain
SG2 spores;   ($\triangle$) spores at 1×10^7/ml;
($\blacktriangle$) spores at 1×10^6/ml.

variable. At some times spores at 1 x 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 x 10^7/ml (Fig. 8). The data in
Fig. 7 and 8 represent the two extremes of density depen-
dence. Subjective observations suggest that the varia-
bility in density dependence may be due to the effects of
humidity. Spores at 1 x 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
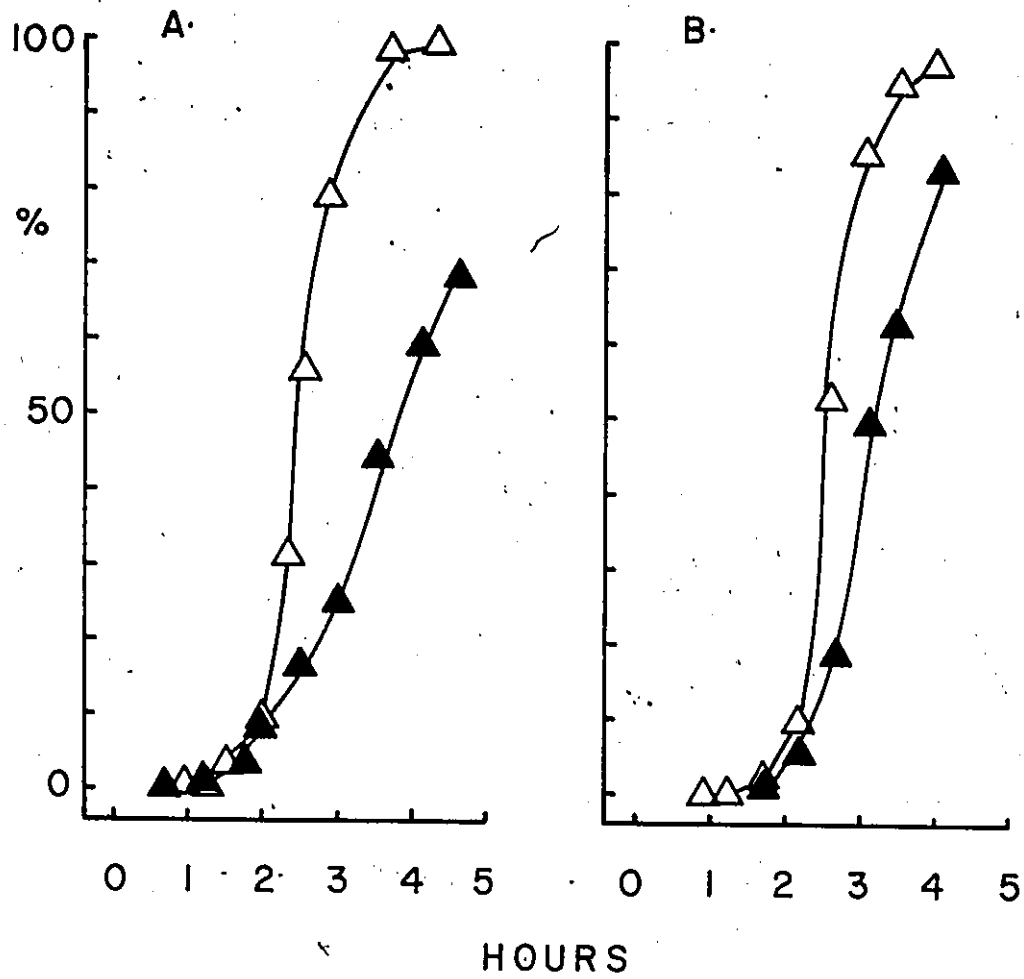
Figure 8. Reduced density dependence of autoactivation. Panel A: strain SG1 spores; Panel B: strain SG2 spores; ($\triangle$) spores at 1×10^7/ml; ($\blacktriangle$) spores at 1×10^6/ml.

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 Bonner, 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 10^7/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 10^6/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 10^7/ml. Activated supernatants also stimulate strain SG2 spores in a similar manner (Fig. 9). Supernatants in which aged strain NC4 spores
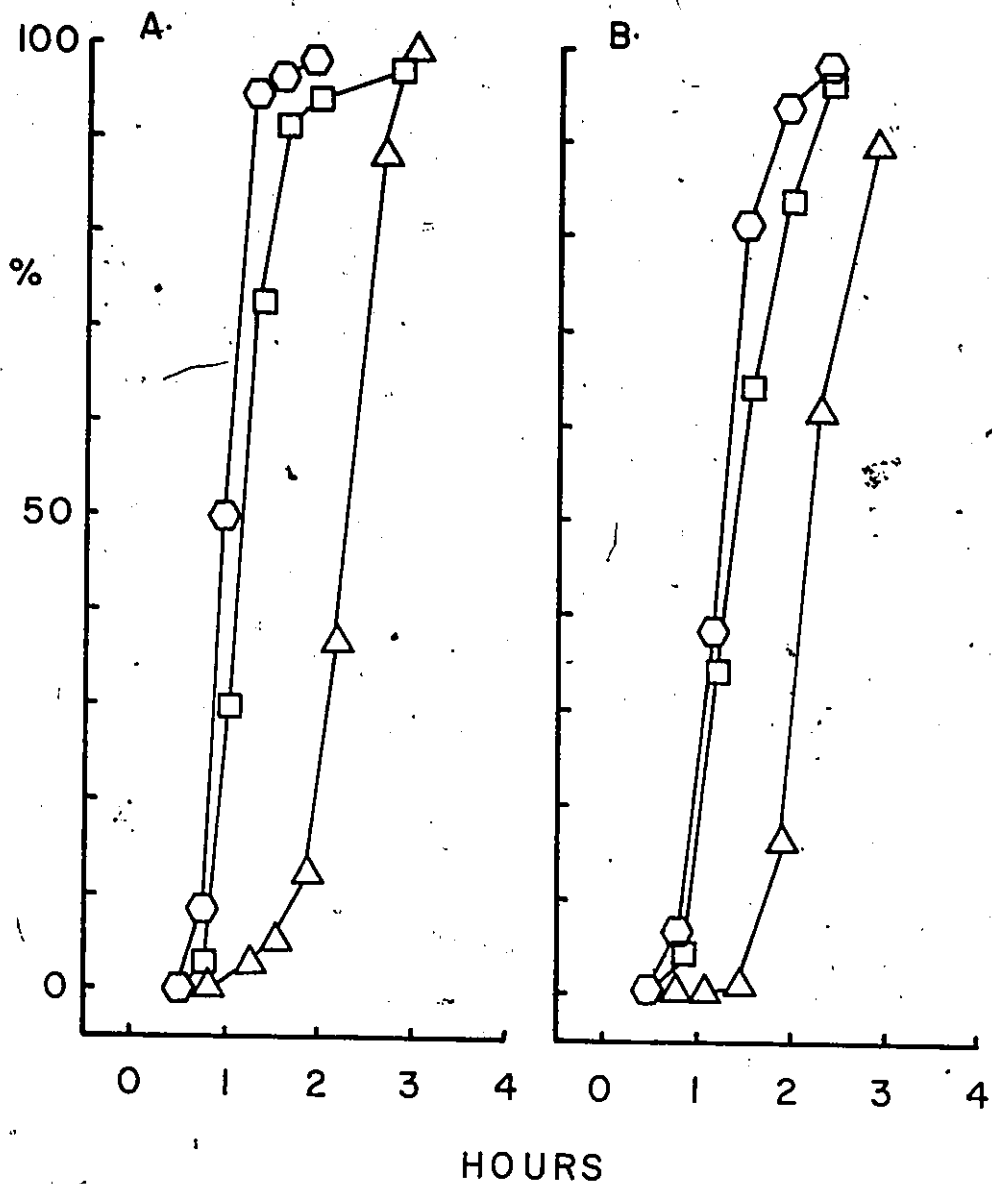
Figure 9. Effect of activated supernatants on strain
SG1 and SG2 spore germination. All data are
plotted as percent germination vs time in
hours after harvesting spores. Panel A:
strain SG1 spores; Panel B: strain SG2 spores;
(△) spores at 1×10^7/ml; (⬡) spores at
1×10^6/ml in a solution in which SG1 spores
have previously autoactivated; (□) spores
at 1×10^6/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
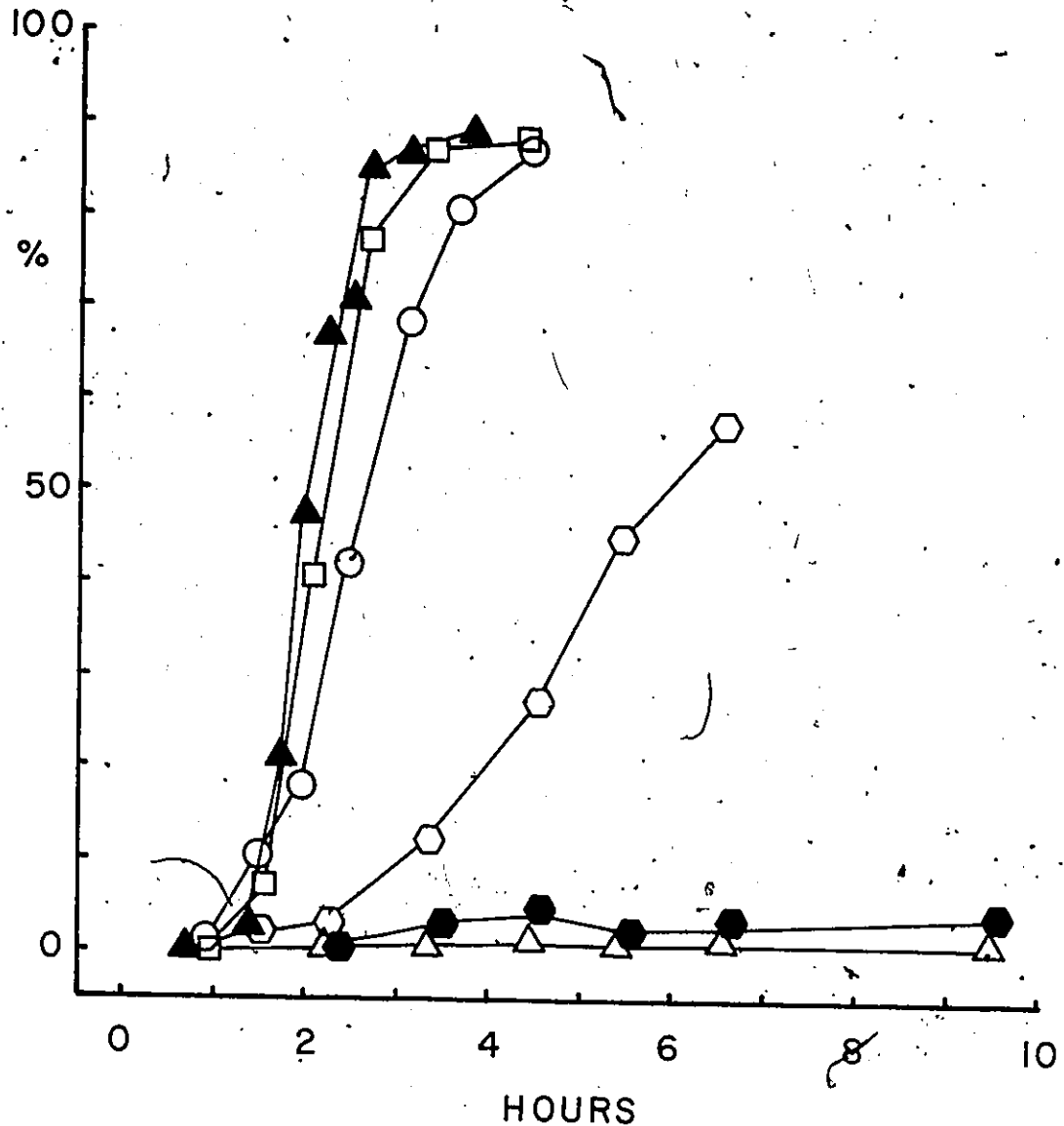
Figure 10. Effect of activated supernatants on strain
NC4 spore germination. Percent spore germin-
ation vs time in hours. ($\triangle$) 2 d old NC4
spores at 1 x 10^7/ml; ($\blacktriangle$) same, heat activ-
ated at 45 C for 30 min. ($\square$) same, plus
activated supernatant from SG1 spores;
($\ominus$) same, plus activated supernatant from
SG1 spores from a different lot added at
t = 0 h; ($\oplus$) same, plus activated supernatant
from SG1 spores from a different lot added
at t = 1 h; ($\bigcirc$) 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.
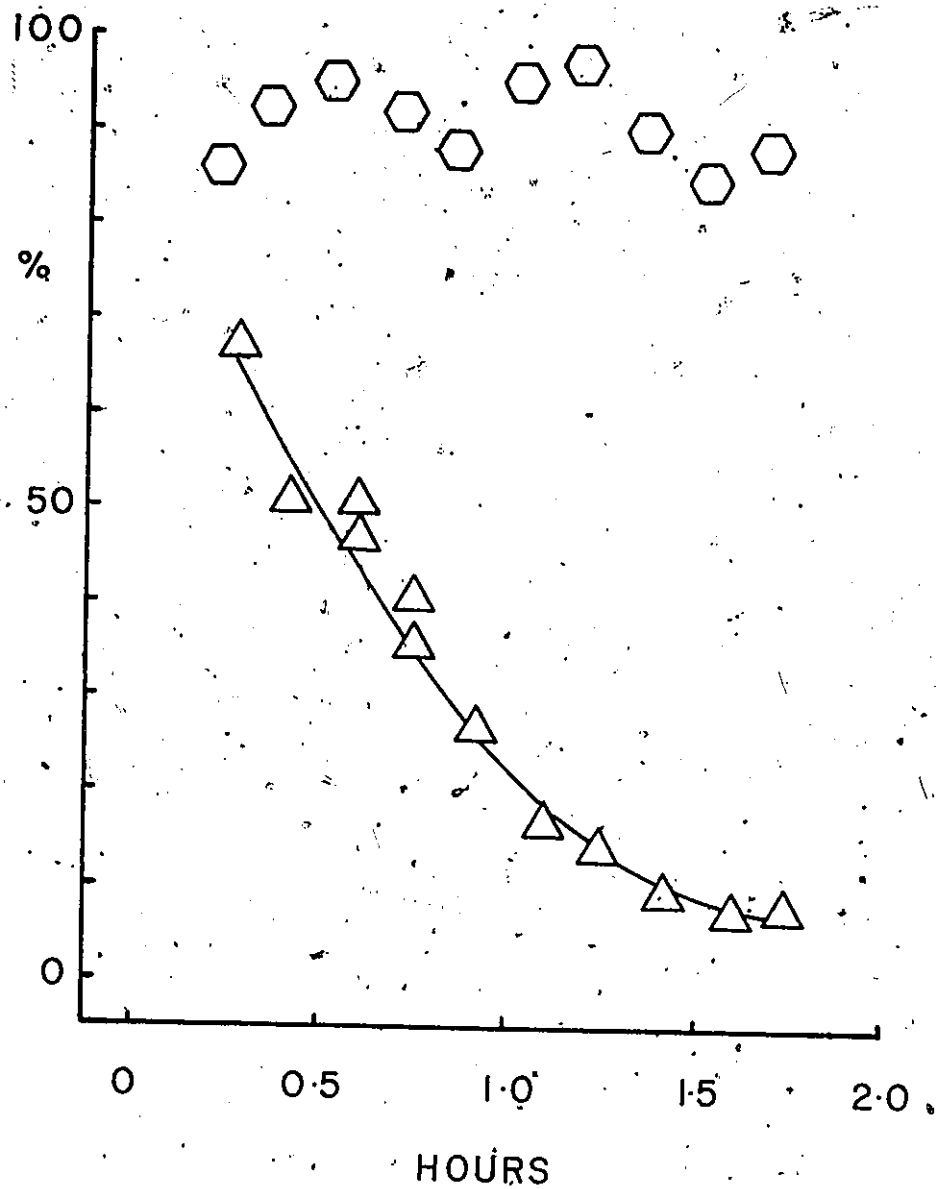
Figure 11. Competence period in response to SG1 auto-
activator preparations. Data represent a
plot of percent germination at 5 h vs time
of addition of SG1 autoactivator preparation.
Spores in both cases were treated with cyclo-
heximide (100 ug/ml final concentration) to
inhibit endogenous spore activation.
($\triangle$) response of NC4 spores; ($\bigcirc$) response
of SG2 spores.

Table 1. Effect of <u>Dictyostelium discoideum</u> strain

NC4 spore age on the competence period

Spore age (d)	% autoactiv-ation at 5 h	% germination[a] at 5 h, "AA" added t = 0 h	% germination at 5 h, "AA" added t = 1 h
3	0.0	97.0	27.0
5	0.0	97.0	58.0
7	0.0	96.0	68.0
9	0.0	99.0	84.0

[a] "AA" refers to addition of autoactivator preparation resulting from strain SG1 spontaneous spore germination at 1×10^7 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 1×10^7 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 ug/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
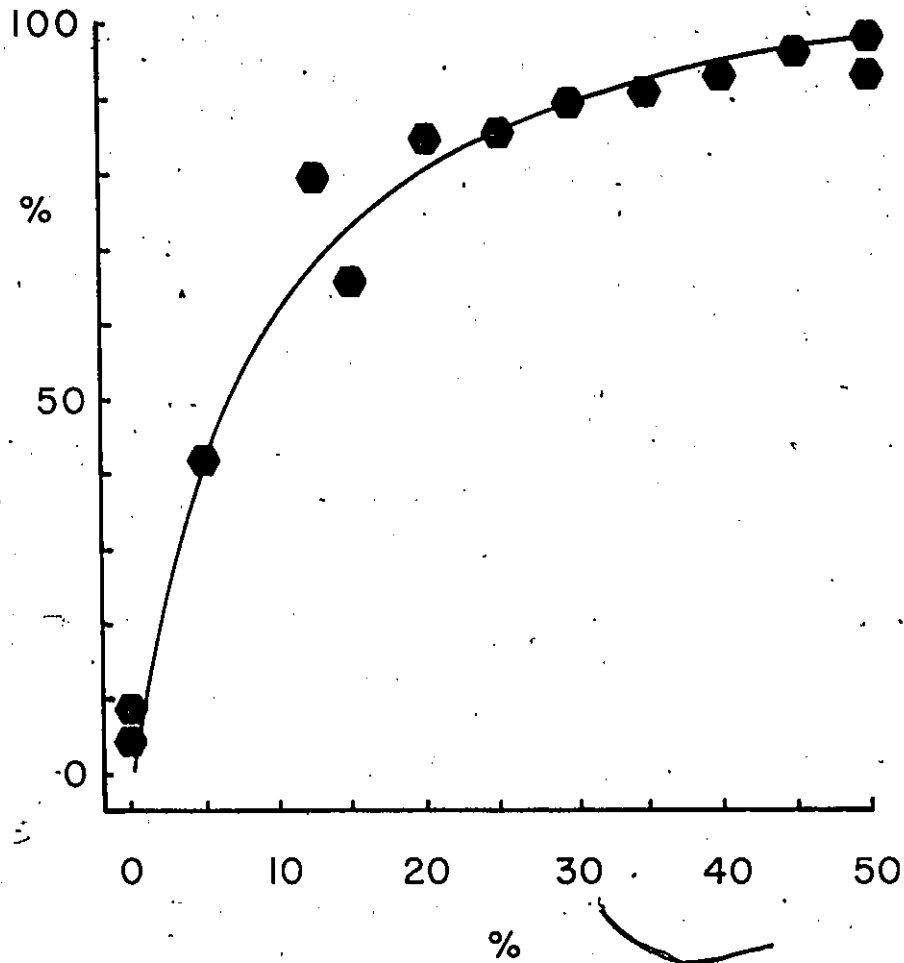
Figure 12. Dilution series on SG2 activated supernatant.
SG2 spores at 2×10^6/ml plus 200 ug/ml
cycloheximide were mixed with an equal
volume of various dilutions of SG2 activated
supernatant. (⬢) represents percent spore
germination at 5 h after harvesting vs
percentage of the suspension as SG2 activated
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).

d. 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
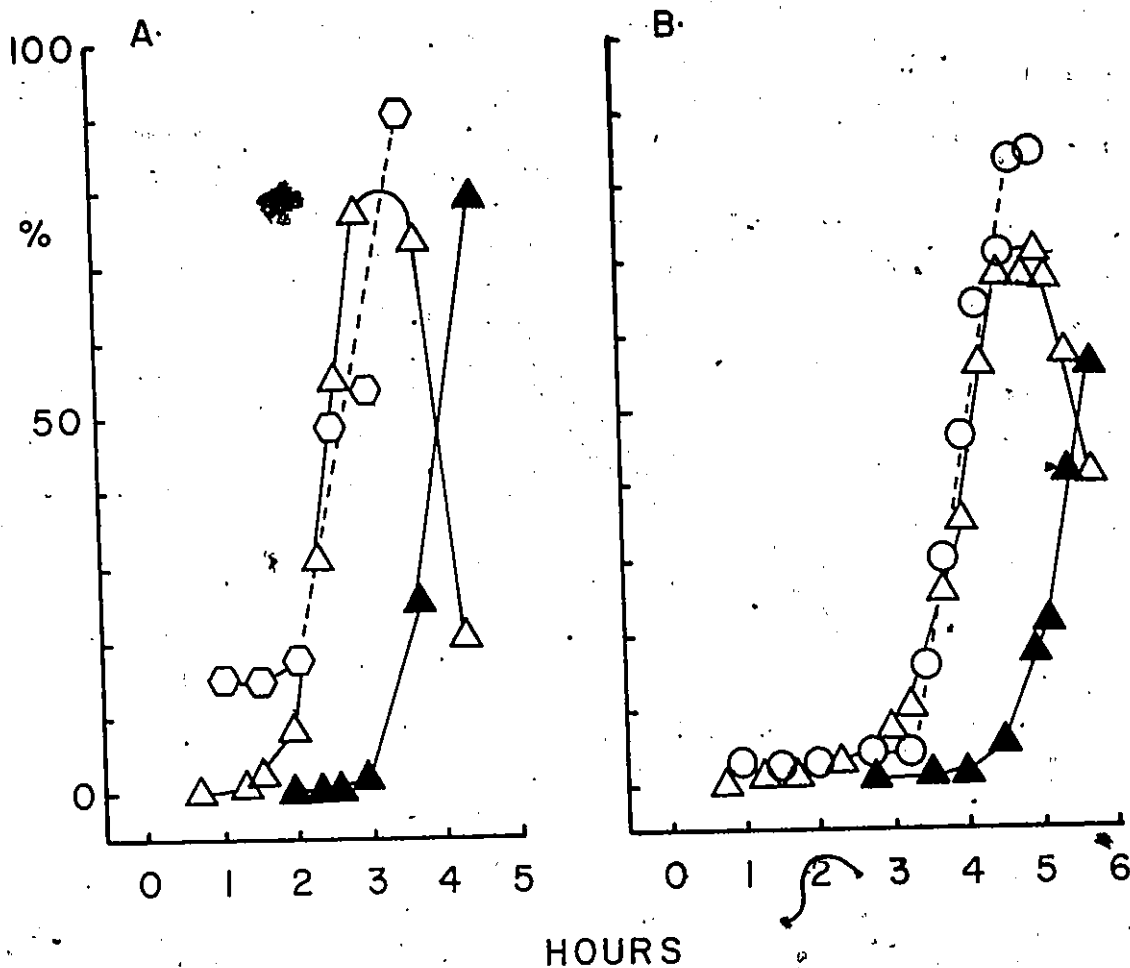
Figure 13. Release of autoactivator factors during spore swelling. Panel A: SG1 spores; Panel B: SG2 spores; (△) percent spore swelling at 1 x 10^7/ml vs·time in hours after harvesting; (▲) percent emergence of myxamoebae; (⬡) and (◯) ability of the supernatants to stimulate subsequent spore germination: 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. Regard-
less 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 inhib-
ited 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 inhib-

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 ug/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

Spores[d]	Drug or treatment	concentration (ug/ml)	+ drug	+AA	45°C, 30 min
SG1	control	---	100.0^a	96.0	95.0
SG1	cyclohex-imide	100	9.0^b	99.0^b	99.0^b
SG1	Edeine	200	18.0^b	94.5^b	98.5^b
SG1	Thiolutin	100^c	2.5^b	81.5^b	93.5^b
SG1	Daunomycin	250	0.0	1.0^b	89.0^b
SG1	4NQO	$---^c$	8.0^b	87.0^b	36.0^b
SG1	UV	---	62.5^b	99.0^b	99.0^b
SG2	control	---	98.0	96.0	94.0
SG2	cyclohex-imide	100	0.0	99.0^b	98.0^b
SG2	Edeine	200	9.5^b	98.5^b	97.5^b
SG2	Thiolutin	100^c	0.5^b	69.5^b	94.5^b
SG2	Daunomycin	250	0.0	3.0^b	4.0^b
SG2	4NQO	$---^c$	3.5^b	96.0^b	58.0^b
SG2	UV	---	47.0^b	99.5^b	99.0^b

[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 1×10^7/ml.
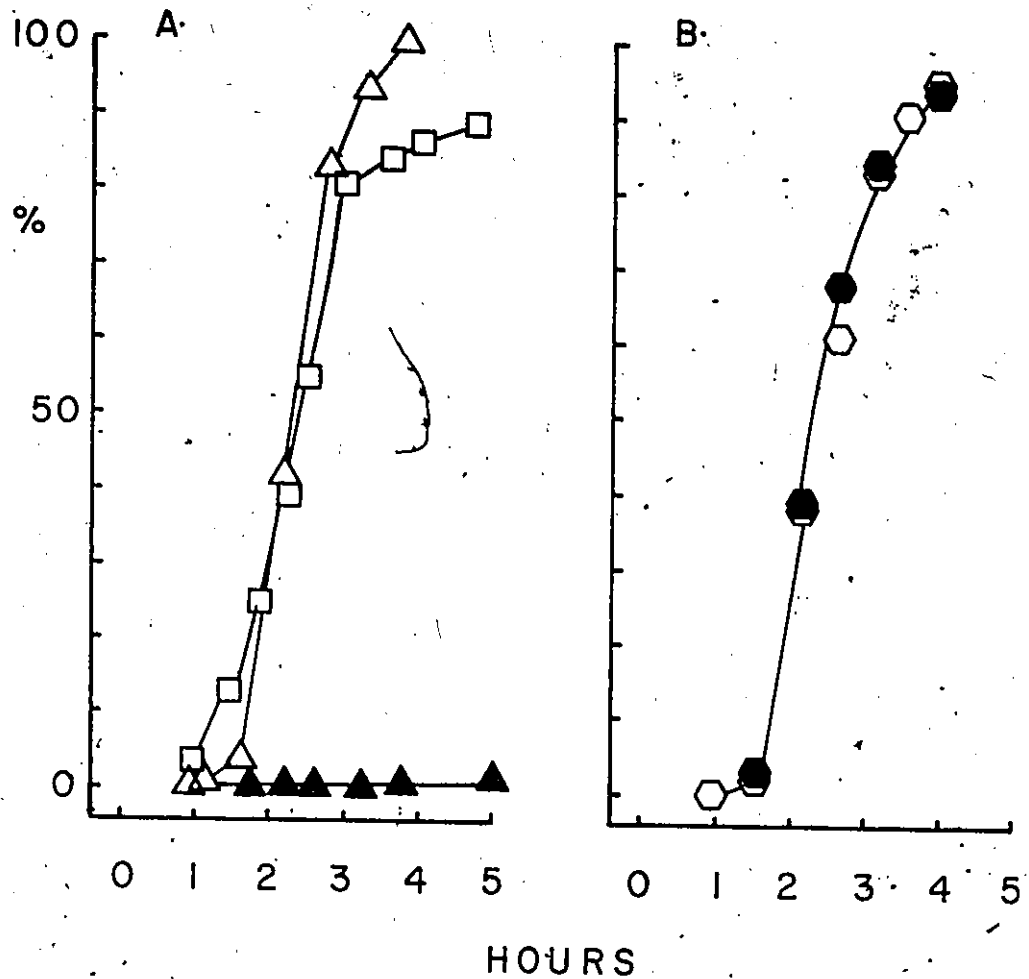
Figure 14. Effect of cycloheximide on SG2 spore germination. All points represent percent spore germination vs time in hours after spore harvesting. Panel A: (△) SG2 spores at 1 x 10⁷/ml; (▲) SG2 spores at 1 x 10⁷/ml in 100 ug/ml cycloheximide; (□) SG2 spores at 1 x 10⁷/ml in 100 ug/ml cycloheximide 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 ug/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 sensitiv-
ity of strain SG1 spores to cycloheximide generally
correlates with the concentration dependence of autoactiv-
ation, and may therefore be attributable to humidity
differences. Accordingly, this topic will be discussed
below in that context.

Cycloheximide inhibition of autoactivation is over-
come 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 auto-
activation 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 cyclohex-
imide until approximately 0.5 h before the onset of spore
swelling (Fig. 15). If the drug is added after this time,
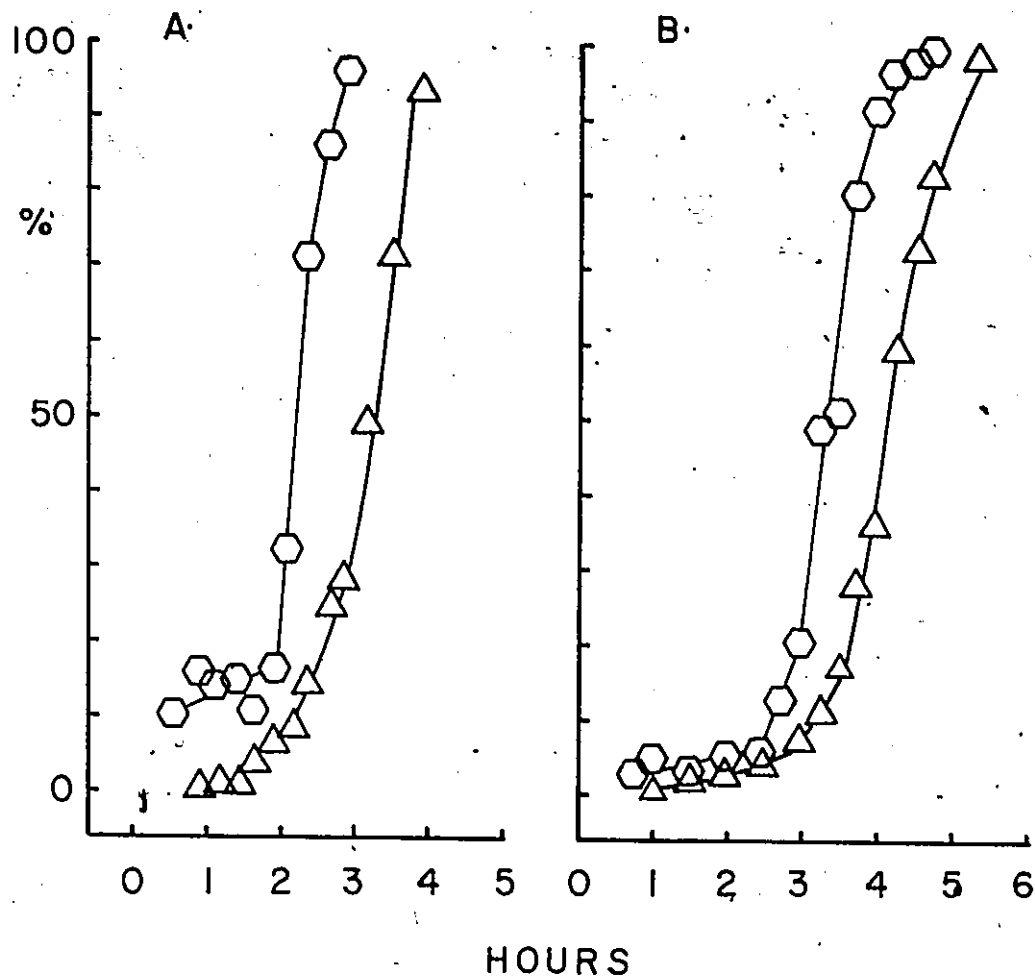
Figure 15.   Time of loss of sensitivity to cycloheximide.
Panel A: SG1 spores;   Panel B: SG2 spores.
($\triangle$) percent spore germination at 1×10^7/ml
vs time in hours after harvesting;   ($\bigcirc$) per-
cent spore germination at 5 h vs time of
addition of cycloheximide to 100 ug/ml.

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$_f$ 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 respir-
atory 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 germin-
ation 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 interchelates 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 equivocally 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 unsuit-
able 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 germina-
tion (data not shown). In contrast, supernatants from
the non-irradiated spores are highly stimulatory. This
observation may suggest that irradiation of spores inter-
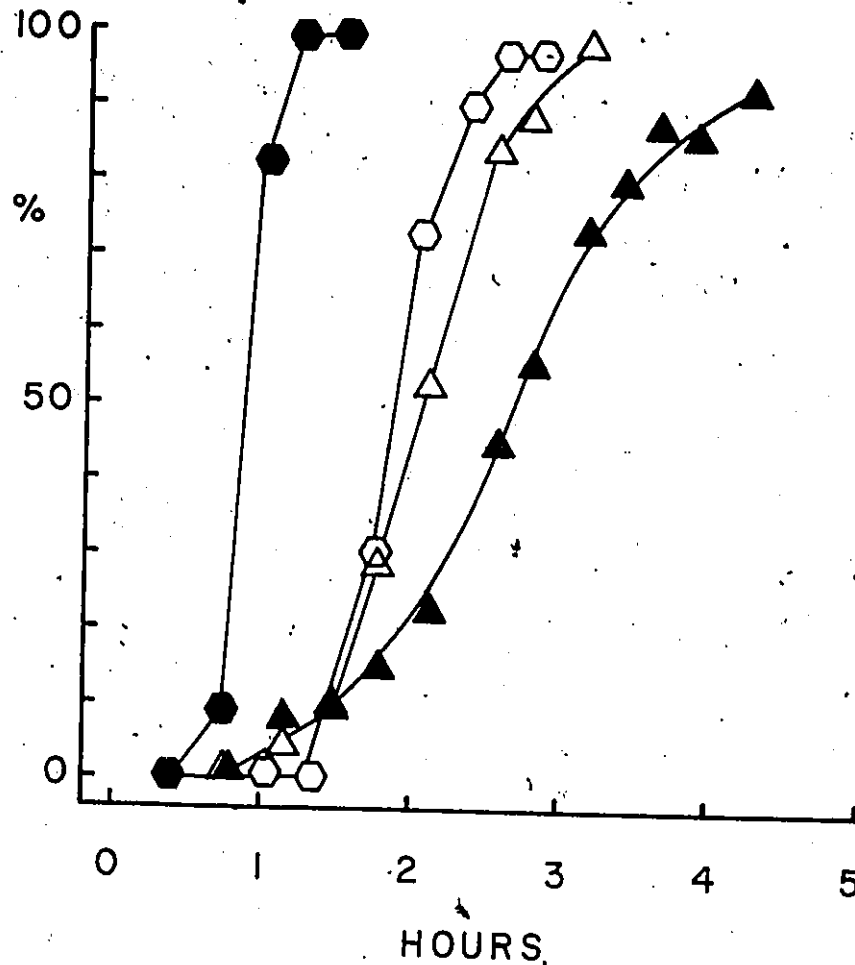
Figure 16. Effect of ultraviolet irradiation on auto-
activation. All data points represent
percent spore germination vs time in hours
after harvesting of spores or end of activa-
tion treatment. (△) SG1 spores at 1×10^7/ml;
(▲) SG1 spores at 1×10^7/ml, UV irradiated
for 2 min at 20 cm lamp height; (⬡) SG1
spores, UV irradiated and heat shocked at
45 C for 30 min; (⬢) SG1 spores, UV
irradiated and treated with SG1 autoactiv-
ator preparation.

feres 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 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 auto-activating by cyanide. This drug is an inhibitor of the mitochondrial electron transport system. In the presence of 2×10^{-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 <u>D. discoideum</u>
strain SG1 spore germination

Treatment[a]	oxygen[b] uptake	% germination at 5 h	% germination at 18 h
control	17.90	98.5	---
CN	8.05	1.5	90.0[c]
SHAM	18.84	10.5[d]	34.0[d]
CN + SHAM	3.45	0.0	32.0[d]
AA	59.67	99.0	---
AA + CN	14.10	48.0	80.0[c]
AA + SHAM	30.77	79.0[d]	79.0[d]
AA + CN + SHAM	3.60	0.0	82.0[d]
HS	--	97.5	---
HS + CN	--	8.5	91.5
HS + SHAM	--	89.0[d]	94.0[d]
HS + CN + SHAM	--	0.0	83.0

[a]Abbreviations: CN = treatment with 2×10^{-3} M cyanide;
SHAM = treatment with 5×10^{-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 O_2/h/1 x 10^7 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 inhib-
it 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 <u>D. discoideum</u>
strain SG2 spore germination

Treatment[a]	% germination at 5 h	% germination at 18 h
control	96.0	---
CN	0.0	60.5[c]
SHAM	2.0[b]	26.0[b]
CN + SHAM	0.0	1.5[b]
AA	99.5	---
AA + CN	14.5	55.0[c]
AA + SHAM	94.0	---
AA + CN + SHAM	0.0	24.5[b]
HS	99.0	---
HS + CN	14.0	62.0[c]
HS + SHAM	85.0[b]	---
HS + CN + SHAM	0.0	12.0[b]

[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]emergence of myxamoebae inhibited.

[c]estimated germination due to clumping of myxamoebae.

Table 5. Effects of cyanide and SHAM on <u>D. discoideum</u>
strain NC4 spore germination

Treatment[a]	oxygen[b] uptake	% germination at 5 h	% germination at 18 h
control	---	0.0	64.0[c,d]
CN	6.59	0.0	2.0
SHAM	---	0.0	0.0
CN + SHAM	1.63	0.0	0.0
AA	---	92.0	---
AA + CN	14.24	1.0	43.0[d]
AA + SHAM	---	45.0[e]	52.0[e]
AA + CN + SHAM	2.12	0.0	2.0[e]
HS	---	96.0	---
HS + CN	---	0.0	0.0
HS + SHAM	---	69.0[e]	---
HS + CN + SHAM	---	0.0	0.0

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

[b]oxygen uptake is expressed as ul O_2/h/1 $\times 10^7$ spores,
and was measured during the first 5 h only.

[c]spores were 5 d old and showed some age induced auto-
activation betweem 5 and 18 h.

[d]estimated due to clumping of myxamoebae.

[e]emergence 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 2×10^{-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 5×10^{-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 auto-activation, 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 auto-activators.

4. Exogenous Activation of Mutant and Wild Type
   Dictyostelium discoideum Spores[1]

   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

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[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

Treatment[a]	Strain		
	SG1	SG2	NC4
45 C, 30 min	99.5	99.0	99..5
37 C, 60 min	63.0	13.5	·0.0
37 C, 30 min	53.5	4.5	0.0
40 C, 60 min	---	80.5	0.0
40 C, 30 min	---	53.0	0.0
no treatment	9.0	0.0	0.0

[a]All spores·were treated with the activation regimen in the presence of 100 ug/ml cycloheximide to inhibit autoactivation. Values represent percent spore germination after 5 h.
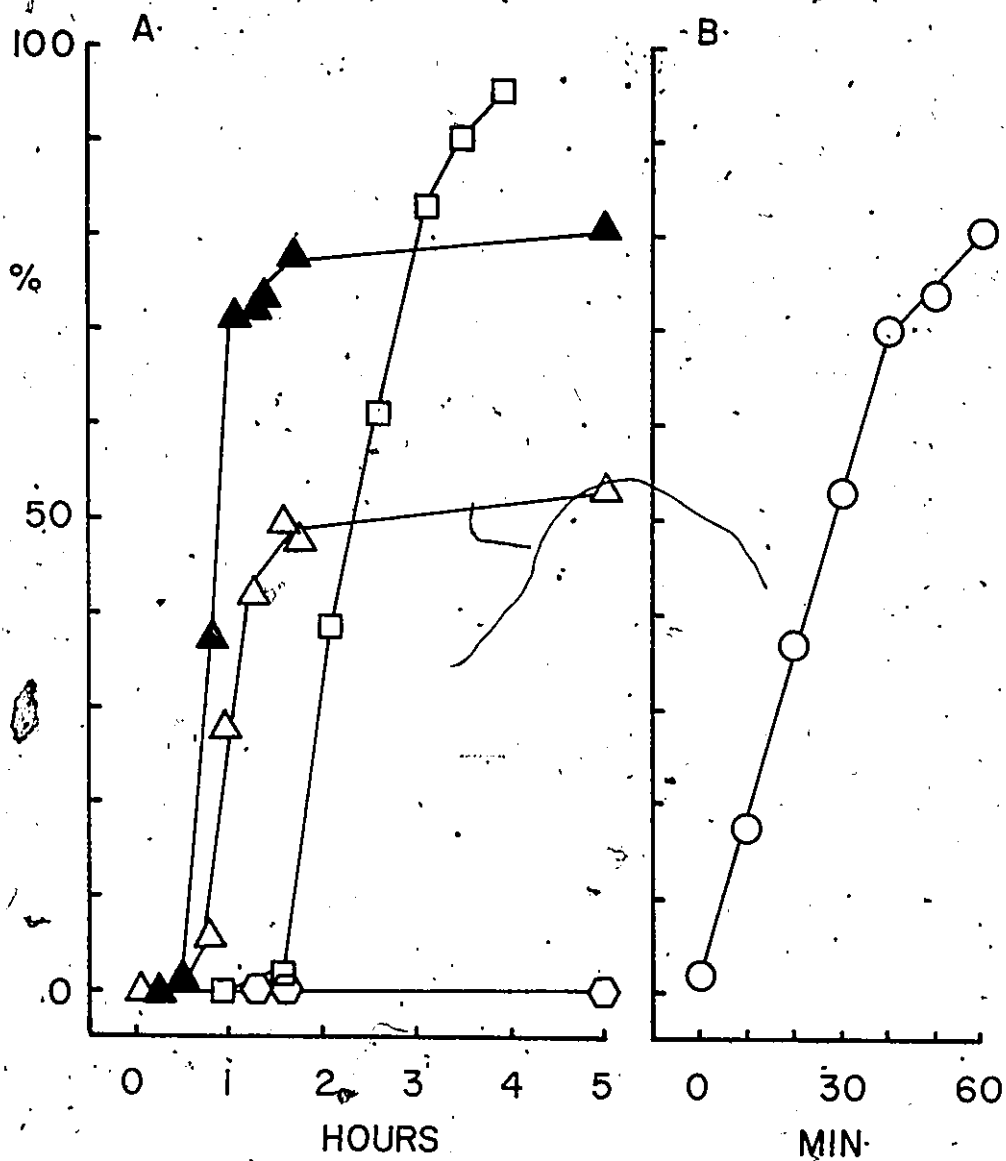
Figure 17. Lowered activation requirements of strain SG2
spores. Panel A: (□) SG2 spores treated at
45 C for 30 min; (▲) SG2 spores treated at
40 C for 60 min; (△) SG2 spores treated at
40 C for 30 min; .(⬡) NC4 spores treated at
40 C for 60 min; all data points represent
percent germination vs time 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 ug/ml cycloheximide.

37 C for 30 min in Table 6).

Spore activation at lower temperatures was studied in the presence of 100 ug/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 1×10^6/ml and 1×10^7/ml

germinate with similar kinetics. Secondly, activation at the lower temperatures occurs despite the presence of 100 ug/ml cycloheximide. It may be recalled that auto-activation is sensitive to cycloheximide while exogenous activation is not affected by the drug. Thirdly, auto-activator 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 gener-
ally 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 sorocarps.
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 germin-
ation of mutant spores is 99%, while wild type spores
germinate to 86.5%. Activated wild type spores return

Figure 18.   Insensitivity of <u>D. discoideum</u> strain SG1
              spores to deactivating conditions.   All
              data are plotted as percent spore germination
              vs time in hours after the end of the activ-
              ation 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 <u>in situ</u> and removed from
              fruiting bodies after 5 h;   (●) SG1 spores,
              same treatment.
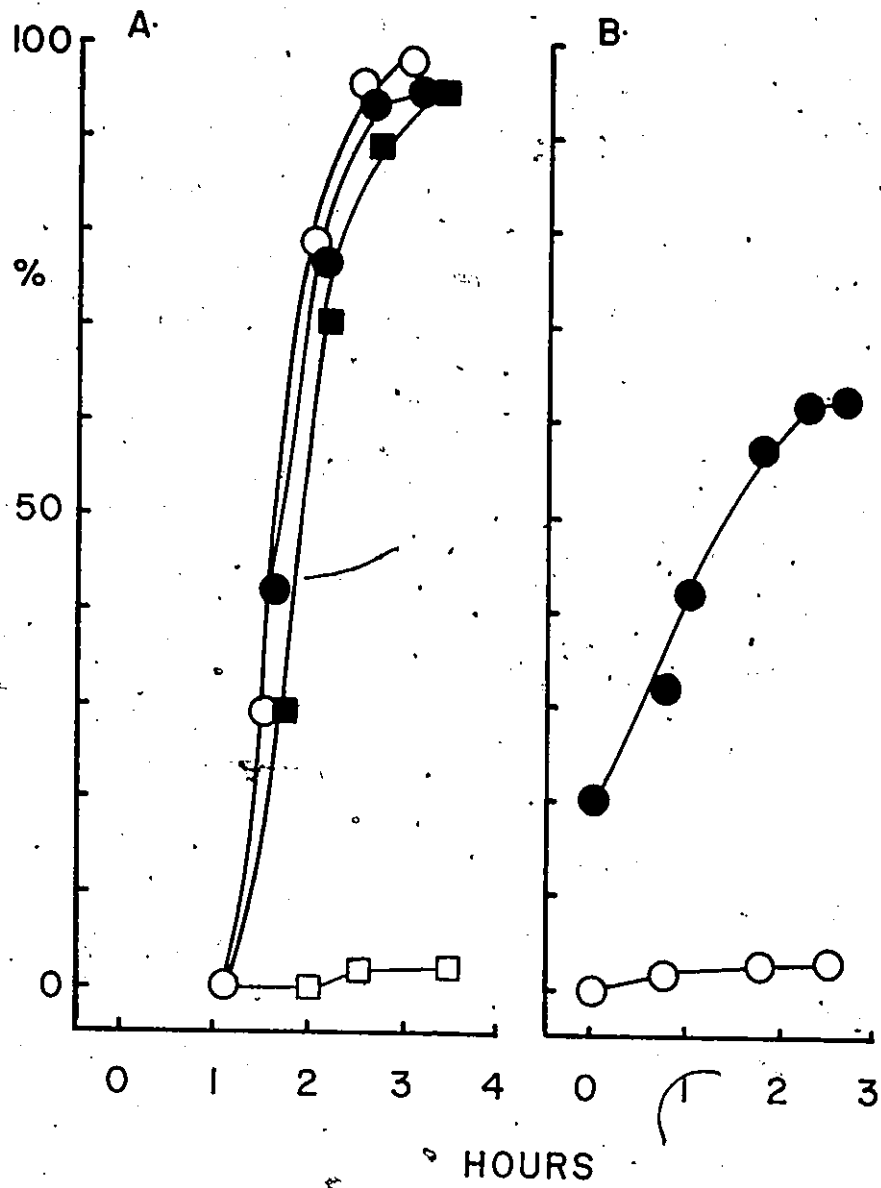
to the dormant state if not washed free of autoinhibitors
and osmotically active materials within the sorocarps
(Cotter and Raper, 1968b). Thus, no germination of
wild type strain NC4 spores is detected under such
conditions (Fig. 18). In contrast, SG1 spores germin-
ate to approximately 20% within the 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 exogen-
ously 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 insensi-
tive 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 deactiv-
ated, spores still autoactivate despite the inhibition

of respiration. In the case of osmotic pressure, spores simply fail to deactivate. The effect of heat activation <u>in situ</u> 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

<u>Dictyostelium discoideum</u> 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 <u>D. discoideum</u> spores may be discadenine, the autoinhibitor of spore germination (Abe et al., 1976). This material
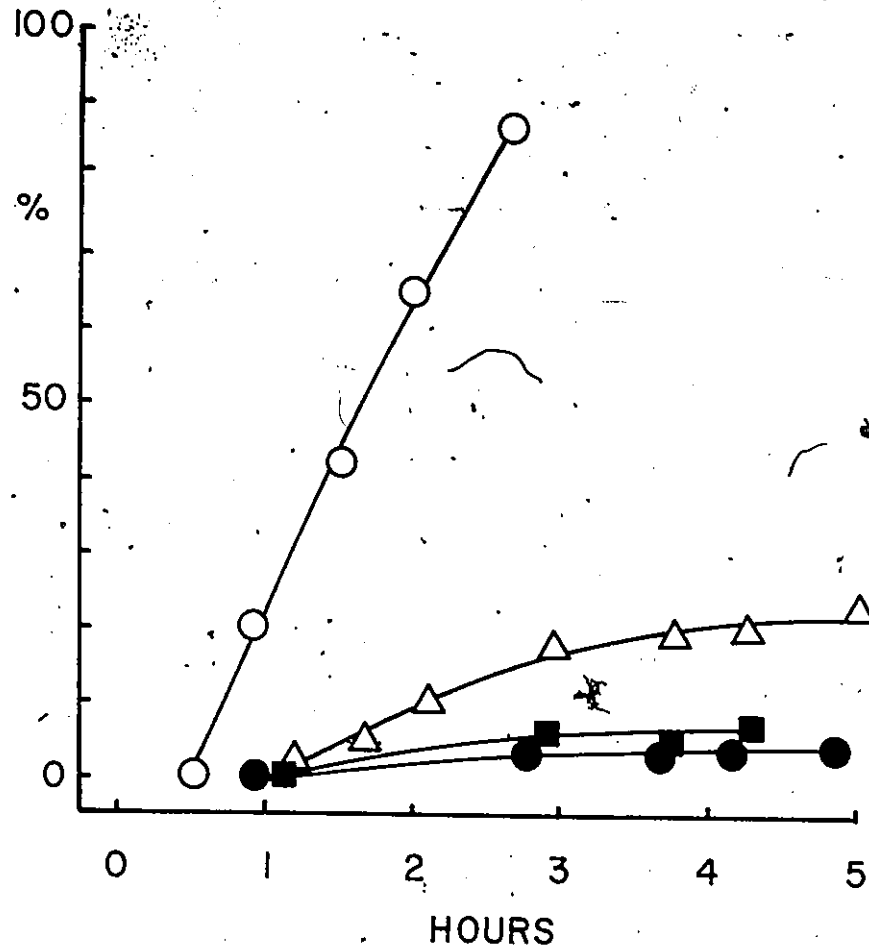
Figure 19. Release of inhibitor from spores activated by heat shock. All data points represent percent spore germination vs time in hours after harvesting. ($\triangle$) SG1 spores at 1 x 10^6 /ml; ($\bigcirc$) SG1 spores in an activated supernatant derived from SG1 spores; ($\blacksquare$) SG1 spores in a solution in which NC4 spores have germinated after heat shock at 45 C for 30 min; ($\bullet$) SG1 spores in a solution in which SG1 spores have germinated after heat shock at 45 C for 30 min.
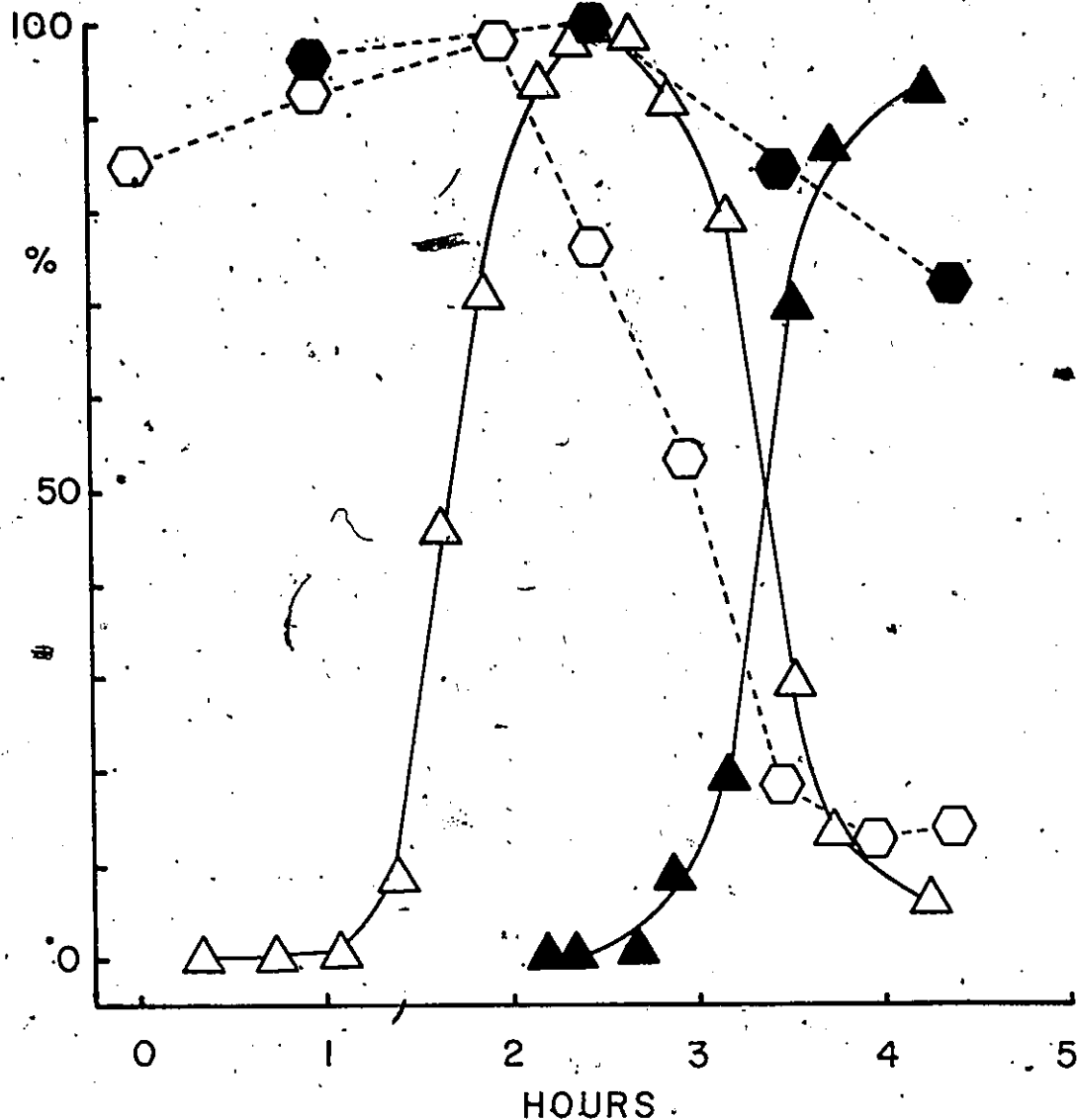
Figure 20: Time of release of inhibitors from heat
activated NC4 spores. (△) percent spore
swelling vs time in hours after end of treat-
ment at 45 C for 30 min; (▲) percent
emergence of myxamoebae; (⬡) percent germin-
ation at 5 h of SG1 spores incubated in
aliquots of the spore suspension vs time of
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 super-
natant during the early swelling stage of spore germina-
tion (Hohl et al., 1978), not late swelling.

Some inhibitory material is also released by spon-
taneously germinating spores. The inhibitor released is
not detected in crude autoactivator preparations, presum-
ably 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 <u>Dictyostelium discoideum</u>
   Autoactivator Factors

   a. General information

   Autoactivator substances resulting from the spon-
taneous germination of <u>D. discoideum</u> 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. Lyophil-
ized preparations are comparitively stable if the prep-
arations 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. Denatur-
ation 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 incorp-
orated as a preliminary purification procedure to rid
the preparations of large proteins and other macromole-
cules. Autoactivator substances appear to be non-dialyz-
able. 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 approx-
imately 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.
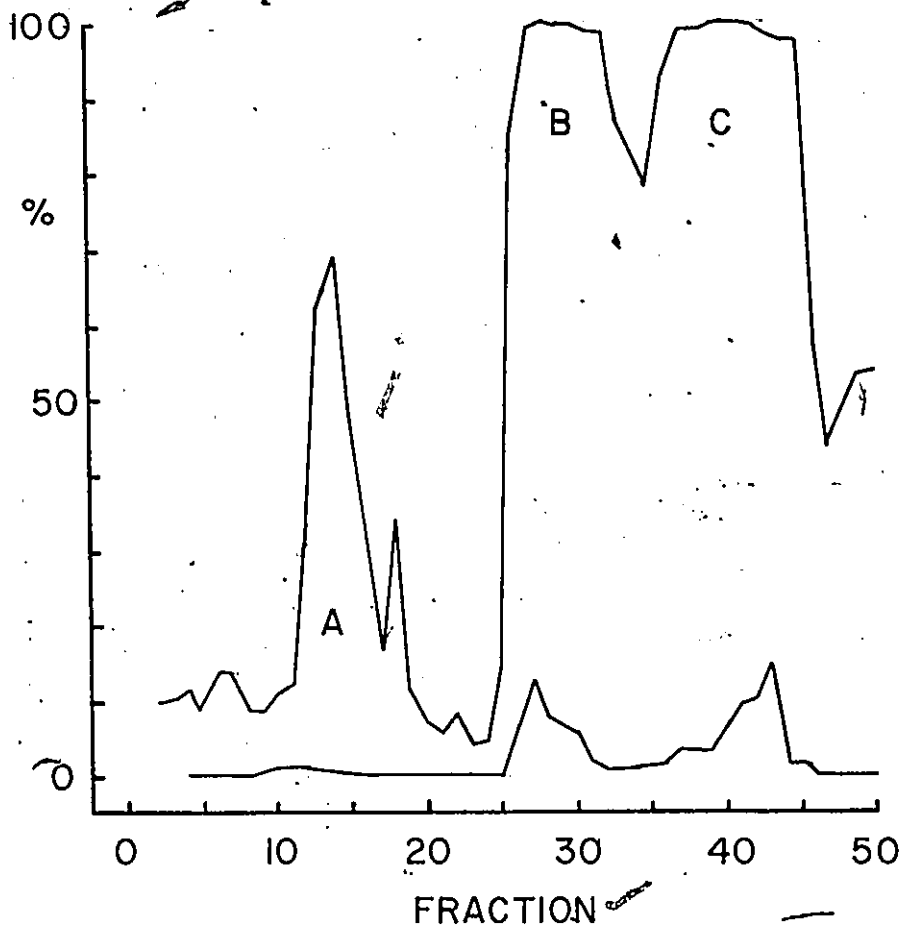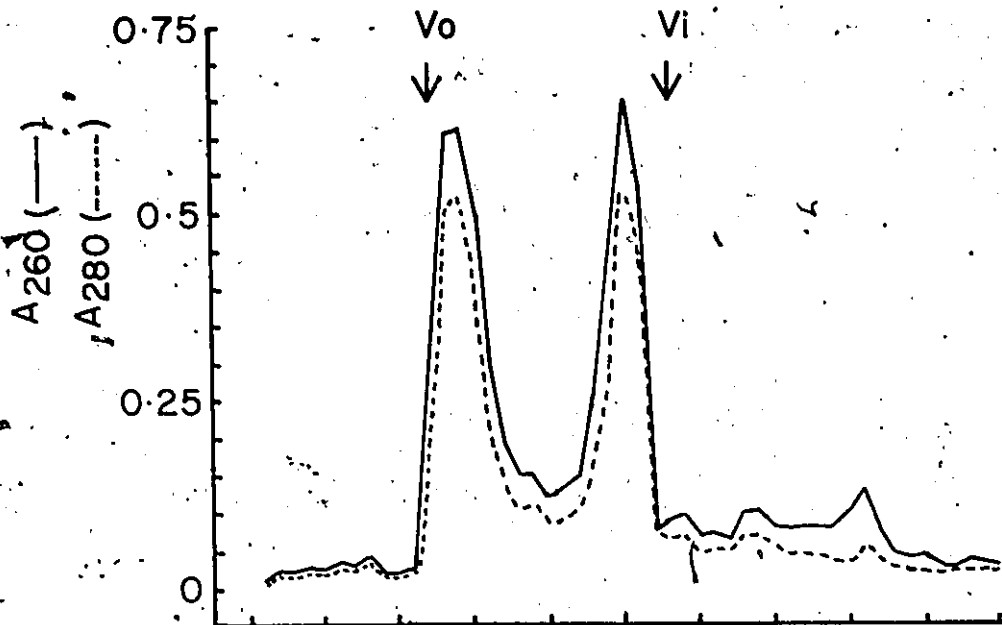
ating by cycloheximide, but shows minimal stimulation of strain NC4 spores. The ability of peak A material to stimulate strain NC4 spores is somewhat variable. Peak B elutes at or slightly after the internal volume of the column. It is associated with minimal UV absorbance. The peak C material elutes 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 componds 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 auto-
inhibitor of D. discoideum spore germination, is a substi-
tuted 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.

FRACTION
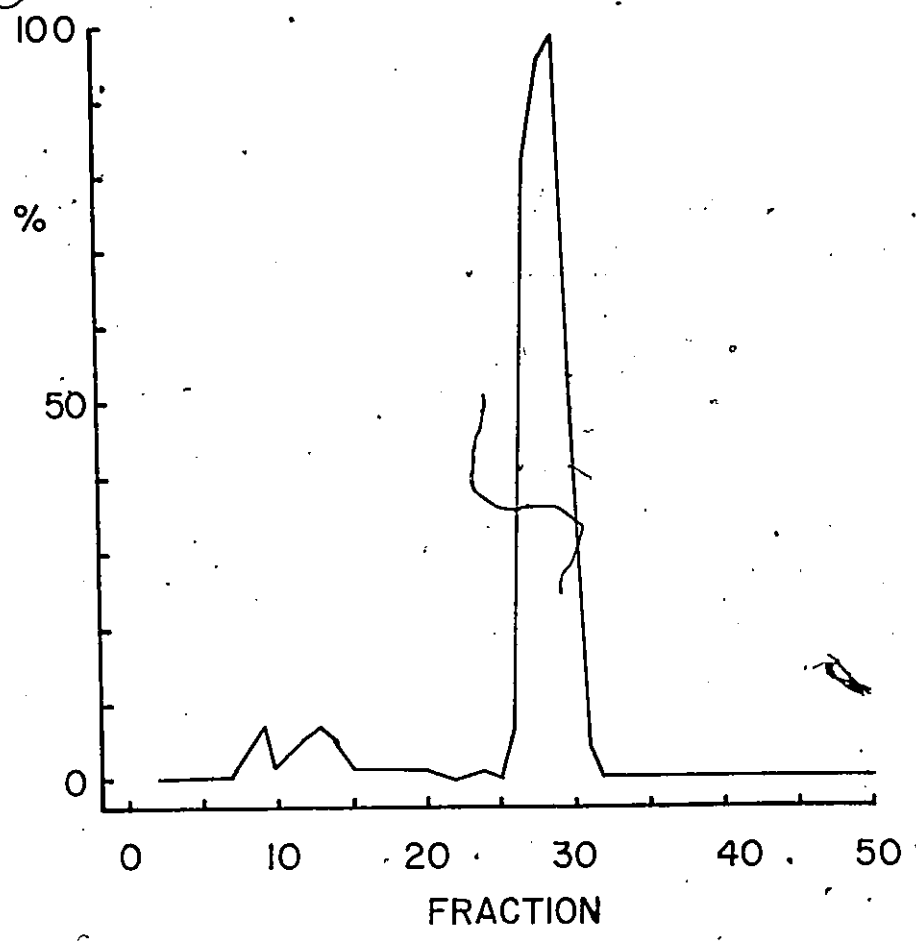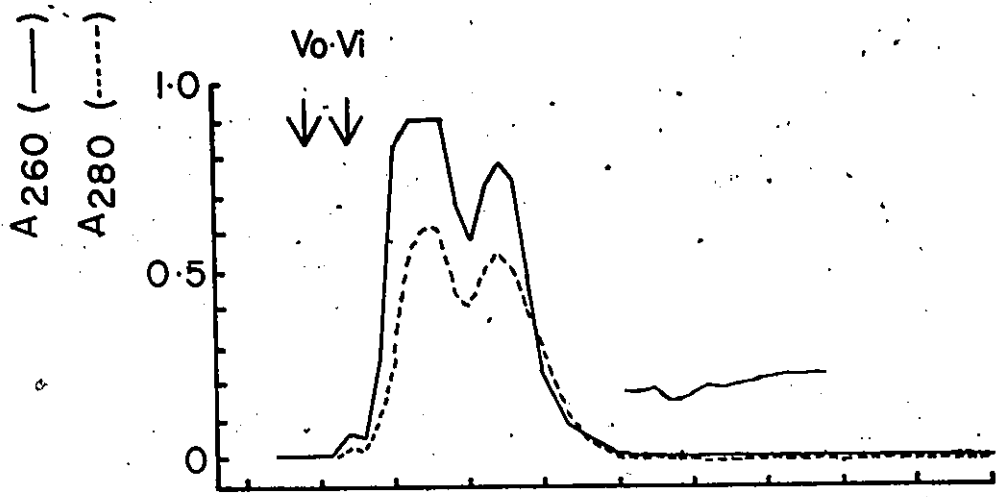
and visually inspected, no residue was observed.

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 absorbtivity coefficient at 260 nm of 17.5×10^3. In such a case, 1.0 A_{260} units equals 5.7×10^{-5} M. Absorbtion 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 5×10^7 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 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 ug/ml cycloheximide to each fraction; lower panel, lower tracing shows response of strain NC4 spores to each fraction.
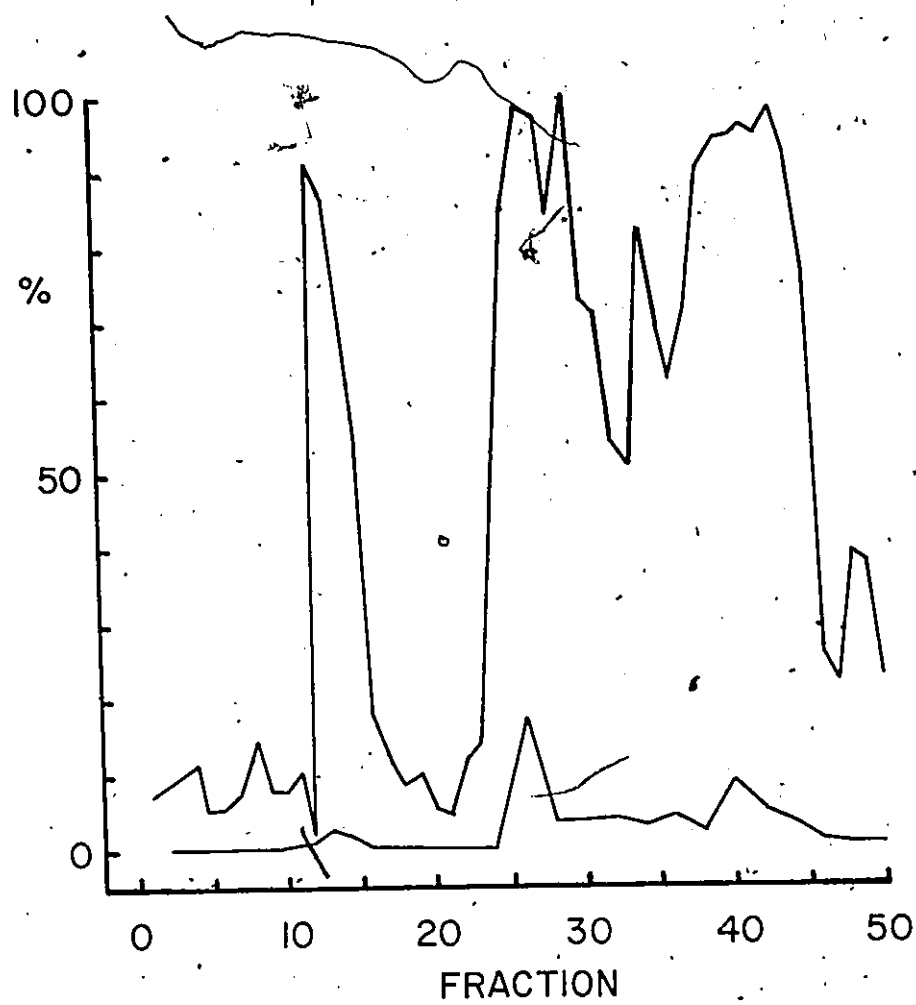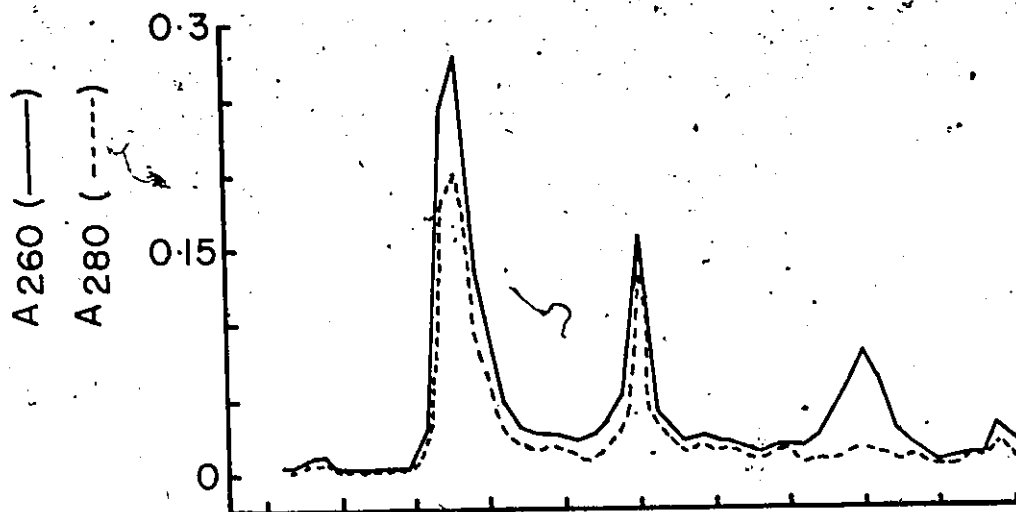
notable. Since strain SG1 is derived from strain NC4,
and aging appears to confer the mutant phenotype on
NC4 spores, it has been assumed that the substances are
identical. Combining of autoactivator peak C materials
from SG1 and aged NC4 preparations and refractionating
on Biogel P2 or Sephadex LH-20 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. discoid-
eum 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 absorb-
of each fraction at 260 and 280 nm;
Lower panel shows percent germination
response of strain NC4 spores to each
fraction.
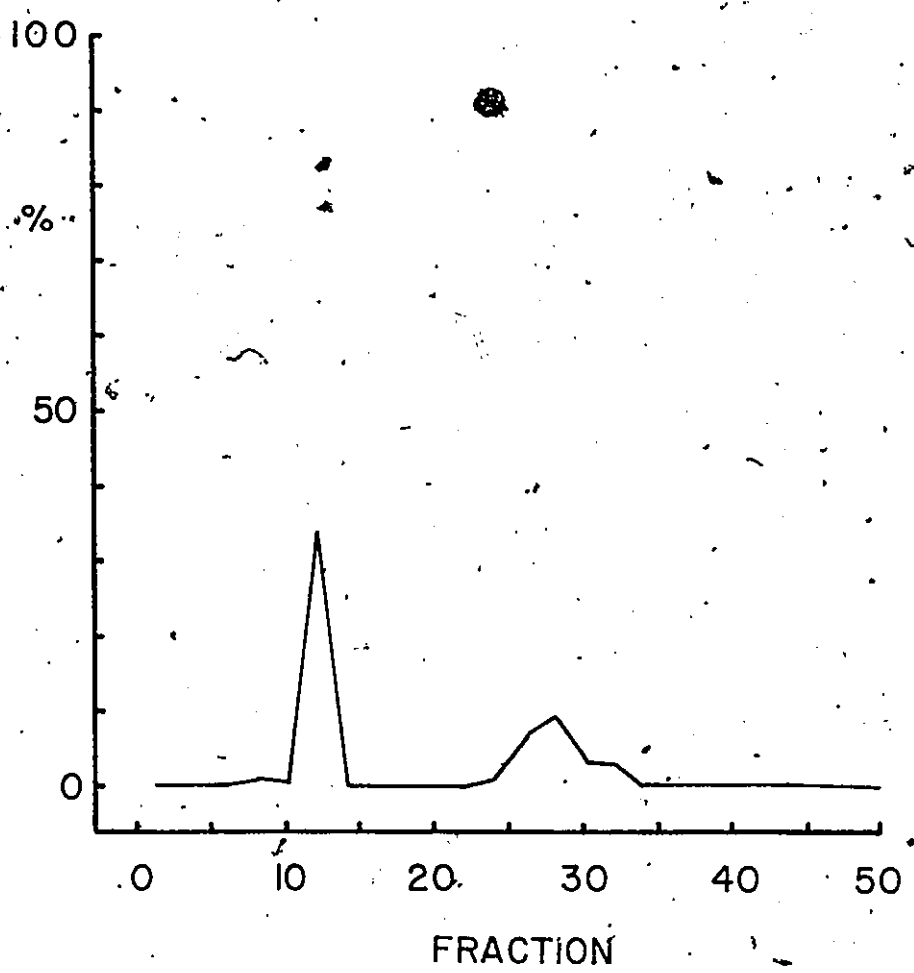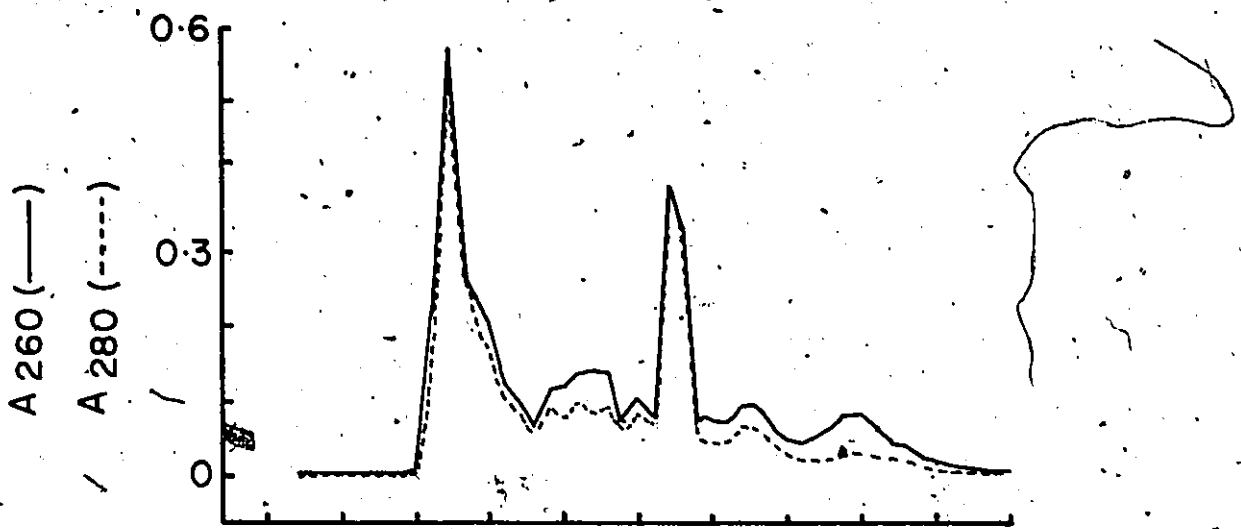
Myxamoebae from strains SG1, SG2, and NC4 were
grown in association with E. coli B/r on glucose-salts
agar plates. Amoebae were washed from the plates during
logarithmic growth with phosphate buffer, washed three
times, and resuspended in fresh phosphate buffer.
Amoebae were either disrupted immediately 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 autoactiva-
tors 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" (con-
tents 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 lyo-
philized 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

Preparation[a]	Autoactivator factor[b]		
	A	B	C
SG1 autoactivator preparation	+	+	+
SG2 autoactivator preparation	+	+	−
NC4 autoactivator preparation	+	+	+
SG1 amoebae	+	+	−
SG2 amoebae	+	+	−
NC4 amoebae	+	+	−
SG1 autoinhibitor preparation	+	+	−
SG2 autoinhibitor prepration	+	+	−
fresh NC4 autoinhibitor preparation	+	+	−
aged NC4 autoinhibitor preparation	+	+	−
SG1 spore extract	−	−	−
SG2 spore extract	−	−	−
fresh NC4 spore extract	+	+	−
aged NC4 spore extract	−	−	−

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 auto-
activator 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 vege-
tative 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 disserta-
tion, 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 autoactiv-
ator 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 cellu-
lar 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}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: $[8-^{14}C]$ 5'AMP, $[^{14}C]$ glycine, $[^{14}C-U]$ algal protein hydrolysate, $[^{14}C-U]$ glucose, and $[^{14}C]$ $NaHCO_3$. 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 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 experi-
ment, petri dishes were incubated in a moist chamber
fitted with a CO_2 trap. During the growth and sporula-
tion 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 auto-
activator factor C. The first, that factor C is inorgan-
ic, is implausible. One would not expect an inorganic
molecule to exhibit the lability observed for the auto-
activator preparations. The second possibility, that
autoactivator factor C is present in undetectably low
amounts, is more reasonable. The possibilty exists that
so little material is present that radioactive incorpor-
ation 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

undoubtably 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 discadenine 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. Undoubtably 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

amino acids	diguanosine tetraphosphate
casamino acids	zeatin
nutrient broth	6-furfurylaminopurine
yeast extract	6-benzylpurine
biotin	indole-3-acetic acid
vitamin B12	gibberellic acid (GA3)
pantothenic acid	S-adenosylmethionine
folic acid	isopentenyl adenine
inositol	isopentenyl adenosine
niacin	adenine
p-aminobenzoic acid	thymine
pyroxidine	guanine
riboflavin	uracil
thiamine	pronase
boric acid	cellulolysin
NAD+	macerase
NADH+H+	cellulase II
succinate	phosphate
pyruvate	molybdenum
maleate	zinc
cAMP	sulfate
cGMP	manganese

<u>discoideum</u> spores.

It should be borne in mind that many of the com-
pounds listed in Table 8 can stimulate <u>D. discoideum</u>
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). Addition-
ally, such activation requires that spores be maintained
at very low densities (fewer than 1×10^6/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 germin-
ation have been detected from sources other than auto-
activating 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. aerog-
enes-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 <u>D. discoideum</u>
spore germination released by <u>E. coli</u>.
<u>E. coli</u> 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 <u>D. discoideum</u> 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.
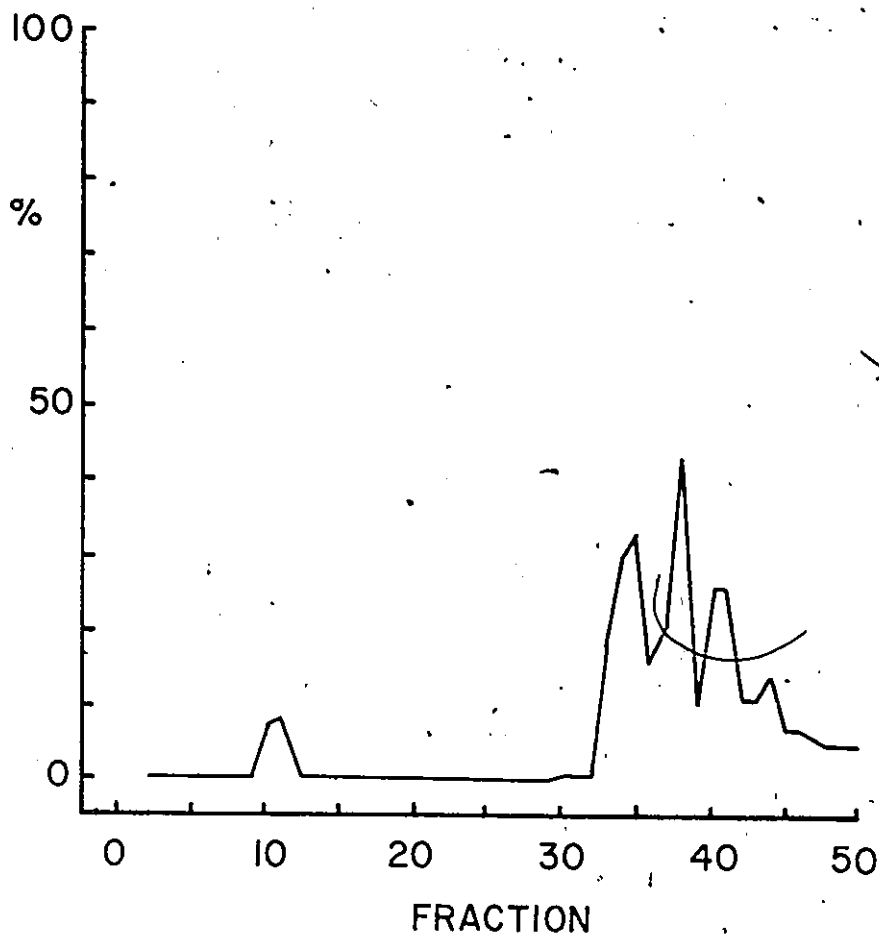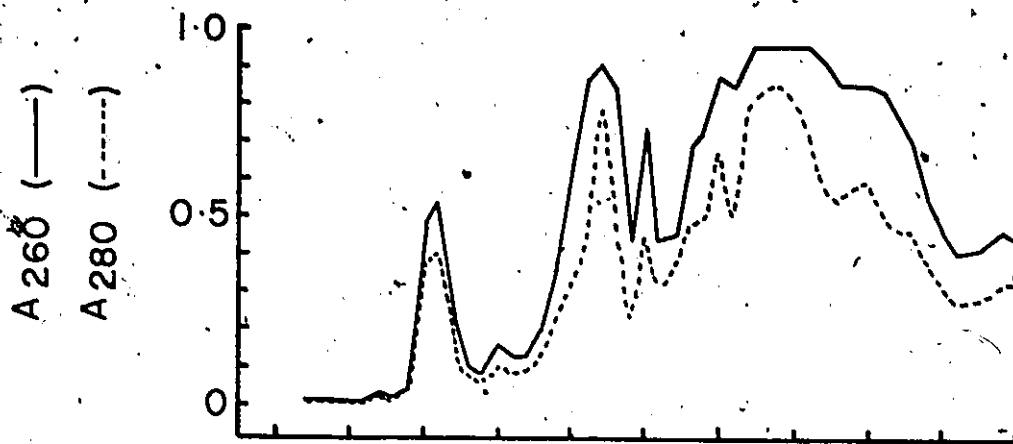
is identical to peak C material since they do not coelute with factor C on a Sephadex LH-20 column (data not shown).

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: sub-
stances 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.

A

B

%

C

D
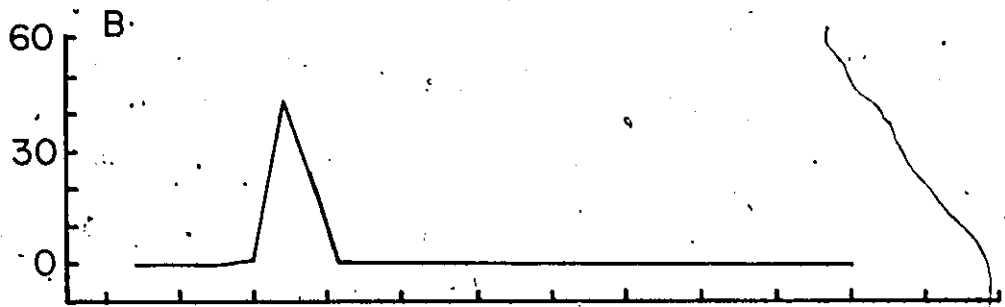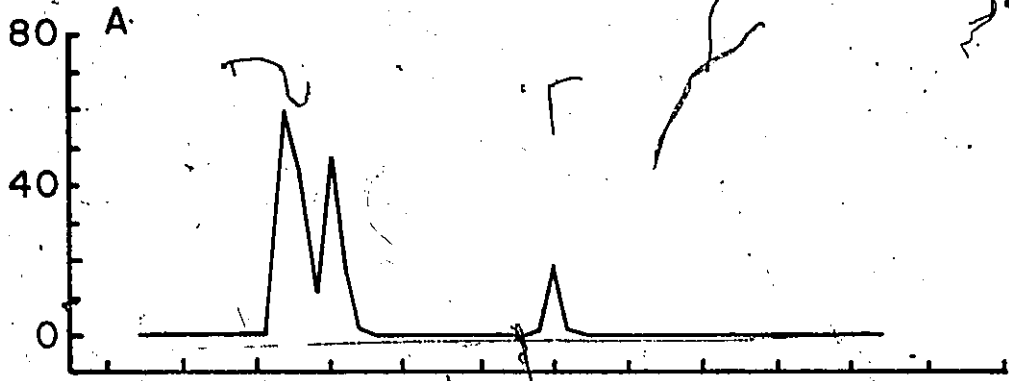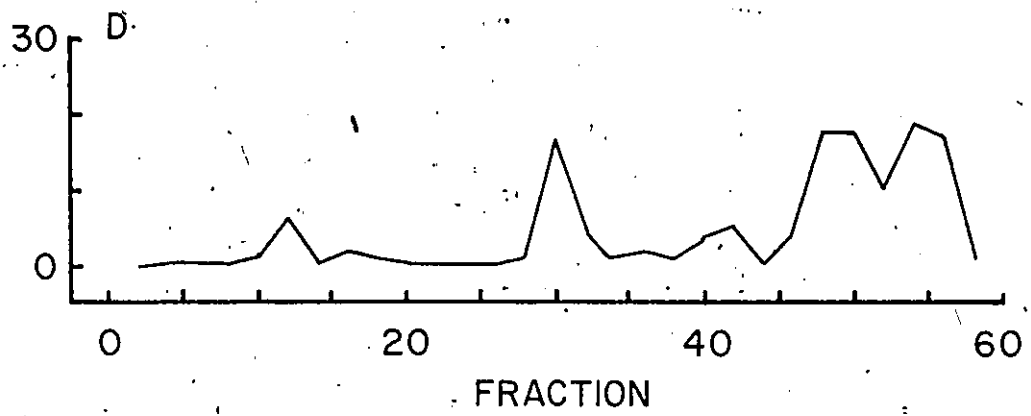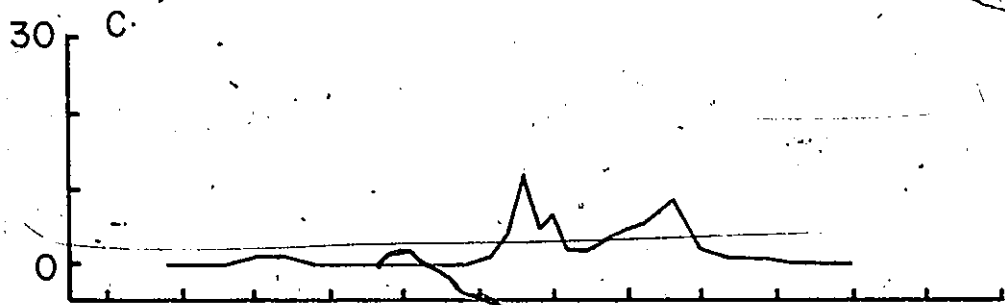
FRACTION

only a single substance corresponding to factor A. The
production of autoactivator factor C-like materials
may correspond to the ability of <u>D. discoideum</u> amoebae
to grow well upon the bacteria. Depratière and Darmon
(1978) have shown that <u>D. discoideum</u> myxamoebae grow
less well upon <u>Sarcina lutea</u> (<u>Micrococcus luteus</u>)than
on other bacteria such as <u>E. coli</u> and <u>E. aerogenes</u>.
Similarly, <u>Pseudomonas aeruginosa</u> produces no type C
material, and also does not support the growth of
<u>D. discoideum</u> amoebae (Fig. 26).

In general then, a variety of bacteria produce
substances which are capable of stimulating <u>D. discoideum</u>
spore germination. While it is unlikely that these
substances are identical to the <u>D. discoideum</u> autoactiv-
ator factors, they may be structural analogs or similar
compounds. The presence of these factors also suggests
a mechanism of <u>D. discoideum</u> 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 <u>Dictyostelium</u> <u>discoideum</u> 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 <u>D.</u> <u>discoideum</u> 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 SG 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 <u>D.</u> <u>discoideum</u> 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 1×10^7/ml germinate synchronously and to high percentages, while spores at 1×10^6/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 1×10^7/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 x 10^6/ml and 1 x 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 autoactiv-

ation 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 synthe-

sis. 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 } x 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 NADH+ H^+
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 require-

ments 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 insen-

sitive 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 synthe-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 comparitively 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 exogen-
ous, 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 distin-
guished 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 continuous-
ly in a state of poised dormancy. Thus, they are capable
of autoactivating at any time they are exposed to per-
missive 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 commun-
ication). 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 consider-
ations 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 altera-
tions in spore permeability. Many dormant spores are
not ametabolic, but rather are hypometabolic. For
instance, dormant _Rhizopus_ _stolonifer_ spores incorporate
^{14}C into various spore components when incubated in an
atmosphere of $^{14}CO_2$ 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 distrib-
ution 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 auto-activator 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 organ-
izes the aggregation of myxamoebae (Loomis, 1975; Newell,
1978), while discadenine is involved in the maintenance
of spore dormancy (Abe et al., 1976). Beyond the specula-
tion 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. Addition-
ally, 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 <u>Dictyostelium</u> <u>discoideum</u> 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 <u>D.</u> <u>discoideum</u>
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 labora-
tory 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 <u>D.</u> <u>discoideum</u> spore germination in nature:
exogenous activation, response to substances released
by bacteria, and autoactivation. Each of these activa-
tion treatments will be considered separately.

    Exogenous activation of <u>D.</u> <u>discoideum</u> 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 (Brock, 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 putre-
scentiae 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 nourish-
ment 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 autoactivation 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. Autoactivation would be delayed by slower maturation rates

due to cooler temperatures. Under such conditions, spores would remain dormant in the soil and would overwinter. Tobler 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.

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