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Effects of restrictive environmental conditions on Dictyostelium discodideum spore germination.

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EFFECTS OF RESTRICTIVE ENVIRONMENTAL CONDITIONS

ON <u>DICTYOSTELIUM</u> <u>DISCOIDEUM</u> SPORE GERMINATION

by

Fred Joseph Garnish

A Thesis
submitted to the Faculty of Graduate Studies
through the Department of
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ABSTRACT

The spore germination process in <u>Dictyostelium</u> <u>discoideum</u> consists of four stages: activation, postactivation lag, swelling and emergence. Spores are reversibly activated by the application of heat and dimethyl sulfoxide. Severe changes in four environmental variables (osmotic pressure, oxygen tension, pH and temperature) are shown to interfere with the germination process. Spores in the lag phase of germination are usually deactivated if exposed to severe environmental conditions and thus do not swell; spores in the swelling and emergence stages may be killed if exposed to severe environmental conditions. The swelling stage of germination is characterized by accelerating rates of respiration and macromolecular syntheses; initiation of trehalase synthesis occurs during this stage. Under all deactivating conditions inhibition of respiration is concomitant with decreased trehalase synthesis in spores which are returning to dormancy. Decreased uridine and amino acid uptake also parallels the reduction of respiratory activity in deactivating spores. Although spores possess a cyanide-resistant electron transport pathway, only the cyanide-sensitive respiratory system is required for germination.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Dr. David A. Cotter for his constant support and guidance throughout the experimental and writing phases of this thesis and for being the unique individual he is.

My appreciation is also extended to my colleagues and friends whose many faceted manners of support made living in Windsor quite tolerable.

Finally, I wish to dedicate this work to my parents whose love and understanding made it all possible.

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INTRODUCTION

History

Dictyostelium discoideum was originally discovered by K. B. Raper (1935) in the decaying leaves of a forest floor in South Carolina. Since then the organism has been found in similar subarboreal environments around the world (Cavender and Raper, 1965a, b, and c, 1968). Raper (1935, 1940a and b, 1941) and Raper and Fennell (1952) were the first to describe the organism's life cycle. Due to the extensive amount of literature that has been compiled concerning the various aspects of the life cycle since then, and because this paper deals primarily with D. discoideum spore germination, only a general overview of the slime mold life cycle is presented here. However, in the case of inspired interest the reader is directed to the fine review of earlier work on the genus Dictyostelium by Bonner (1967) and the more recent review of Loomis (1975) which deals exclusively with D. discoideum.

Life Cycle of D. discoideum

A diagrammatic representation of the cellular slime mold life cycle is presented below for reference (Fig. 1). The vegetative cells of D. discoideum are uninucleate amoebae which feed upon the bacterial flora living in the decaying organic material on the floor of temperate forests. These amoebae replicate by binary fission as other soil amoebae but possess a social aspect to their life cycle which differentiates them from the protozoan genera. Upon depletion of the immediate food supply the cells of D. discoideum congregate in a streaming pattern to form a single mass which may contain up to 10^5 cells. This aggregate integrates itself by producing a cellulose sheath as it or-

1
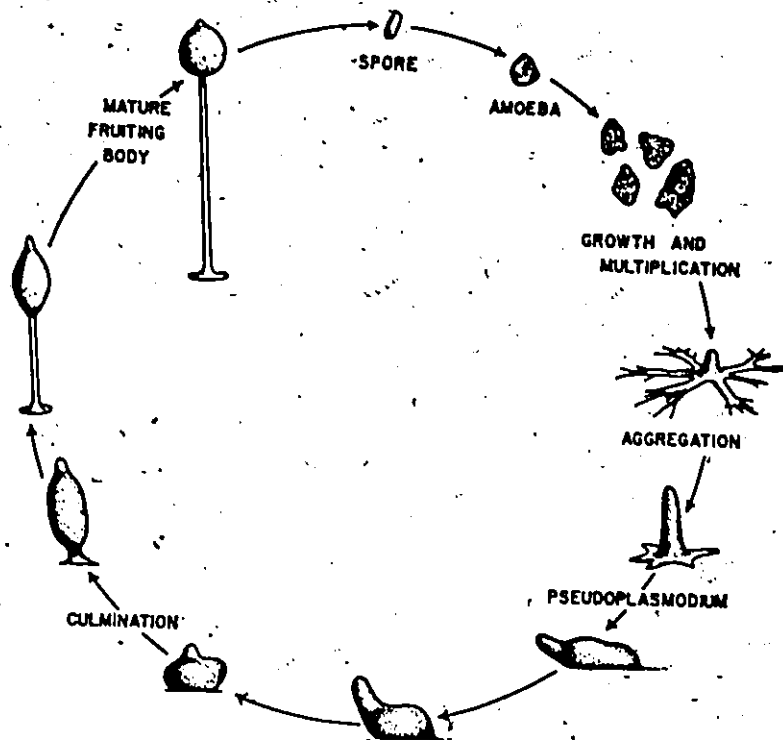
Fig. 1. The Life Cycle of D. discoideum after Wright (1963).

ganizes into a fingerlike structure. This fingerlike mass of cells in-itially rises almost perpendicular to the surface of the substrate and quickly topples over. This aggregate mass is refered to as a pseudo-plasmodium, grex, or slug. After regaining contact with the substra-tum the slug initiates a period of horizontal migration of variable duration which is directionally sensitive to gradients of light, temp-erature and moisture. During the course of late aggregation and early migration the cells in positionally defined areas of the slug become reversibly committed to their final role in the formation of the fruiting body. Specifically, the cells at the tip are tentatively pre-stalk cells and the cells towards the posterior (up to 85% of the to-tal mass) are fated to be spores. When the tip of the pseudoplasmodium comes to a halt the cells in the posterior region migrate directly un-

der the prestalk cells to form a structure likened in appearance to a mexican hat. The anterior cells then begin to produce the outer sheath of the stalk while pushing through the prespore mass, forming the inner sheath of the stalk as they proceed. Final differentiation of the stalk cells entails swelling of the cell while producing a cellulose wall after which death ensues. Prespore cells are carried up the tapering stalk en masse by a mechanism resembling a fountain running backwards. While ascending the developing stalk, the prespore cells differentiate from amoeboid cells into elliptical spores.

General Characteristics of D. discoideum Spores

The spores of D. discoideum are constitutively dormant when formed in the fruiting body. Constitutive dormancy is defined by Sussman (in Sussman and Halvorson, 1966) as:

> "..... a condition in which development is delayed due to
> an innate property of the dormant stage such as a barrier
> to the penetration of nutrients, a metabolic block, or
> the production of a self inhibitor."

In relating this definition to D. discoideum spores one finds that, although these spores are empirically known to be extremely impermeable as compared to vegetative cells, a barrier to the penetration of nutrients per se is not a key factor in dormancy. This holds true because these spores contain all the required energy sources for the completion of germination (Cotter and Raper, 1966). In regard to a metabolic block there is some evidence for the presence of a regulatory protein which acts to restrict respiration of dormant spores (Cotter, 1973, Cotter and George, 1975, Cotter et al., 1976). Self inhibitor substances are also produced by this organism (see Cotter, 1975, Katilus and Ceccarini, 1975, Abe et al., 1976, Nomura et al.,

1977). Such an inhibitor of spore germination has recently been report-
ed to be 3-(3-amino-3-carboxy-propyl)-6-(3-methyl-2-butenylamino)pu-
rine (refered to as discadenine), which inhibits spore germination at
a concentration of 10^{-8}M (Nomura et al., 1977). Recently, a mutant des-
ignated SG1 which stems from a natural mutation, was found to spontane-
ously germinate without an activation treatment when washed free of
autoinhibitory substances (Cotter and Dahlberg, 1977). This finding
demonstrates that the definition of constitutive dormancy may not hold
completely even in the same species because of natural variation. For
example, the SG1 mutant produces self inhibitor substance(s) but lacks
the metabolic block which acts as a secondary mechanism in the mainten-
ance of dormancy in wild type spores.

Aspects of Spore Germination

Optimal conditions for spore germination in a laboratory situation
have been reported by Cotter and Raper (1966). These authors concluded
that spores should be washed and activated in 10 mM phosphate buffer
(pH 6.5); after activation the spores should be incubated at 23.5 C
(this temperature being a compromise between optimal temperatures ob-
served for swelling and emergence). With these parameters in mind then,
germination consists of four stages; activation, postactivation lag,
swelling and emergence. The first stage, activation, may be defined as
those events occuring during the germination inducing treatment. Spore
activation may be accomplished by heat shock, addition of a racemic
mixture of the amino acids tryptophan, phenylalanine and methionine
(Cotter and Raper, 1966, 1968a), gamma irradiation (Hashimoto and Yana-
gisawa, 1970, Khoury et al., 1970, Hashimoto, 1971), addition of eth-

ylene glycol, dimethyl sulfoxide, urea and urea derivatives (Cotter and O'Connell, 1976, Cotter et al., 1976). All of the latter treatments, except for the amino acids, have a common property of acting as protein denaturants. However, as pointed out by Cotter (1975), these amino acids may induce protein conformational shifts with their hydrophobic side chains. In relation to activation, the theoretical site of activation is a set of regulatory proteins (Cotter, 1973, Cotter et al., 1976) located on the inner mitochondrial membrane (Cotter and George, 1975). According to the "multistate model for spore germination" (Cotter, 1973, Cotter et al., 1976), activation treatments induce a partial helix-coil transition in a "dormant" regulatory protein which is responsible for the inhibition of oxidative phosphorylation in dormant spores. The induced transition leads to a "relaxed" state of the protein. The relaxed conformation then allows function of the mitochondrial electron transport system to produce energy in the form of adenosine triphosphate (ATP) required for germination.

Sussman (1976) presented an alternate hypothesis in which he does not disagree with Cotter but postulates a possible role for membrane-associated lipids in spore activation. Using Neurospora and other fungal spore studies as evidence Sussman argued that phase transitions in the lipids of the mitochondrial inner membrane may be responsible for the regulation of respiration during dormancy and spore activation.

Another hypothesis profferred by Bacon and Sussman (1973) suggests that protein synthesis mediated by stable messenger ribonucleic acid (RNA) (which is formed during sporulation) is stimulated through activation. Inhibition of this early protein synthesis by a presumptive autoinhibitor (eg. N, N'-dimethylguanosine) reportedly prevented spore

germination (Bacon and Sussman, 1973). However, attempts to duplicate these results using purified N, N'-dimethylguanosine failed to produce the previously reported inhibition of spore germination (Cotter, 1975). Hohl (1976) suggested a combination of the latter hypotheses which stated that activation allows ATP production which in turn is required to synthesize tryptophan. This amino acid is reportedly absent in dormant D. discoideum spores (Bacon and Sussman, 1973). The tryptophan is then utilized for the synthesis of a spore "swelling" enzyme.

The postactivation lag stage may be divided equally into two phases (Cotter, 1975): early and late. Cotter reported that heat activated spores in early lag phase are prevented from entering late lag phase by incubation in 5% dimethyl sulfoxide (DMSO). Heat activated spores stored at 4 C enter late lag but are prevented from continuing past this phase (Cotter and Raper, 1968b). Spores blocked in such a manner fail to swell even after several days of incubation at 4 C. However, upon release from these conditions, a small fraction of the spores began to swell within 15-30 minutes; the majority of the spores did not swell (Cotter and Raper, 1968b). The majority of the spores treated at 4 C were "deactivated", which is the reversible return of activated spores to dormancy. Thus, spores which enter late lag phase are capable of reacting in one of two alternative ways; they either continue to swell under permissive conditions or deactivate under restrictive environmental conditions (Cotter, 1975). Furthermore, activated spores on the verge of swelling are quickly deactivated when placed under restrictive environmental conditions, whereas activated spores in early lag phase require a considerably longer time period in order to deactivate (Cotter, 1975).

The later stages of germination, swelling and emergence, are marked by distinct morphological changes. Swelling begins with a small lateral protuberance which eventually spreads longitudinally, resulting in an approximate two fold increase in cellular volume. Completion of this stage requires about 90 minutes (Cotter and Raper, 1968a). The optimal temperature for this stage of germination was reported to be near 28 C (Cotter and Raper, 1968a). Emergence constitutes the liberation of a single myxamoeba from the ruptured spore case. The duration of this stage varies with the activation treatment used. For instance, with heat activated spores the emergence stage of germination lasts for about 2 hours, whereas with DMSO activated populations the emergence stage may last up to 5-6 hours. Emergence of myxamoebae is favored at a lower temperature (eg. 22 C) in contrast to spore swelling (Cotter and Raper, 1968a).

Ultrastructural Changes During Germination

There are no observable ultrastructural changes in freshly activated spores (Cotter et al., 1969). The mature spore is surrounded by a three layered wall (Cotter et al., 1969; Hohl and Hamamoto, 1969). Swelling constitutes the production of a lateral protuberance which results in a longitudinal split in the outer two layers of the spore coat (Cotter et al., 1969). During the swelling stage of germination, the mitochondria lose their peripheral coat of ribosomes and their crenated appearance; the tubular cristae become prominent, resembling the vegetative state of this organelle (Cotter et al., 1969). Also during this period, large roundish vacuoles which contain flocular material arise from stacks of flat cisternae. The outer electron dense layer

and the thick middle layer of the spore wall begin to degrade (Cotter et al., 1969, Gregg and Badman, 1970, Hemmes et al., 1972). This degradation may be aided by cellulase present in the spore (Rosness, 1968). Near completion of the process the cell contains one or more contractile vacuoles which function as water expulsion vesicles (Cotter et al., 1969).

There are two hypotheses which attempt to explain the swelling process in D. discoideum spores: one is based upon the synthesis of a specific "swelling" enzyme and the other upon a naturally occuring osmotic pressure increase after activation. The dilation of the vacuoles observed within the swelling spore may play a role in the latter mechanism since this change alone may increase the internal osmotic pressure sufficiently by virtue of an increased concentration of osmotically active molecules. Spore swelling may be induced artificially by treatment of dormant spores in 6 M urea for 24 hours at 23.5 C (Cotter and O'Connell, 1976). Resuspension of spores treated in this manner into buffer without urea results in approximately 86% of the population producing protoplasts surrounded only by the innermost wall layer. This demonstrates that a change in osmotic pressure alone is able to cause the morphological event of swelling. However, this mechanism in naturally swelling spores may be complemented by a spore "swelling" enzyme which increases the internal osmotic pressure through degradation of endogenously stored polymers into their respective monomers (ie. proteins to amino acids or glycogen to glucose) (Hohl, 1976). In fact, there is protein turnover during this stage (Cotter and Raper, 1970). Also noteworthy is the fact that spores of the mutant strain SG1, do not swell in the presence of cycloheximide (Cotter and Dahlberg,

1977). Thus, these two hypotheses are by no means mutually exclusive and may even complement each other.

The emergence stage of germination is marked by the degradation of the innermost layer of the spore coat which releases the myxamoeba (Cotter et al., 1969). The amoeba characteristically clings to the spore case for a short time before becoming completely liberated (Cotter and Raper, 1968b, Cotter et al., 1969, Gregg and Badman, 1970). During this stage the lipoidal bodies with associated electron transparent vesicles and the proteinaceous crystals of the spores degrade, leaving the vegetative amoebae devoid of these structures (Cotter et al., 1969, Maeda and Takeuchi, 1969, Gregg and Badman, 1970). Examination of the discarded spore case reveals that the outermost wall layer remains largely intact, whereas only fragments of the inner and middle layers remain (Cotter et al., 1969, Gregg and Badman, 1970, Hemmes et al., 1972). It is thought that either the spore wall is synthesized with a single line of structural weakness or the protoplast is able to degrade the wall enzymatically without producing gross damage elsewhere (George et al., 1972).

Respiration and Molecular Aspects of Germination

Germination of the vast majority of dormant systems is accompanied by increased respiration and macromolecular syntheses. Oxygen consumption rises markedly after activation of D. discoideum spores (Cotter and Raper, 1968b, Bacon and Sussman, 1973, Cotter and George, 1975, Cotter et al., 1976, Kobilinsky and Beattie, 1977). Activated spores subjected to treatment with any nonpenetrating carbohydrate or polyalcohol at 0.2 M show severely restricted oxygen uptake, eventually re-

sulting in a return to the dormant state (Cotter, 1977). Similarly, the inhibitors of cytochrome oxidase, azide and cyanide also deactivate spores (Cotter and Raper, 1968b, Cotter et al., 1976). Incubation of germinating spores anaerobically also inhibits the continuation of this developmental process (Cotter and Raper, 1968b). These observations strongly suggest that mitochondrial respiration is necessary for the germination of activated spores. In addition, Weber (cited as a personal communication in Cotter, 1975) found that although sporulation may occur on media containing azide, the spores were unable to germinate.

The respiratory competence of D. discoideum spores was recently investigated by Kobilinsky and Beattie (1977). These authors found that dormant spores contained all the structural components necessary for mitochondrial respiration, some of which even exhibited higher activities than the equivalent vegetative counterpart. Several enzymes of the citric acid cycle were also present with specific activity levels comparable to vegetative amoebae. Further, oligomycin-sensitive adenosine triphosphatase (ATPase) activity was found to be approximately equal in both dormant and vegetative cells although total ATPase activity was decreased by 56% in the dormant system. The addition of various respiratory substrates plus oxidized nicotinamide dinucleotide (NAD) to sonically treated spores produced similar oxygen consumption rates when compared to the addition of the same components to sonically treated amoebae (Kobilinsky and Beattie, 1977). The latter observation implies that the lack of cyanide-sensitive respiration in dormant spores may be due to lack of respiratory substrate and/or NAD. On the other hand, the finding by Wright and Wasserman (1964) that pyridine nucleotide levels of vegetative amoebae and fruiting bodies are virtually equal conflicts

with such a conjecture. Alternatively, compartmentalization may function to physically separate these metabolites from intracellular sites where they are normally utilized.

Protein and RNA syntheses in activated D. discoideum spores seem to initiate just prior to swelling and in early swelling respectively (Bacon and Sussman, 1973, Yagura and Iwabuchi, 1976, Giri and Ennis, 1977). Dormant spores of a related species, D. purpureum have been shown to contain small amounts of polyribosomes which increase dramatically upon the entry of activated spores into the late swelling phase (Feit et al., 1971). Giri and Ennis (1977) reported that polyribosomes are detected in activated D. discoideum spores upon the initiation of swelling, which corresponds with the beginning of protein synthesis. Puromycin, cycloheximide, chloramphenicol and amino acid analogs have been reported to block the entry of activated spores into the emergence stage without affecting spore swelling (Cotter and Raper, 1970, Kobilinsky and Beattie, 1977). Trehalase, the enzyme responsible for splitting trehalose (which is a major storage compound in D. discoideum spores) into its glucose monomers, increases in activity during late swelling, thus supplying the required energy for the completion of germination (Ceccarini, 1966, Cotter and Raper, 1970). This activity increase is suppressed in the presence of cycloheximide (Cotter and Raper, 1970). The synthesis of mitochondrial ATPase may also be affected by such inhibitors, since its components are synthesized by both cytoplasmic and mitochondrial ribosome systems, as is found in yeast (Tzagoloff et al., 1973). The ultrastructurally observed detachment of cytoplasmic ribosomes from the mitochondrial periphery and the appearance of inner mitochondrial tubuli still occur in the presence

cycloheximide. However, mitochondria remain crenated (Cotter et al.,
1969). The drug does not inhibit the rupture of the outer and middle
wall layers nor does it affect the reappearance of endoplasmic retic-
ulum and autophagic vacuoles. Breakdown of the innermost wall layer is
prevented by cycloheximide (Cotter et al., 1969). The electron trans-
parent vesicles and associated lipid bodies, as well as proteinaceous
crystals which usually disappear in late swelling or early emergence,
are retained in cycloheximide treated spores (Cotter et al., 1969).
Relief from cycloheximide induced inhibition by washing the spores and
resuspending them in fresh buffer without the antibiotic allows an in-
crease of trehalase activity, breakdown of the innermost wall layer
and finally, the emergence of viable amoebae (Cotter and Raper, 1970).
Myxamoebae may also be artificially released by treatment of the cyclo-
heximide inhibited spores with cellulase and pronase (Hemmes et al.,
1972). These results seem to suggest that a proteolytic enzyme which
is responsible for degradation of the innermost wall layer is synthe-
sized late in the swelling stage. This enzyme allows the emergence of
the myxamoebae (Cotter et al., 1969, Hemmes et al., 1972).

Actinomycin D, an inhibitor of RNA synthesis, has been observed
to inhibit 20% of an activated spore population from emerging at a con-
centration of 1 mg/ml after 5 hours of incubation at 23.5 C (Cotter,
PhD. Dissertation, Univ. Wisconsin, 1967). In spite of one report to
the contrary (Bacon and Sussman, 1973), lower concentrations of actino-
mycin D are ineffective at producing any overt inhibition of D. dis-
coideum spore germination (Cotter and Raper, 1966, 1970, Yagura and
Iwabuchi, 1976, Giri and Ennis, 1977). However, de novo ribosomal RNA
(rRNA) synthesis is specifically inhibited in vegetative amoebae of

strain AX3 treated with a relatively low concentration of the drug
(Firtel and Lodish, 1973). Yagura and Iwabuchi (1976) have presented
evidence that predominently messenger-like RNA (mRNA) and 4-5 S RNA
species are formed in the earlier phase of swelling whereas rRNA syn-
thesis is initiated in the later phase of spore swelling. This presump-
tion is corroborated by another recent observation that in the early
swelling phase the activity of the deoxyribonucleic acid (DNA)-depend-
ent RNA polymerase II, which is thought to function in non-rRNA synthe-
sis, is much higher in activity than that of polymerase I, which is
responsible for rRNA synthesis. This trend is reversed in the late
phase of spore swelling (Yagura et al. in Yagura and Iwabuchi, 1976).
Giri and Ennis (1977) concluded that RNA synthesis is required for
swelling of activated spores. However, thiolutin, the one drug which
did prevent swelling, also inhibited the incorporation of ^{3}H-uracil by
97%. Since thiolutin is a relatively new drug perhaps the observed in-
hibition of uracil uptake was not due to specific prevention of RNA
synthesis but an inhibition of transport systems. Likewise an unexpect-
ed inhibition of some other cellular mechanism(s) may have been respon-
sible for the observed inhibition of spore swelling. Two other inhibi-
tors of RNA synthesis used in their study, lomofungin and daunomycin
also decreased incorporation of ^{3}H-uracil by 98 and 99% respectively,
with no effect upon the swelling of the treated spores. If one believes
that the incorporation of labelled precursors is a true reflection of
the events occuring during such a developmental process, then the con-
clusion that RNA synthesis is an absolute requirement for spore swel-
ling would seem to be erroneous. Heat activated spores devoid of func-
tional DNA have been observed to swell but were prevented from emerging

(Cotter, 1975). This could indicate that <u>de novo</u> RNA synthesis is required for the production of the protein(s) which mediate the final stage of germination (eg. emergence) but would refute the necessity of such synthesis being required for the swelling stage.

Purpose of This Study

The proposed mechanism of reversible activation along with other observations in the above text (including the almost immediate increase of respiration of activated spores) implicates the mitochondrion as a prime candidate for being the crucial site which regulates dormancy in <u>D</u>. <u>discoideum</u> spores. Once initiated, spore germination seems to require some <u>de novo</u> protein and RNA syntheses for completion of only the later stages of development. With these premises in mind, the purpose of this study was to examine the physiological consequences of various defined restrictive environmental conditions in an attempt to characterize the phenomenon of reversible spore activation.

MATERIALS AND METHODS

Media Used

Cultures of <u>Dictyostelium discoideum</u> were grown in conjunction with their bacterial host <u>Escherichia coli</u> strain B/r on glucose salts agar (Adams, 1959). This medium contains the following components; 1.0 g NH_4Cl, 0.13 g $MgSO_4$, 3.0 g KH_2PO_4, 6.0 g Na_2HPO_4 and 20.0 g bacto agar (Difco) per liter of distilled water. A total of 4.0 g of glucose, which was sterilized separately in solution, was added to the medium after autoclaving. The medium was cooled to 50 C and dispensed into petri dishes (Fisher brand, 100 X 15 mm).

The phosphate buffer used consisted of; 1.04 g KH_2PO_4, in 1 liter

of distilled water; the pH of this 10 mM solution was adjusted to 6.5
with concentrated KOH prior to autoclaving.

Production of Spores

Spores of <u>Dictyostelium discoideum</u> (Raper, 1935) strain NC4 (dip-
loid) were picked from a stock culture with an inoculating loop and
added aseptically to sterile 100 ml culture bottles containing 20-60 ml
of distilled water bringing the spores to approximately 10^4/ml. A loop-
ful of <u>E</u>. <u>coli</u> strain B/r was also added to this suspension and mixed
thoroughly; 2 ml of this mixture was then transferred to glucose salts
agar plates and spread over the surface. The cultures were shaken at 24
h and 48 h after initial plating to ensure even and synchronous fruiting
of the slime-mold. By the third day aggregation centers were apparent
and on the fourth day mature fruiting bodies had been produced. Fruit-
ing bodies were allowed to age for an additional day before spores were
harvested; this ensured that all spores used in experimental work were
dormant.

Preparation of Spores for Experimental Manipulation

Cultures of <u>D</u>. <u>discoideum</u> NC4 (diploid), 1-3 days old, were har-
vested by passing a glass slide over the agar surface in such a way as
to contact and thereby collect only the spores from the stalks of the
fruiting bodies. The spores were suspended in 5 ml of sterile distilled
water and poured into a 15 ml conical or round bottom centrifuge tube
(Corex brand). Any spore clumping was then eliminated by vortexing and
the suspension was subjected to centrifugation in a Damon/IEC clinical
centrifuge (model CL) at setting 6 for 5 min. The resulting supernatant
was saved for possible studies on the autoinhibitor substance(s)

while the pellet was resuspended in fresh 10 mM phosphate buffer (referred to as buffer hereafter). This suspension was again centrifuged for 5 min. as before and resuspended in fresh buffer; the cycle was repeated once more for a total of 3 washings (Cotter and Raper, 1966).

Activation of Dormant Spores

Washed dormant spores were activated by either of two methods: heat shock or treatment with dimethyl sulfoxide (DMSO) (Fisher Scientific Co.).

Heat shock involved the immersion of the 5 ml spore suspension, usually contained in a 15 ml conical bottom centrifuge tube, into a circulating waterbath preheated to 45 ± 0.1 C. The tube was adjusted in the bath so that the meniscus of the spore suspension was at least 2 cm below the surface of the water to ensure uniform heating for the required 30 min. treatment (Cotter and Raper, 1968a). After the activation period the suspension was quickly cooled and the spores adjusted to the desired concentration by addition of buffer.

DMSO activation was accomplished in the following manner; after the third washing of the newly harvested spores the pelleted spore mass was resuspended in 5 ml of a premixed 20% DMSO-buffer solution. This suspension was then incubated for 60 min. at ambient temperature. At 55 min. into the activation period, the spores were centrifuged at setting 6 for 5 min. and the supernatant containing DMSO was discarded. The sides of the tube were wiped with paper towelling to remove residual DMSO. The pelleted spores were then resuspended in fresh buffer to terminate the activation treatment at 60 min. and subsequently washed 2 times to remove any trace of DMSO (Cotter et al., 1976).

Time zero is defined here as the time at which the activation treatment was terminated.

A Brightline hemacytometer was used in all determinations of spore concentration.

In all experiments spores were kept in suspension by small (3 mm) stirring bars (Tri-R Corp.) placed into the tubes which were propelled by a submersible stirring unit (Tri-R Corp., model MS-7). Stirrer speed was such as to prevent settling of spores and to facilitate gas exchange.

Monitoring Spore Germination

In all experiments samples of the incubating spore suspensions were removed with short tipped Pasteur pipets (Fisher brand) and sandwiched between a cover glass and slide. At least 200 individuals were counted under a Zeiss phase contrast microscope with a final magnification setting of 320 X. The individuals enumerated were tallied into one of three classes; i) unswollen, phase bright spores, ii) swollen phase dark spores, or iii) emerged myxamoebae.

Production of Restrictive Environmental Conditions

Restrictive environmental conditions are defined as those conditions which if imposed after activation do not allow normal developmental stages of spore germination (eg. swelling and emergence) to occur. These include; high (37 C) and low (0 C) temperature, pH extremes of 2 and 12, high osmotic pressure (0.25 M sucrose), anaerobic conditions and the presence of the inhibitors sodium azide and potassium cyanide (refered to as azide and cyanide hereafter).

To treat spores at 37 C (whether activated or not), the spores

were placed in a preheated circulating waterbath (Exatherm, model P5)
or for oxygen uptake studies, into a YSI model 5301 standard bath assembly connected to a Haake model FE circulator set at 37 C. Only 2-4 min.
were required for spore suspensions of 2-5 ml to reach equilibrium with
the surrounding 37 C water. Low temperature (0 C) was achieved by placement of the sample tube into an ice bath which was left under refrigeration (4-7 C). Under these conditions it was necessary to add more ice
every 12 h.

The pH extremes were created by the addition of half strength HCl
or concentrated KOH to a 50 mL beaker containing at least 30 ml of buffer. The pH was monitored with an Orion (model 407A) ionalyzer while
the solution was being mixed by a magnetic stirrer. These pH-adjusted
solutions were added to activated spores which had been pelleted by
centrifugation.

In the osmotic pressure experiments 0.5 M sucrose (Fisher Scientific Co.) in buffer was added to activated spore suspensions to yield a
final concentration of 0.25 M.

Azide and cyanide (Fisher Scientific Co.) were mixed in buffer to
a concentration of 2×10^{-2}M. These stock solutions were added to activated spore suspensions to produce a final concentration of 2×10^{-3}M
(2 mM) except in the trehalase assay experiments. In the latter, a final concentration of 3 mM was used for both azide and cyanide to ensure
100% inhibition of the concentrated spore populations.

Anaerobic conditions were produced by the addition of preboiled
buffer (cooled under a nitrogen atmosphere) to activated spores which
had been pelleted by centrifugation. Upon resuspension to a volume of
8 ml (8 ml being the maximum volume accommodated by the YSI system for

efficient monitoring of dissolved oxygen) and a spore concentration of 10^7 spores/ml or greater, the spore mixture was poured into a YSI sample chamber and N_2 gas (99.53% N_2 - 0.47% CO_2 mixture) was bubbled through the liquid for 2 min. The oxygen probe was then placed into the chamber under a constant stream of the gas mixture to prevent introduction of laboratory air. A constant stream of N_2 gas was also passed into the sample chamber through the access slot of the probe via a 20 ga. hypodermic needle; this ensured against the introduction of oxygen into the treated suspension during incubation. Under these conditions it was possible to obtain and hold a 2% or less (<0.15 μl O_2/ml) air saturation level in the chamber at 23.5 C. Samples were taken from the chamber with pipets under a stream of N_2 gas, which maintained the low air saturation level.

Deactivation Experiments

Spores were shocked in 20% DMSO for 60 min. and washed as previously described. Upon final resuspension of the spore population in fresh buffer, 1-2 ml aliquots were taken immediately after the final wash and at 10, 20, 30, 40 and 50 min. from time zero. These aliquots of the original spore suspension were transfered to glass tubes (10 x 100 mm) and subjected to the various restrictive conditions under study. In the case of 0.25 M sucrose treatments spores were heat shocked, otherwise the procedure was the same as above. The remainder of the stock suspension was used as a control. In all cases the spores were kept in suspension by small stirring bars as mentioned previously. Incubation temperature was maintained at 23.5 C in all cases except for temperature shift experiments by a circulating waterbath.

After the appropriate incubation period under restrictive conditions (1-24 h depending upon the particular experiment) the spores were relieved from their restrictive environmental condition by washing the population two times in fresh buffer or by a temperature shift to 23.5 C. The treated spores were scored for germination by microscopic count immediately after washing or temperature shift and again after 2-4 h incubation at 23.5 C to determine the final percent germination. Samples in which the majority of the population remained unswollen were tested for viability the following day. These spores were heat shocked and allowed to germinate for at least 3 h at 23.5 C. The samples were then scored for percent germination by microscopic examination.

Survival Experiments

Activated spores were adjusted to a concentration of 2-4 x 10^6/ml in a conical bottom centrifuge tube. An 8 ml volume was placed into a 23.5 C circulating waterbath for most experiments (37 C was used for high temperature experiments and a 0 C ice bath was utilized in low temperature experiments) and stirred as in previous manipulations. Aliquots of 2 ml were removed at three time periods during the course of the germination process, these were, specifically, during the following stages: i) early swelling, 10-20% of the population being swollen, ii) mid-swelling, 45-55% of the population being swollen and iii) late swelling, 90-100% of the population being swollen. These aliquots were placed under the various restrictive conditions for a period of 5 h. The spores were relieved from restrictive environmental conditions by washing the inhibitory components out or by a shift back to 23.5 C after the treatment period. Vegetative amoebae removed from the remaining stock suspension were also treated under the restrictive cond-

itions for 1 or 5 h periods and subsequently manipulated similarly to the treated spores. Once relieved, 0.5 ml of the suspension, which was readjusted to a cell concentration of $1-2 \times 10^6$/ml, was transferred to non-nutrient purified agar (Difco, 2%) plates and spread over the surface by agitation. The plates were incubated at room temperature for a period of 14 h and then were examined under a compound microscope at 125 X. The first 200 objects were counted (excluding spore cases) and the respective percentages of unswollen, swollen and emerged amoebae calculated. Unswollen spores may be considered as viable provided they responded to a second activation treatment in separate experiments. Swollen spores, on the other hand, were considered as nonviable since they did not complete germination after such a prolonged incubation period under permissive conditions. Amoebae represent spores which were able to complete the germination process and hence were considered as viable survivors.

Controls included activated spores plated directly from non-treated populations and dormant spores plated after the third wash of the post-harvest treatment.

Respiration Experiments

Oxygen uptake of D. discoideum spores was recorded by a YSI model 53 oxygen monitor which was calibrated to 100% with air-saturated buffer and to 0% by the addition of a few crystals of dithionite (Baker Chem. Co.) to nitrogen-saturated buffer. Temperature was maintained in a YSI model 5301 standard bath assembly coupled to a Haake model FE circulator. Temperatures remained stable to within ± 0.1 C in all cases. All experiments were maintained at 23.5 C except for 37 C treatments.

In these experiments, the spore suspensions used were adjusted to 5 ml volumes containing a spore concentration of $3-5 \times 10^6$/ml. Oxygen consumption was recorded as a function of percent air saturation at 30 min. intervals. During the course of an experiment if the air saturation fell below 55%, the sample was reaerated by bubbling filtered laboratory air through the spore suspension for a period of 30 sec. With this procedure saturation levels of up to 98% were reached, alleviating the problem of possible inhibition of spore respiration due to low oxygen tension.

The inhibitor of the alternate electron transport system, salicylhydroxamic acid (Aldrich Chem. Co.) was dissolved to a final concentration of 5×10^{-2}M in alkaline buffer (0.5 ml of 6N KOH in 4.5 ml buffer) and subsequently titrated to pH 7 with 6-10 drops of concentrated HCl. The uncoupler, 2,4-dinitrophenol (Nutritional Biochem. Corp.) was also dissolved in a slightly alkaline buffer solution (5 drops of 6 N KOH in 10 ml buffer) to a concentration of 2.5×10^{-2}M. Dilution of this stock solution to a working concentration of 3.75 mM did not shift the final pH of the spore suspension.

In all experiments spores were kept in suspension by small stirring bars (Tri-R Corp.) placed inside the sample chamber rather than the stirring discs supplied with the instrument by YSI. The small stirring bars were efficient and had a smaller mass which avoided the possible problem of spore breakage.

All results were corrected to represent the respiration of spore populations at a concentration of 10^7 spores/ml. The values reported for percent reduction in cumulative oxygen uptake were computed by subtracting the amount of oxygen taken up by the electrodes from the a-

mounts of oxygen utilized by the spore samples and then calculating a comparative ratio from the corrected values.

Radiolabeling Experiments

Algal protein hydrolysate (^{14}C-labelled, ICN Pharmaceuticals) and ^{3}H-uridine (ICN Pharmaceuticals) were used at 1 µCi/ml to determine the uptake kinetics of heat activated spores incubated at 23.5 C.

Protein and RNA syntheses of activated spores under the various restrictive environmental conditions were evaluated by incorporation of ^{14}C-leucine and ^{3}H-uridine (ICN Pharmaceuticals) respectively. After activation the spores were adjusted to approximately 10^{7} spores/ml. Labelled precursors were used at 1 µCi/ml. Streptomycin sulfate (Sigma Chem. Co.) was added to all samples at a final concentration of 25 µg/ml to inhibit uptake by bacterial contamination that may have been present. Zero time and 5 h samples were taken in triplicate. Each sample constituted 0.2 ml of the spore suspension removed by a 200 ul Eppendorf micropipet which was injected into 5 ml of 10% trichloroacetic acid (TCA) (Fisher Scientific Co.)- 5% acetone solution. The insoluble material was precipitated in hot TCA (90 C, for 20 min. for protein studies) or cold TCA (0 C, for RNA studies) as described by Yagura and Iwabuchi (1976). The precipitated material was collected on 24 mm glass fiber filters (Whatman, GF/A) and washed with four 5 ml aliquots of cold 5% TCA including 1 mM leucine (Sigma Chem. Co.) or 1 mM uridine (Sigma Chem. Co.). The filters were dried under a 250 watt Sylvania infrared heat lamp attached to a base with rotating disc holders (Atomic Development Corp.) and placed into glass scintillation vials (Isolab, 24mm) containing 5 ml of scintillation fluid. The particular fluid used was

toluene-based with 0.4% (w/v) 2,5-diphenyloxazole as a primary fluor
and 0.01% (w/v) p-bis-(2(5-phenyloxazolyl))-benzene (ICN Pharmaceutic-
als) as a secondary fluor. Each sample was counted for a period of 10
min. in a Nuclear-Chicago Mark II liquid scintillation counter set on
the desired preset channel (eg. ^{14}C or ^{3}H). All results were standard-
ized to a 10^{7} spore/ml concentration and corrected for background for
purposes of comparison.

Trehalase Assays

Spores were harvested, washed and heat activated. In these exper-
iments it was necessary to obtain final spore concentrations of 2-3 x
10^{7}/ml in each treated sample since the assay was dependent upon the
amount of protein in the reaction mixture.

In the case of control spores and those treated with sucrose, az-
ide, cyanide and pH 2 and 12, incubation periods of 6 h at 23.5 C were
utilized. Other activated preparations were incubated at 37 C for 6 h
and in the case of 0 C treatment, spores were allowed a 72 h incubation
period before further manipulation. After the incubation period the
samples were centrifuged at setting 7 in an IEC clinical centrifuge for
at least 4 min. The supernatants were discarded and the pellet resus-
pended in 0.1 M citrate buffer (pH 5.5). The spore suspensions were
then subjected to disruption using an Aminco French pressure cell
which had been precooled in crushed ice for a period of at least 30 min.
The pressure in the Aminco device was raised to 20,000 psi and then
slowly released; the procedure was then repeated to ensure spore break-
age. This treatment resulted in greater than 95% spore breakage in
every case except for pH 12 treated samples. In the case of pH 12 treat-

ed samples it was necessary to disrupt the spores with glass beads according to the method of Dr. J. Van Etten (unpublished). In this treatment, 5 g of solid glass beads (0.25-0.30 mm in diameter; Arthur H. Thomas Co.) were added to pelleted spores in a round bottom centrifuge tube (Corex brand). Citrate buffer (0.1 M) was added to the tube so that the meniscus of the liquid reached approximately 1 mm above the surface of the beads. The tube was then vortexed on the fastest setting of a Scientific Products deluxe mixer for a period of 2 min. At least 50% breakage was accomplished by this method.

The disrupted samples, which were stored in ice between manipulations, were centrifuged at 8,200 x g for 10 min. in a Sorvall model RC2-B centrifuge fitted with a small rotor. The supernatant was decanted off into clean tubes and stored in ice prior to the enzyme assay.

The enzyme trehalase was assayed according to the method of Ceccarini (1966), later modified by Cotter and Raper (1970). Further modification of the procedure was necessary here and is noted in the protocol. The assay procedure was as follows;

1. The incubation mixture contained: 0.3 ml of 0.1 M citrate buffer (rather than 0.6 ml) at pH 5.5, 0.5 ml of 50 μM/ml trehalose (Sigma Chem. Co.) and 0.4 ml (instead of 0.1 ml) of enzyme extract.

2. Incubation was for 30 min. at 35 C.

3. The reaction was stopped by boiling for 10 min.

4. The mixture was cooled and brought to pH 7 with 0.8 ml of 0.1 M potassium phosphate.

5. To each sample, 2.0 ml of Glucostat Special reagent (Worthington) was added.

6. Incubation was for 45 min. at 35 C.

7. The absorbance of the samples was read at 400 nm in a Beckman model DB spectrophotometer (instead of a Spectronic 20) using 1.5 ml quartz cuvettes (Thermal Syndicate Ltd.).

Controls included in each assay run were: i) heated enzyme, which was used as a measure of the amount of glucose present in the spore extracts, ii) blanks without enzyme but containing all other components and iii) a standard with 1 μM of glucose.

One unit of enzyme activity was equivalent to 1 μM of glucose released in 30 min. at 35 C. The specific activity was determined by dividing the milligrams of protein in the extract into the units of activity.

Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin (Sigma Chem. Co.) as the standard protein.

RESULTS

Kinetics of Spore Germination

The kinetics of germination elicited by the two activation treatments used in this study (eg. heat and DMSO) are shown by the data of Fig. 2. The postactivation lag phase constituted a 30-40 min. period with DMSO treatment, whereas an extended lag of 55-65 min. was observed for heat activated spores. The diagrammatic scheme of spore morphology included in Fig. 2 represents the temporal sequence of development induced by heat activation. In DMSO activated cultures the spore swelling phases would be condensed to accomodate the shortened lag while emergence would extend over a longer period of time, since this stage was asynchronous.

Fig. 2. The germination kinetics of spores activated by heat and DMSO treatments. Spores 1-2 days old were activated by heat (45 C for 30 min.) or 20% DMSO (for 60 min.) and incubated at 23.5 C. Spore swelling and emergence were monitored at the indicated time intervals by microscopic examination. Included is a diagrammatic representation of the respective morphology of the organism as a function of time in a heat shocked population. Note that swelling is slightly accelerated in the DMSO activated culture, however, emergence is asynchronous. Symbols represent: ○, percentage of swollen spores in the heat shocked culture; ●, percentage of emerged myxamoebae in the heat shocked culture; △, percentage of swollen spores in the DMSO activated culture; ▲, percentage of emerged myxamoebae in the DMSO activated culture.
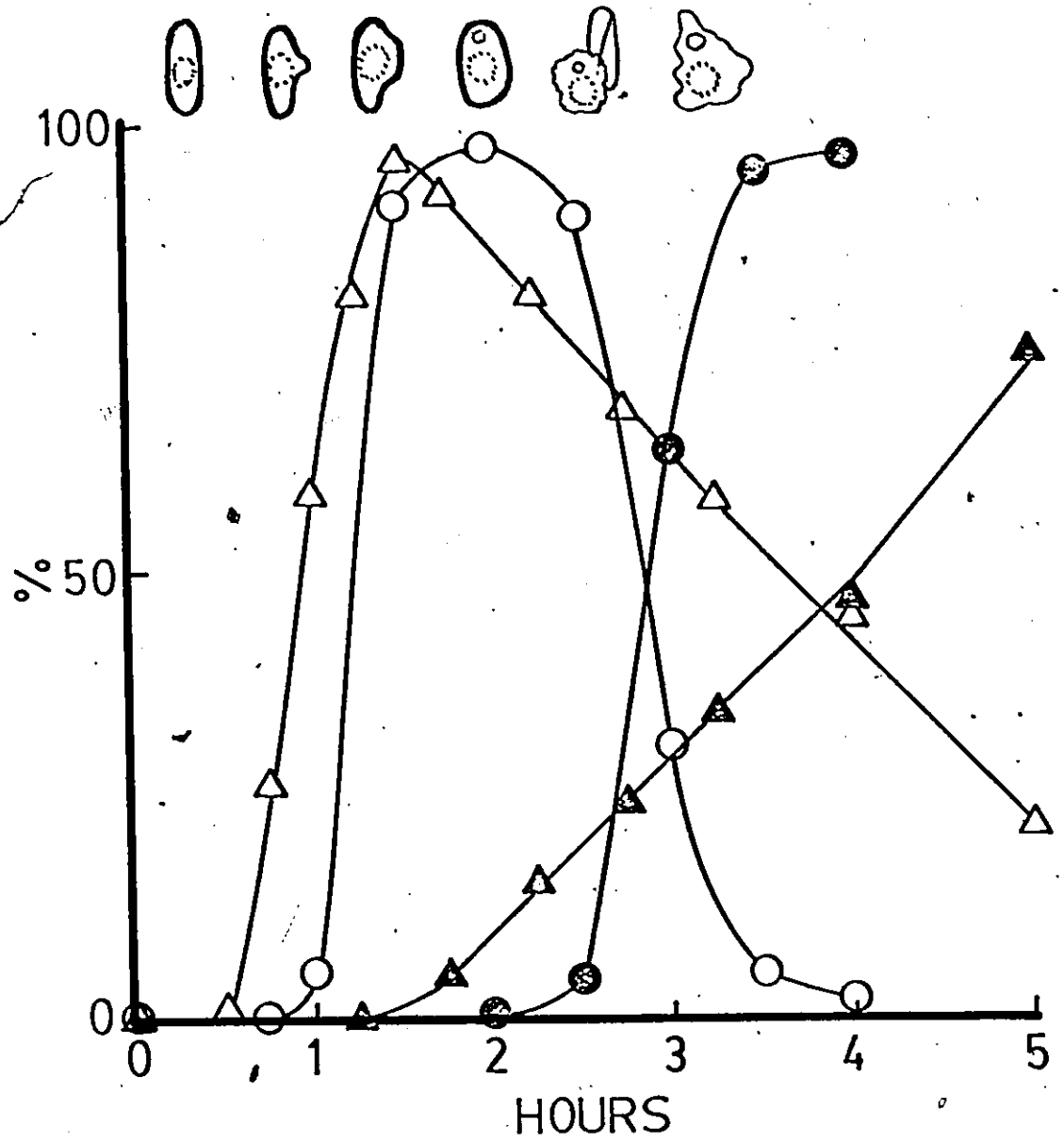
Deactivation Experiments

Treatment of activated spores with low temperature results in their return to dormancy (deactivation) as previously shown by Cotter et al. (1976). Cotter (1977) observed that activated spores within 7 min. of the initiation of swelling were insensitive to unfavorable shifts in their environment and thus the spores continued to swell. Spores within the time period just prior to 7-10 min. of the initiation of swelling were most sensitive to the temperature shift from 23.5 C to 0 C; that is, the greatest spore deactivation occurred in the 20 min. sample (Fig. 3). Spores shifted into 0 C before this point did not deactivate to the same extent. Conversely, spores incubated for 30 min. or more at 23.5 C continued to swell after their shift to 0 C indicating that the spores were committed to swell. Deactivated spores were able to be reactivated by a second activation treatment (45 C for 30 min.).

High temperature (37 C) incubation of DMSO activated spores caused deactivation more quickly than low temperature incubation. The data of Fig. 4 demonstrate that after 1 h treatment at 37 C the majority of the treated population was deactivated. As with low temperature incubation the spores that were near the initiation of swelling were most sensitive to the temperature shift and again the 20 min. sample yielded the highest percent of deactivated spores; the relationship was not as clear as that shown in the low temperature experiments because of the rapid spore deactivation at 37 C. The spores which had returned to dormancy were viable as demonstrated by a high percentage of germination after a second activation treatment.

The effect of pH on the germination of DMSO activated spores is

Fig. 3. Deactivation of spores incubated at 0 C. Spores were activated

with DMSO and incubated at 23.5 C. Aliquots of this activated stock

suspension were removed as close as possible to time zero for the first

sample and subsequently every 10 min. for up to 50 min. and placed at

0 C. After 24 h each aliquot was shifted back to 23.5 C and incubated

for 2 h at which time the final percent germination was determined.

Symbols represent: ○ , control spores at 23.5 C; △ , aliquots of

spores shifted to 0 C for 24 h which were then incubated at 23.5 C for

2 h.

Fig. 4. Deactivation of spores incubated at 37 C. Spores were activated
with DMSO and incubated at 23.5 C. Aliquots of this activated stock
suspension were removed as close as possible to time zero for the first
sample and subsequently at 10 min. intervals for up to 50 min. and
placed at 37 C. After a 1 h incubation at 37 C each aliquot was shifted
back to 23.5 C and incubated for 4 h; the final percent germination was
then determined. Symbols represent: ◯ , control spores at 23.5 C;
 △ , aliquots of spores shifted to 37 C for 1 h which were then in-
cubated at 23.5 C for 4 h.

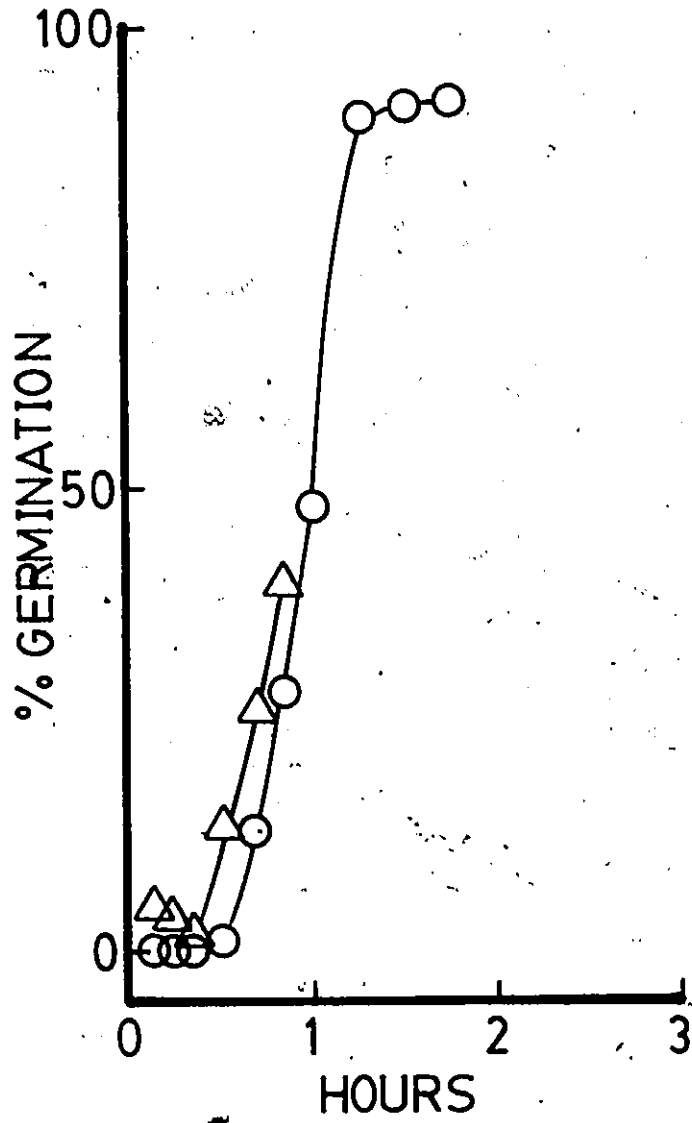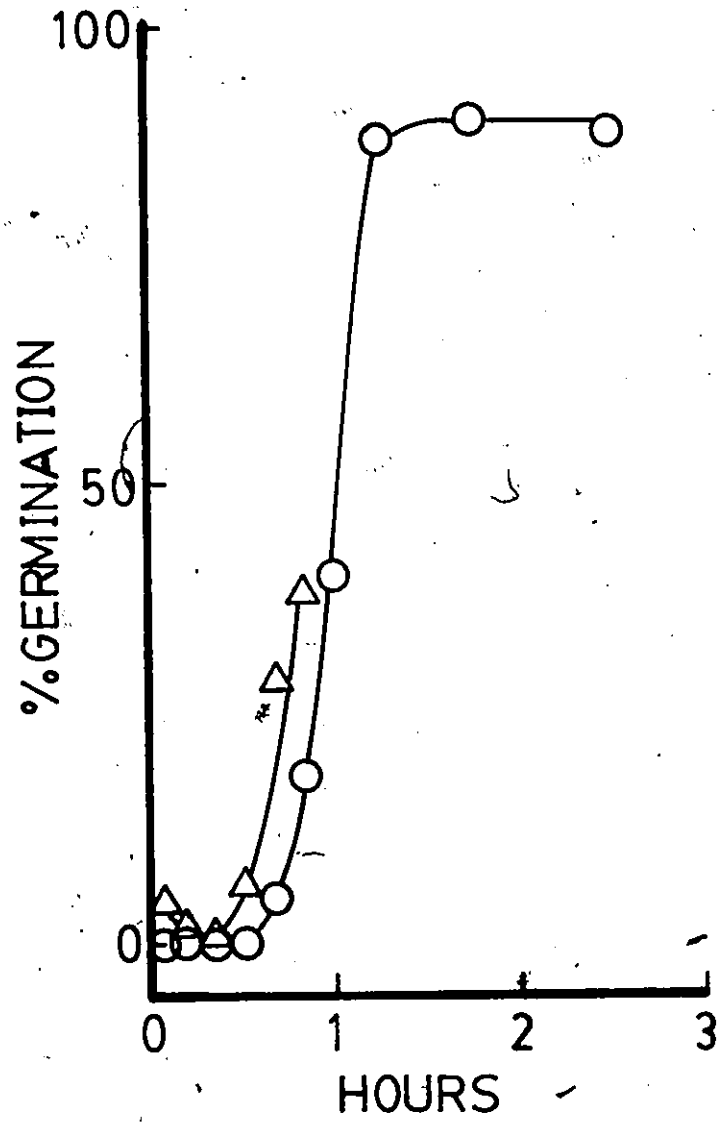shown in Fig. 5. Only the extremes of pH 2 and 12 seemed to efficient-
ly hold activated spores from swelling. The slightly less extreme pH
values of 3 and 11 considerably slowed swelling and caused some deacti-
vation of the treated population. Below pH 10 or above pH 4 little ef-
fect upon the rate of swelling was observed (data not shown). However,
slight lowering of the emergence rate was noted near the latter pH val-
ues. Even more pronounced than the lowering of the rate of swelling was
the decline in the rate of emergence in the pH ranges of 3-4 and 10-11.
Treatment of activated spores at the extremes of pH 2 and 12 resulted
in loss of viabilty in the majority of the population (ca. 80%). The
remainder of the population (ca. 20%) was able to germinate after a
second activation treatment.

The deactivation profile generated by 0.25 M sucrose treatment
(Fig. 6) agrees with the data of Cotter (1977), who also demonstrated
similar effects with other nonpenetrating carbohydrates and polyalco-
hols. Once again the spores closest to the period 7-10 min. prior to
the initiation of swelling were most sensitive to deactivation, as
shown in Fig. 6. Deactivated spores were viable since they were able to
germinate upon a second activation treatment.

Heat activation was utilized in this particular experiment since,
unlike heat activated spores, a fraction (ca. 20%) of the spores acti-
vated with DMSO and introduced into 0.25 M sucrose continued to swell.
This phenomenon was not unique to this study but had also been observed
in previous work (Cotter, personal communication). Since 20% DMSO may
change the permeabilty of the spores it was suggested that such a
change was responsible for the above enigma. Further investigation re-
vealed that 0.5 M sucrose prevented 100% of DMSO activated spores from

Fig. 5. Effect of pH on the kinetics of spore germination. Spores were
activated with DMSO and placed into tubes containing buffer adjusted
to various pH values. Symbols represent: ○ , control spores at pH
6.5; ● , spores at pH 2; △ , spores at pH 3; □ , spores at pH
4; ⬡ , spores at pH 12; ■ , spores at pH 11 and; ▲ , spores at pH
10.
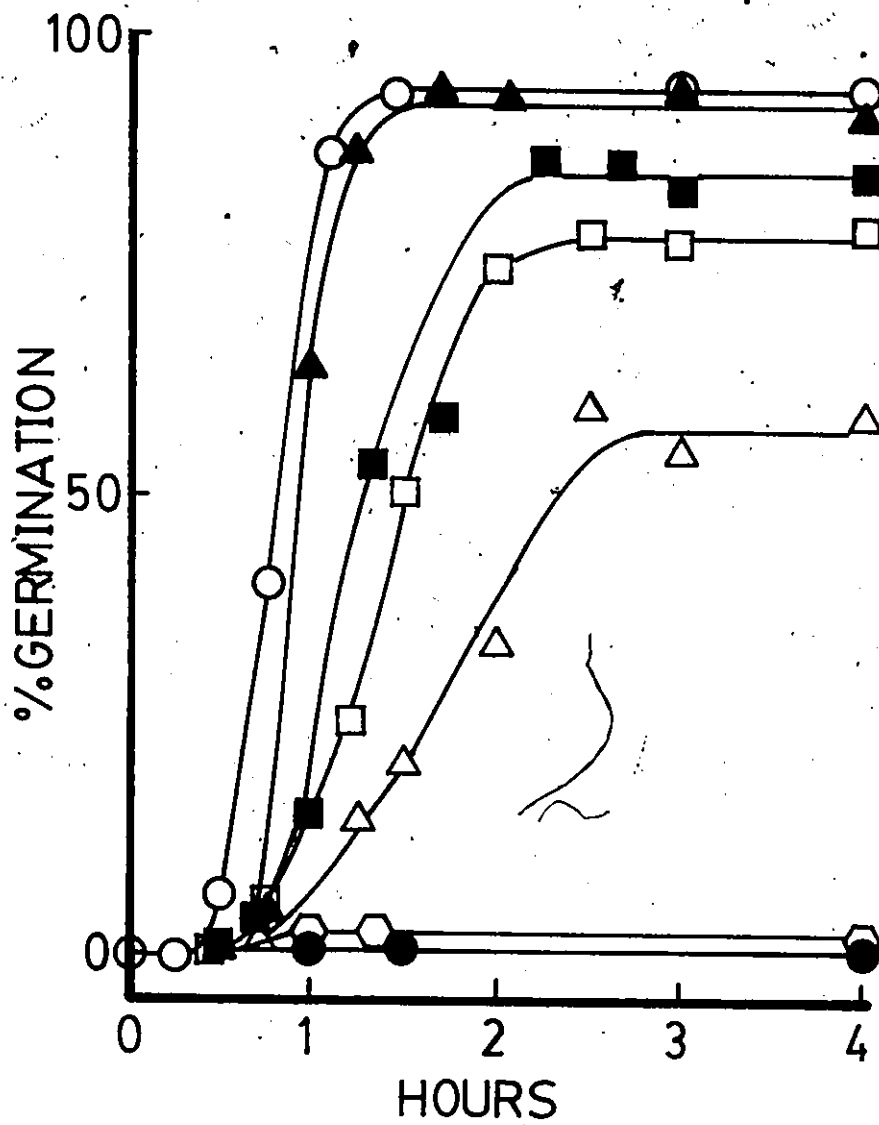
Fig. 6. Deactivation of spores incubated with 0.25 M sucrose. Spores

were heat activated and incubated at 23.5 C. Aliquots of this stock

suspension were removed as close as possible to time zero for the

first sample and subsequently every 10 min. for up to 60 min. and

placed into buffer containing sucrose in order to obtain a final

concentration of 0.25 M. After a 3 h incubation each aliquot was

washed free of the disaccharide and allowed to incubate for 2 h at

23.5 C after which the final percent germination was determined.

Symbols represent: $\bigcirc$, control spores in buffer; $\triangle$, 0.25 M treat-

ed spores.

38
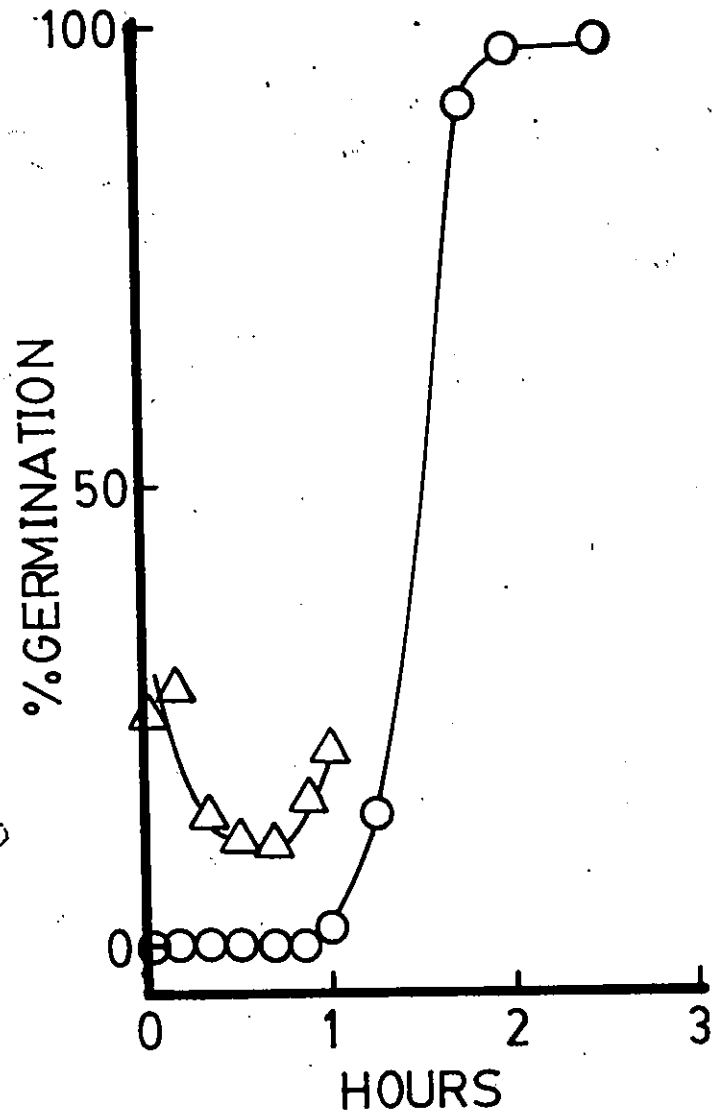
swelling and resulted in the deactivation of the majority (>90%) of
the treated population after 5 h of incubation. Deactivated spores, as
in other experiments, were viable as demonstrated by a high percentage
of germination after a second activation treatment.

Lowered oxygen tension restricts emergence of swollen spores and
causes death of emerged myxamoebae (Cotter and Raper, 1968a). Spores
may be heat activated in the absence of oxygen and germinate normally
after aeration following the activation treatment (data not shown).
However, incubation of activated spores under anaerobic conditions
caused deactivation of these individuals (Fig. 7). Due to technical dif-
ficulties a deactivation profile such as that produced by sucrose treat-
ment, for instance, was not generated for anaerobic conditions. However,
azide and cyanide artificially produce a similar physiological state
and were easily manipulated, thus these compounds were utilized to in-
hibit electron transport. Since these inhibitors produce typical deacti-
vation kinetics (Figs. 8 and 9) it may be assumed that anaerobic condi-
tions would create a similar effect upon activated spores.

As in temperature shifts and 0.25 M sucrose treatment of activated
spores, azide and cyanide treated spores were most vulnerable to deact-
ivation near the time in the postactivation lag phase where swelling
begins. This was seen most clearly in azide treated spores (Fig. 8).
The synchronization of the spore population at this point seemed to be
at a maximum since the majority of the spores reacted to the imposition
of adverse conditions in a similar manner within a short time span. In
contrast, spores earlier in the lag phase were unable to achieve this
synchronization and were subsequently unable to deactivate as efficient-
ly. The majority of the spores which had begun swelling or were within

Fig. 7. Time-dependent deactivation of spores under anaerobic condi-
tions. Spores were heat activated and placed under anaerobic condi-
tions for up to 5 h as described in Materials and Methods. Samples
of these spores were removed within the 5 h time period at specified
times, reaerated and allowed to germinate for a 2 h period. Symbols
represent: $\triangle$, final percent germination versus the time in hours
that spores were incubated under anaerobic conditions.
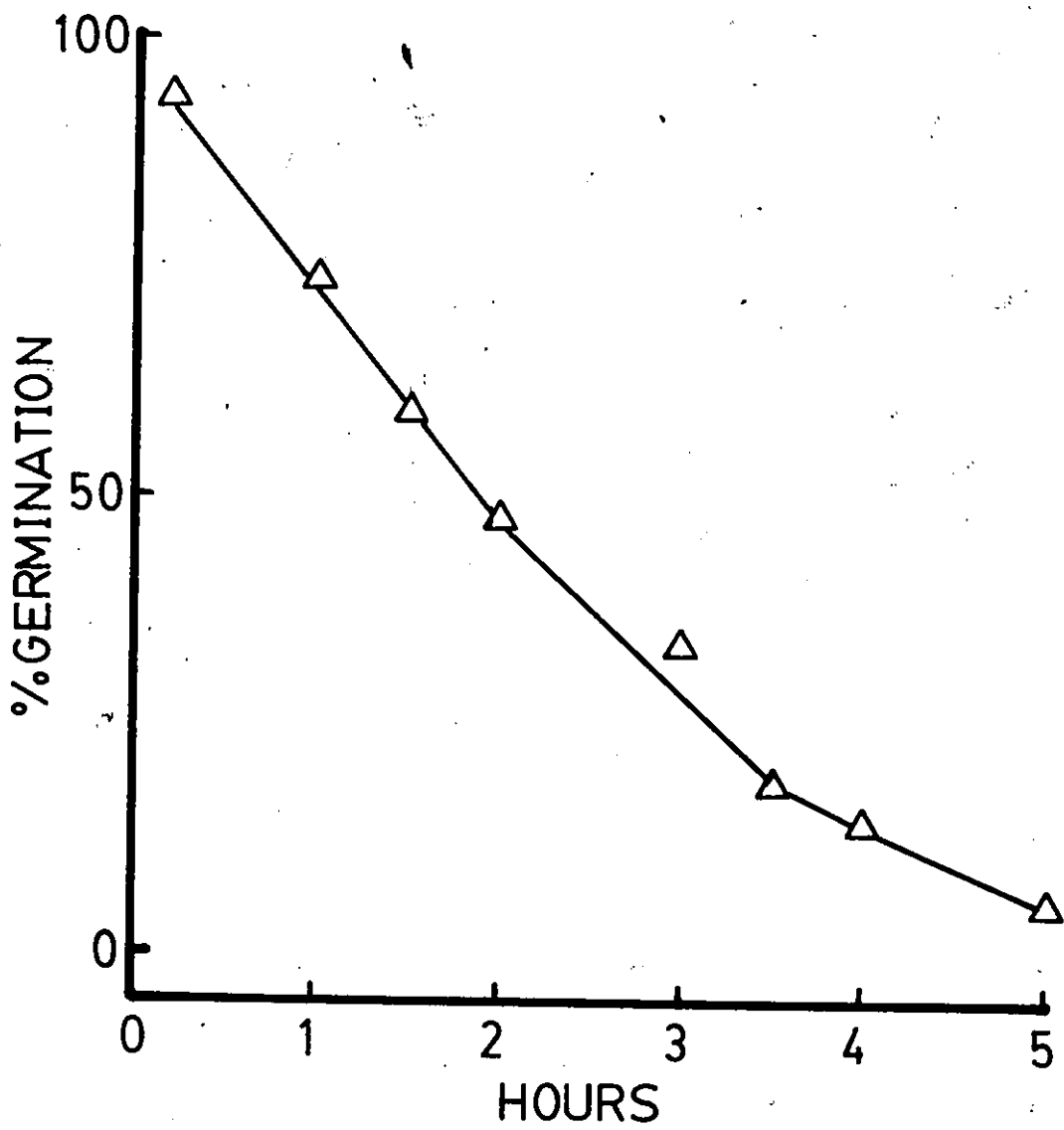
Fig. 8. Deactivation of spores incubated in 2 mM azide. Spores were

activated with DMSO and incubated at 23.5 C. Aliquots of this activa-

ted stock suspension were removed as close as possible to time zero

for the first sample and subsequently every 10 min. for up to 50 min.

and placed into buffer containing azide in order to obtain a final

concentration of 2 mM. After a 2 h treatment each aliquot was washed

free of the inhibitor and incubated for 3 h at 23.5 C; after which

the final percent germination was determined. Symbols represent:

$\bigcirc$, control spores in buffer; $\triangle$, 2 mM azide treated spores.
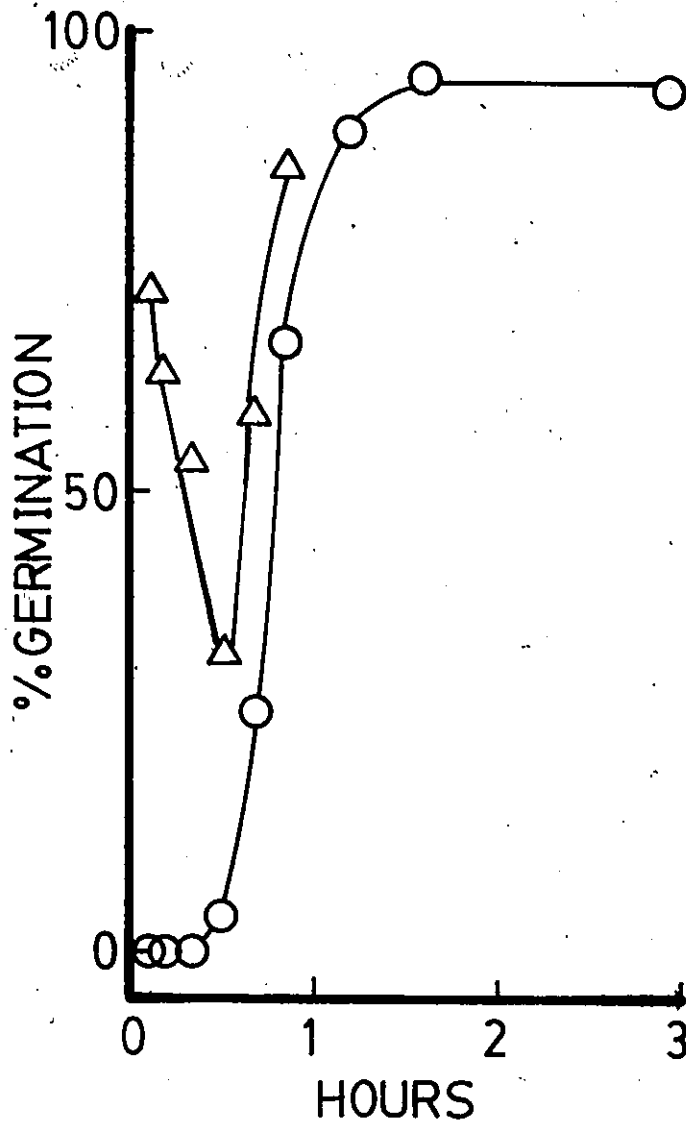
Fig. 9. Deactivation of spores incubated in 2 mM cyanide. Spores were activated with DMSO and incubated at 23.5 C. Aliquots of this activated suspension were removed as close as possible to time zero for the first sample and subsequently every 10 min. for up to 60 min. and placed into buffer containing cyanide in order to obtain a final concentration of 2 mM. After a 2 h treatment each aliquot was washed free of the inhibitor and incubated for 3 h; after which the final percent germination was determined. Symbols represent: $\bigcirc$, control spores in buffer; $\triangle$, 2 mM cyanide treated spores.

7 min. of beginning to swell were past this crucial point and may be considered committed to the continuation of this germination stage. Deactivated spores washed free of the inhibitors were reactivatable and germinated with high efficiency when subjected to a second activation treatment.
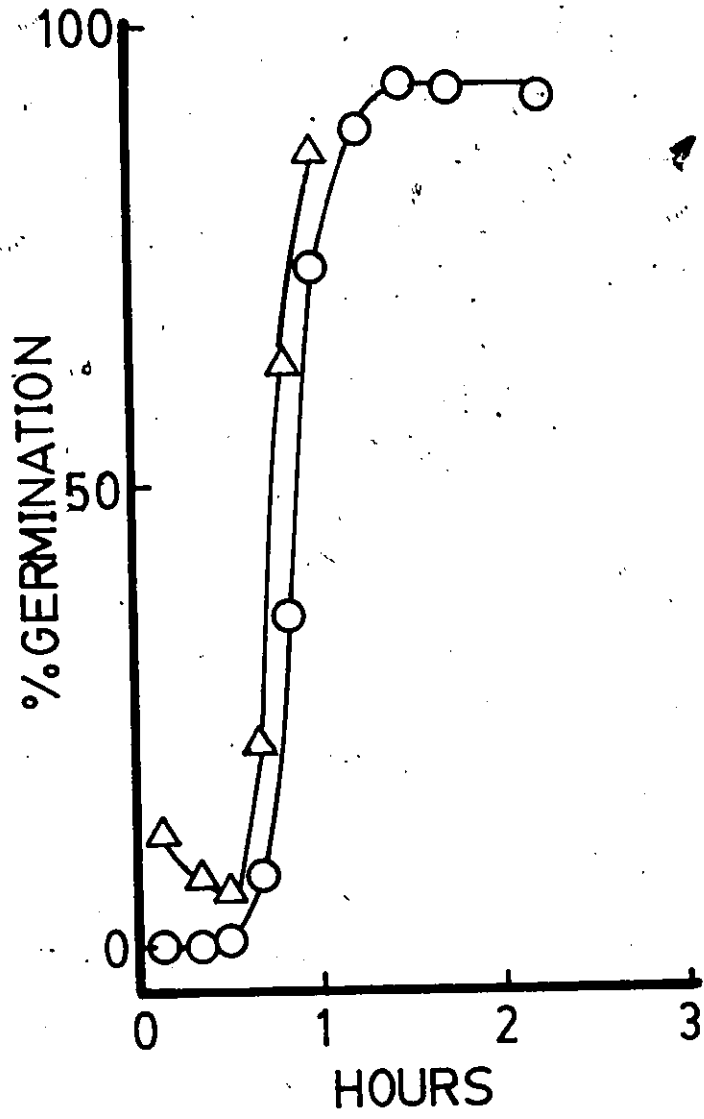
The effect of salicylhydroxamic acid (SHAM); an inhibitor of cyanide-resistant electron transport systems of various other organisms, was examined to determine if D. discoideum spores may require the function of such an alternate respiratory system during germination. The data in Fig. 10 demonstrate that although the rate of swelling and the rate of emergence was slowed in the presence of 5 mM SHAM no deactivation occurred.

Survival Experiments

These experiments were aimed at determining whether activated spores in various phases of the temporal sequence of swelling were progressively more susceptible to environmental stress. The data of Table 1 exemplified the type of results observed in these experiments. Nonswollen spores which were capable of deactivating returned to dormancy. However, those spores which were unswollen, but committed to swell prior to treatment, continued to swell during the treatment (Table 1). As an exception, the pH extremes of 2 and 12 not only prevented most unswollen spores from swelling but resulted in the death of the majority of these individuals along with the death of the swollen fraction in every phase of swelling. The nonswollen spores in the remaining treatments were considered as deactivated since the majority were viable when reactivated. The swollen spore fraction remaining after the 14 h

Fig. 10. The influence of SHAM on spore germination. A population of heat activated spores was split in half; one half remained in buffer while SHAM was added to the other half to a final concentration of 5 mM. Both cultures were incubated at 23.5 C and the kinetics of germination was followed until the majority of the populations had emerged. At 3.5 h the sample containing SHAM was split in half; one half was allowed to continue germination in the presence of the drug while the other was washed free of the drug and incubated in fresh buffer for the remainder of the experiment. Symbols represent: $\bigcirc$, percent of swollen spores in cultures incubated in buffer; $\bullet$, percent of e-merged amoebae in cultures incubated in buffer; $\triangle$, percent of swol-len spores in cultures containing SHAM; $\blacktriangle$, percent of emerged amoe-bae in cultures containing SHAM; $\square$, percent of swollen spores in cultures washed free of SHAM at 3.5 h; $\blacksquare$, percent of emerged amoe-bae in cultures washed free of SHAM at 3.5 h.
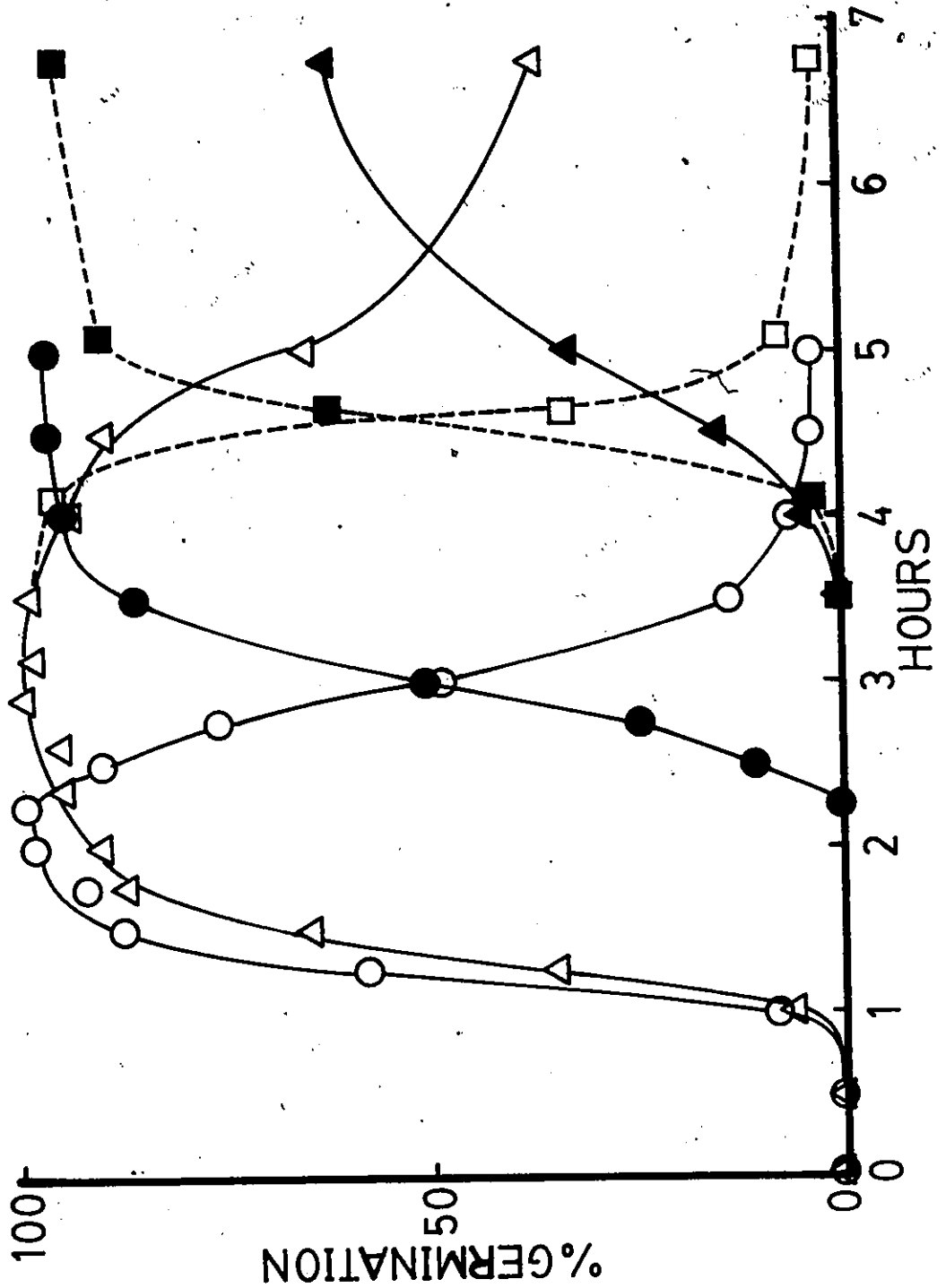
Table 1. Effect of 2 mM azide on the viability of swollen spores.

Swelling phase of spores in treated sample	Spore morphology prior to treatment			Spore morphology directly after the 5 h treatment			Final percentages of unswollen, swollen spores and amoebae after the 14 h under permissive conditions		
	D	S	E	D	S	E	D	S	E
Early	87.0	13.0	0.0	32.5	67.5	0.0	35.5	31.0	33.5
Mid	45.0	55.0	0.0	17.5	82.5	0.0	17.5	31.0	51.5
Late	6.5	93.5	0.0	10.0	90.0	0.0	3.5	52.0	44.5

Samples of spores in early, mid and late swelling phase were removed from DMSO activated stock cultures and placed into buffer containing azide to obtain a final concentration of 2 mM. After a 5 h treatment period in azide the spores were transfered to permissive environmental conditions and incubated for 14 h as described in Materials and Methods. Percentages of dormant, unswollen (D), swollen (S) spores and viable (intact and morphologically normal) amoebae (E) were determined via microscopic examination.

incubation under permissive conditions were considered as a nonviable
fraction since emergence was usually completed in a 6 h period under
identical incubation conditions. The emerged amoebae represented viable
individuals which were able to complete the developmental sequence of
germination after the respective treatment. Thus, the data in Table 1
indicate that swollen spores had partially lost their resistance to en-
vironmental stress to which dormant spores were resistant. There was
little difference between the 45% loss in viability (31.0/67.5 x 100)
of swollen spores from the early phase of swelling and the 58% loss in
viability (52.0/90.0 x 100) observed in the spores shifted to restric-
tive environmental conditions during the late phase of swelling (Table
1). Therefore there seems to be little correlation between vulnerabil-
ity to environmental stress and the respective phase of swelling. Ex-
ceptions to the latter data were observed for the environmental condi-
tions of 0 C and 0.25 M sucrose treatments; these two stresses merely
retarded the completion of swelling and prevented emergence until
spores were returned to permissive environmental conditions. Low tem-
perature and high osmotic pressure were tolerated by both swollen
spores (data not shown) and emerged myxamoebae (refer to Table 2). How-
ever, emerged myxamoebae were more vulnerable than swollen spores, under
the other unfavorable environmental conditions utilized, since a 1 h
treatment resulted in the death of virtually all of these individuals
(Table 2).

Respiration Experiments

The typical kinetics for oxygen uptake of D. discoideum spores
(activated by heat shock or DMSO treatment) are shown in Fig. 11. In-

Table 2. Effect of stressful environmental shifts on myxamoebal survival.

Treatment	Percent of viable amoebae in treated samples after the 14 h incubation	Percent of viable amoebae in nontreated samples after the 14 h incubation
0 C for 5 h	93.0	94.5
37 C for 1 h	0.5	90.5
pH 2 for 1 h	0.0	84.0
pH 12 for 1 h	0.0	84.0
0.25 M sucrose for 5 h	99.0	95.0
2 mM azide for 1 h	0.0	91.5
2 mM cyanide for 1 h	0.0	91.5

Emerged amoebae were removed from the DMSO activated stock cultures 6 h after the activation treatment and placed under stressful environmental conditions. After a 1 or 5 h treatment the amoebae were transfered to permissive environmental conditions and incubated for 14 h as described in Materials and Methods. Only intact, morphologically normal myxamoebae were enumerated.

Fig. 11. Kinetics of endogenous respiration of activated and dormant
spores. Suspensions of dormant, heat activated and DMSO activated
spores were monitored for endogenous respiration with Clark-type oxy-
gen electrodes. These experiments were done at a constant temperature
of 23.5 C for a 5 h period. Buffer without spores was also included to
gauge the oxygen consumption of the electrodes. The averaged data from
10 experiments is shown. Symbols represent: $\bigcirc$, DMSO activated
spores; $\triangle$, heat activated spores; $\square$, dormant spores and; $\bigcirc$,
buffer without spores.
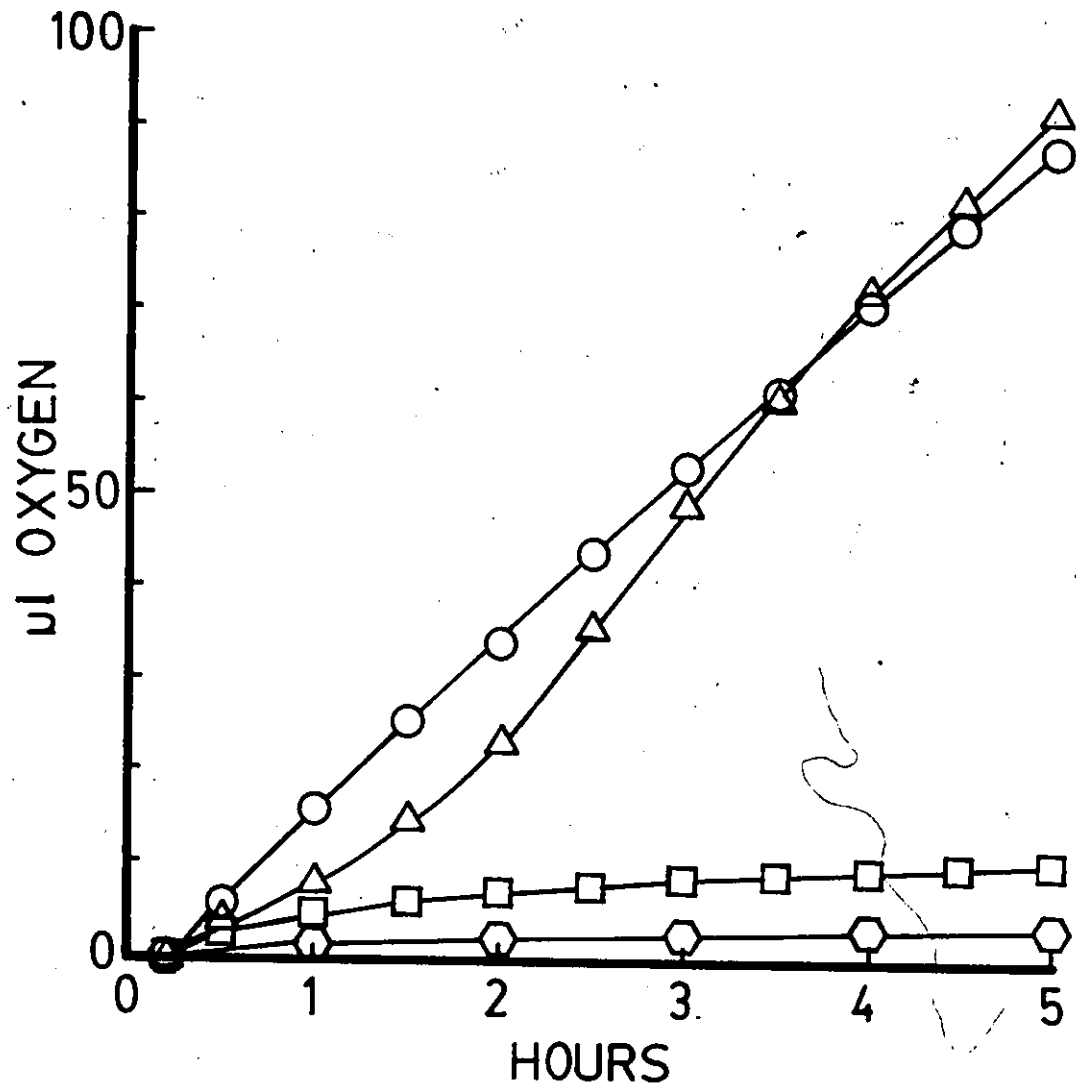
cluded in this figure are the kinetics of dormant spore respiration
and the level of oxygen that was consumed by the oxygen electrodes
used to measure respiration. The difference between the heat activated
and DMSO activated spore respiratory profiles is due to the different
germination kinetics elicited by these two activation techniques. As
shown previously in Fig. 2, heat activated spores have a postactivation
lag of 55-65 min. before swelling begins whereas DMSO activated spores
have a postactivation lag of 30-40 min. Thus, the initiation of the
swelling event correlates with a marked increase in the rate of oxygen
consumption. The increase is especially noticable in the case of heat
activated spore populations where respiration shows a biphasic profile.
Heat activated spores emerge at a faster rate than DMSO shocked spores
which may explain the slightly higher cumulative volume of oxygen taken
up by these spores towards the later portion of the incubation period.

The majority of DMSO activated spores were deactivated after only
1 h of incubation at 37 C. Activated spores held at 37 C exhibit virtu-
ally identical respiratory rates as nonactivated spores incubated at
the same temperature (Fig. 12). In contrast to the 63% reduction of tot-
al oxygen consumed by DMSO activated spores held at 37 C, heat activa-
ted spores retain a higher respiratory rate during incubation at 37 C,
which resulted in a 30% reduction after 5 h.(Fig. 13). Heat activated
spores, unlike DMSO activated populations, were not deactivated even
after 5 h of incubation at 37 C. Thus, there seems to be a correlation
between deactivation and a concomitant decreased respiratory rate of
deactivating spores.

The respiratory profile for activated spores incubated at 0 C was
not done since this temperature is out of the working range of the oxy-

Fig. 12. Effect of 37 C incubation on the respiration of DMSO activated spores. DMSO activated spores and dormant spores were monitored for endogenous oxygen consumption with a YSI oxygen monitor at 37 C. The controls included buffer without spores to measure the oxygen uptake of the electrodes at 37 C and DMSO activated spores which were incubated at 23.5 C and monitored for oxygen uptake. Symbols represent: ◯ , activated spores incubated at 23.5 C; △ , activated spores incubated at 37 C; ▢ , dormant spores incubated at 37 C and; ⬡ , buffer incubated at 37 C without spores.
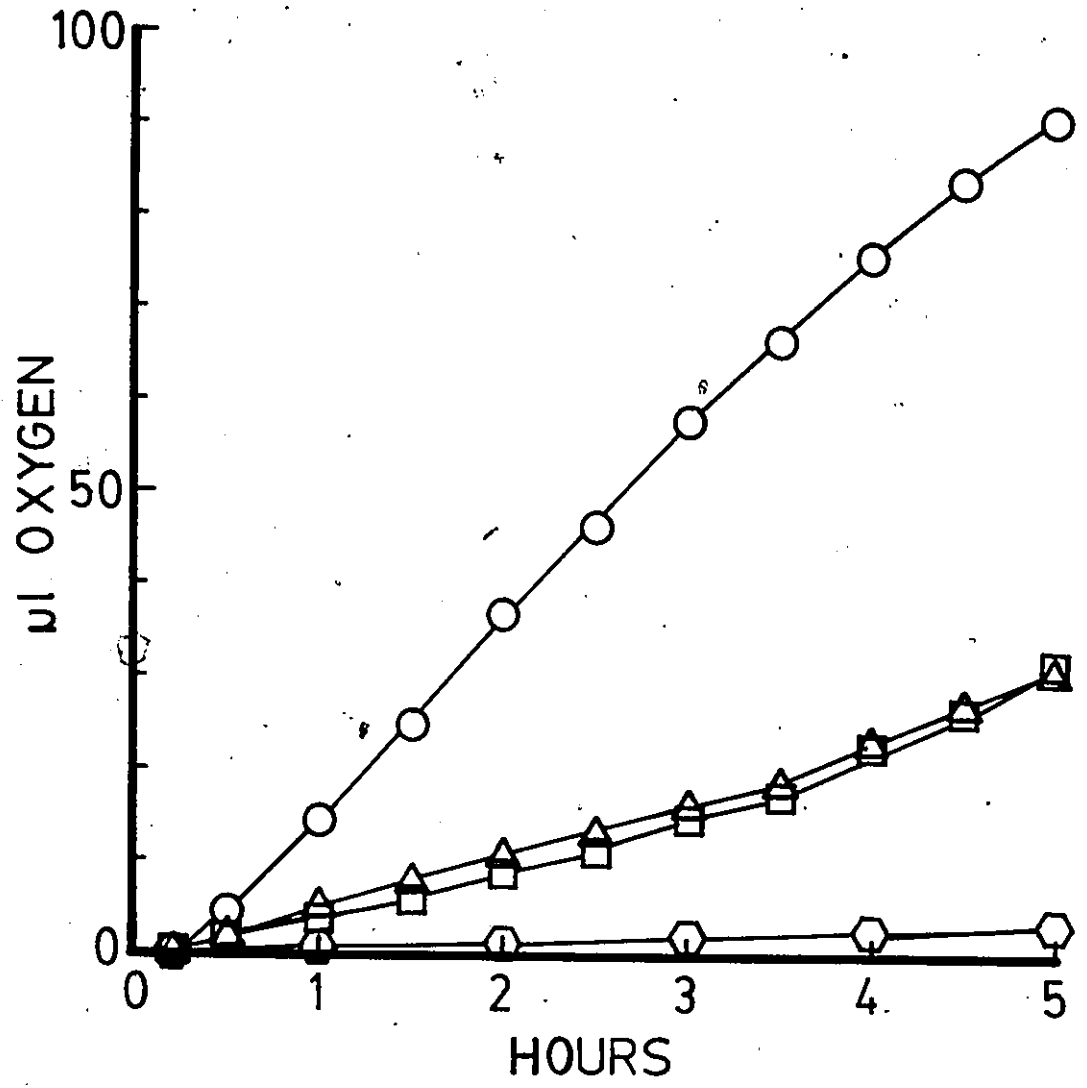
Fig. 13. Comparative effect of 37 C incubation on the respiration of
heat and DMSO activated spores. Aliquots of spores from the same popu-
lation were activated either by heat shock or DMSO treatment. These
activated spores were split into 2 equal volumes; one half was incu-
bated at 37 C and the other half was incubated at 23.5 C. Respiration
was monitored for a 5 h period. Symbols represent: ◯ , DMSO activa-
ted spores at 23.5 C; ☐ , heat activated spores at 23.5 C; △ ,
DMSO activated spores at 37 C and; ⬡ , heat activated spores at 37 C.
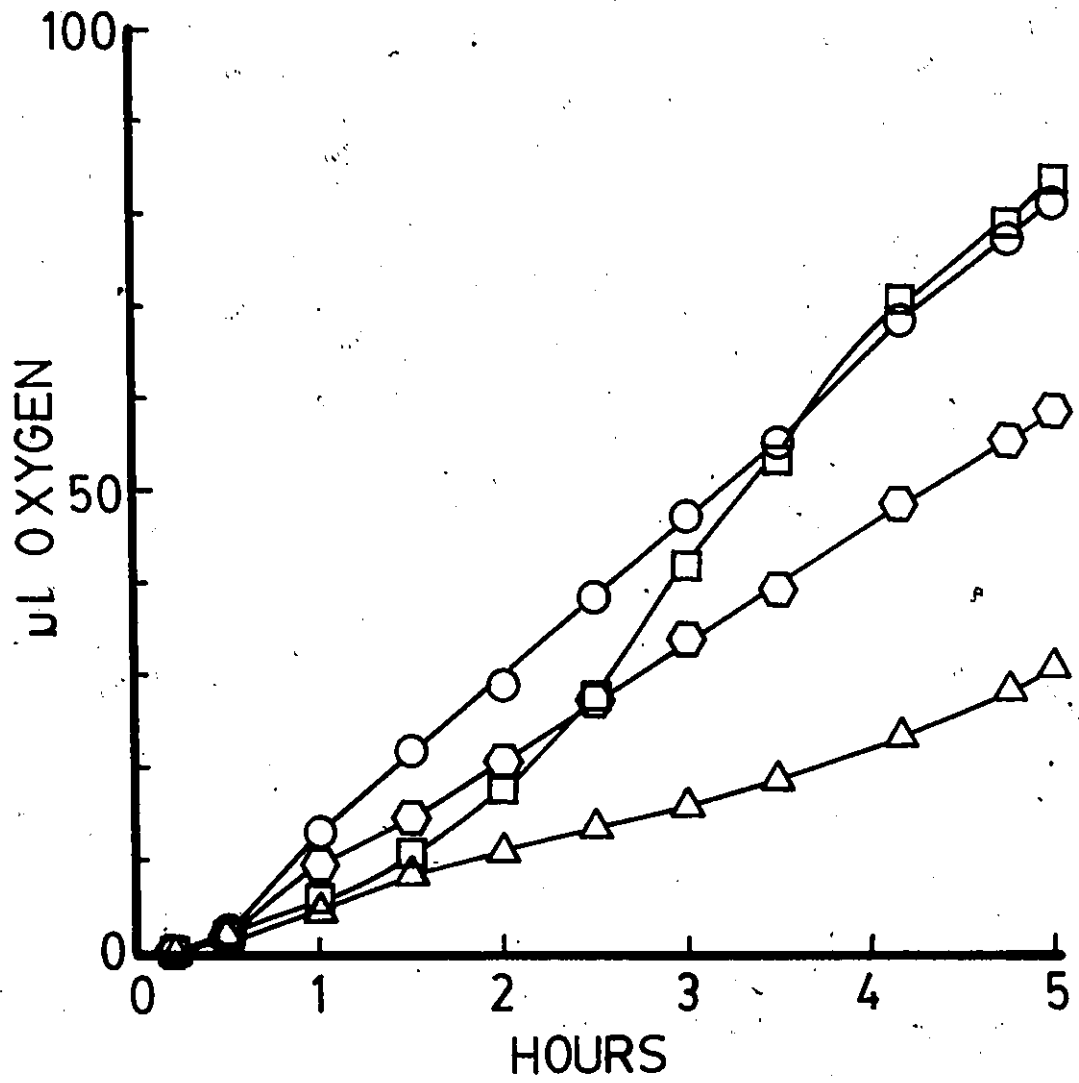
gen monitoring system. Similar results to those reported for the other
treatments however, would be expected since spores were deactivated
under low temperature incubation.

The pH extremes of 2 and 12 drastically reduced the oxygen consump-
tion of DMSO activated spores (Fig. 14). As shown previously, these pH
regimes do not allow normal development of activated spores and in fact
kill the majority of the treated population within a 5 h period. Due to
the latter effect the decrease in respiration was attributed mainly to
the reduction in viability rather than deactivation of the population.

High osmotic pressure imposed by 0.25 M sucrose also caused a de-
creased respiratory efficiency (Fig. 15) resulting in a 72% overall re-
duction of oxygen consumption. Treatment of heat activated spores with
0.25 M sucrose resulted in deactivation of the majority of the popula-
tion within a 5 h period. The respiratory rate of the deactivating
spores decreased from 7.8 μl O_2/h (in the second hour of incubation) to
2.3 μl O_2/h (in the fifth hour of incubation). The final rate of respir-
ation was approaching that of dormant spores incubated in buffer at
23.5 C which was 1 μl O_2/h (in the fifth hour).

The drugs azide and cyanide, which exert specific inhibitory ef-
fects on the function of cytochrome oxidase, also markedly decrease the
respiration of activated spores (Fig. 16). As in earlier experiments
done by Cotter and Raper (1968a), these inhibitors did not allow devel-
opment of activated spores and eventually the compounds caused deacti-
vation. Unlike the results reported by Bacon and Sussman (1973) cyanide
and azide consistently reduced cumulative oxygen uptake by 60-80% (de-
pending upon the activation treatment of the spores treated) of spores
after a 5 h period rather than the 100% rate decrease noted by these

Fig. 14. Effect of pH extremes on the respiration of activated spores.
DMSO activated spores were divided equally into 3 aliquots prior to
the last wash after the activation treatment. The pelleted spores were
then resuspended in buffer (pH 6.5) and buffer adjusted to pH 2 or pH
12. Respiration was monitored at 23.5 C for a 5 h period. Symbols
represent: ◯ , spores at pH 6.5; △ , spores at pH 2 and; ☐ ,
spores at pH 12.
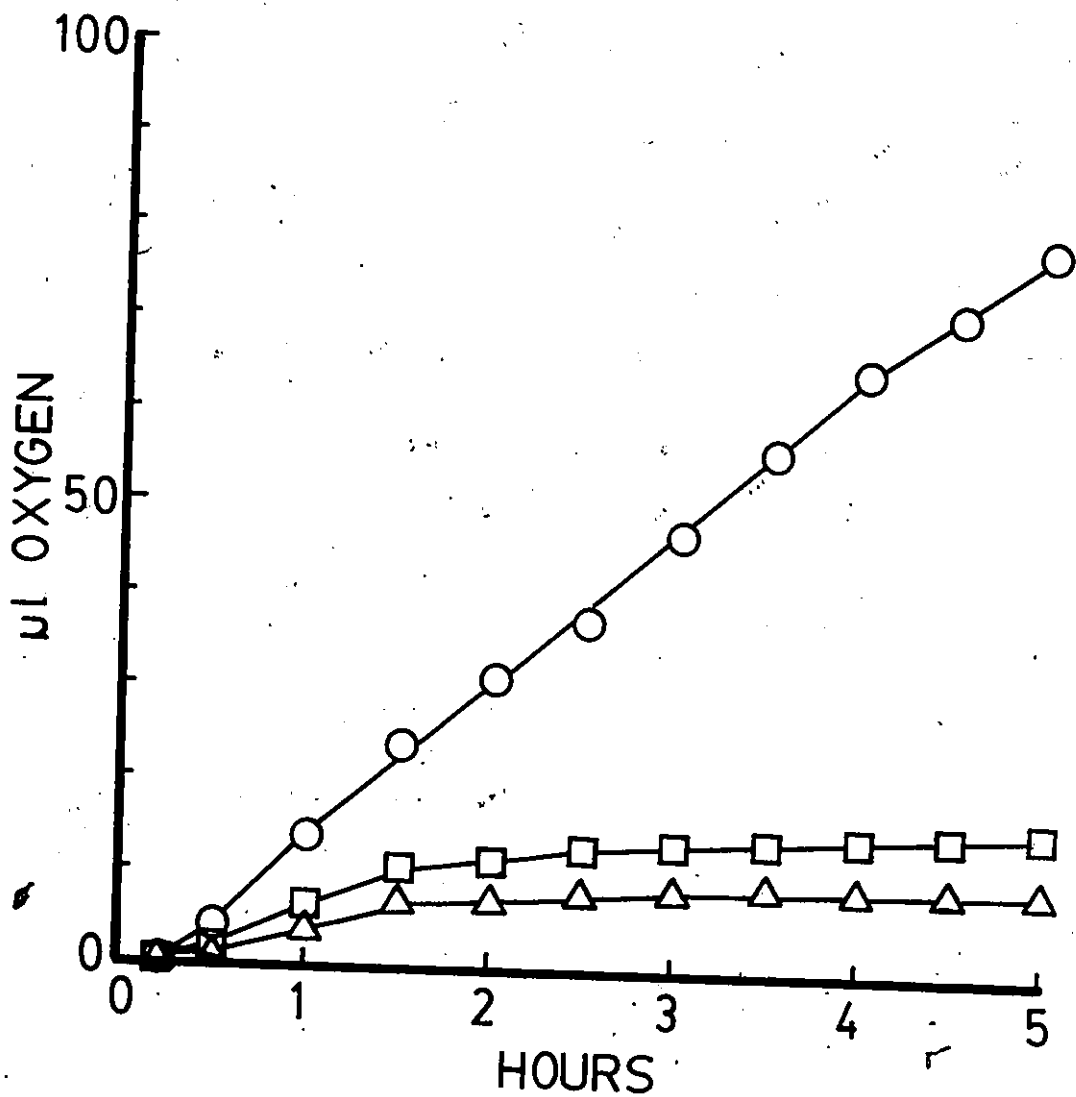
Fig. 15. Effect of 0.25 M sucrose on the respiration of activated

spores. Heat activated spores were placed in buffer or in buffer

containing sucrose to yield a final concentration of 0.25 M. These

suspensions were monitored for oxygen uptake for 5 h at 23.5 C.

Symbols represent: ○ , spores in buffer and; △ , spores in 0.25 M

sucrose.

Fig. 16. Effect of azide and cyanide on the respiration of DMSO acti-
vated spores. A suspension of DMSO activated spores was divided equal-
ly into 3 aliquots. One aliquot was suspended in buffer while the
other aliquots received buffer containing azide or cyanide to yield
a final concentration of 2 mM. Respiration was monitored for 5 h at
23.5 C. Symbols represent: $\bigcirc$, spores in buffer; $\square$, spores in
2 mM azide and; $\triangle$, spores in 2 mM cyanide.
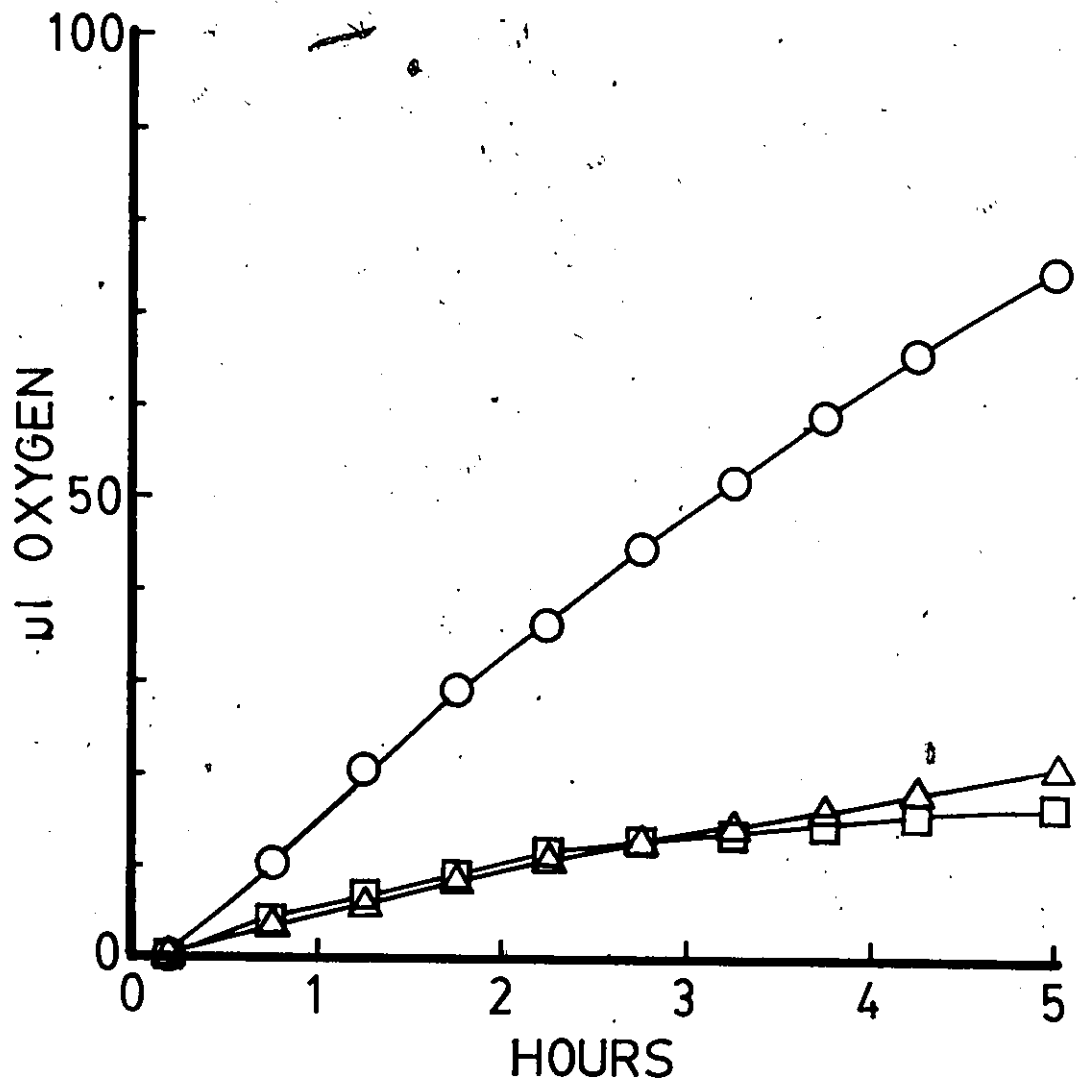
authors.

Azide, like 0.25 M sucrose, elicited a decreasing pattern of respi-ration (Fig. 16). The respiratory rate dropped from 5.0 μl O_2/h (in the second hour) to 1.5 μl O_2/h (in the fifth hour) which is nearly equiv-alent to the endogenous respiratory rate of dormant spores. Cyanide e-licited a decreased spore respiration which dropped from 4.7 μl O_2/h (in the second hour) to 3.2 μl O_2/h (in the fifth hour). The final drop in total oxygen consumed observed was a 76% reduction in 2 mM cy-anide- and an 81% reduction in 2 mM azide-treated populations of DMSO activated spores. Azide and cyanide had similar overall effects upon heat activated spores in that a 75% and a 63% reduction of the cumula-tive amount of oxygen was observed for 2 mM treatments respectively, after a 5 h period (Fig. 17). The rate decreases of 4.6 μl O_2/h (in the second hour) to 3.2 μl O_2/h (in the fifth hour) for azide and 6.9 μl O_2/h (in the second hour) to 5.7 μl O_2/h (in the fifth hour) for cy-anide treated spores were not quite as marked as those observed for DMSO activated spores. Further, heat activated spores were not deacti-vated by a 5 h treatment in the presence of the inhibitors.

Salicylhydroxamic acid (SHAM) is known to be an inhibitor of cy-anide-resistant respiration. Heat activated spores which were incubated in the presence of 5 mM SHAM demonstrated an 18% reduction in the total amount of oxygen consumed over a 5 h period when compared to activated spores from the same population free of the drug (Fig. 18). No deacti-vation was observed in the SHAM-treated spores.

Exposing activated spores to a mixture of 2 mM cyanide and 5 mM SHAM resulted in a 97% reduction in total oxygen taken up over a 5 h
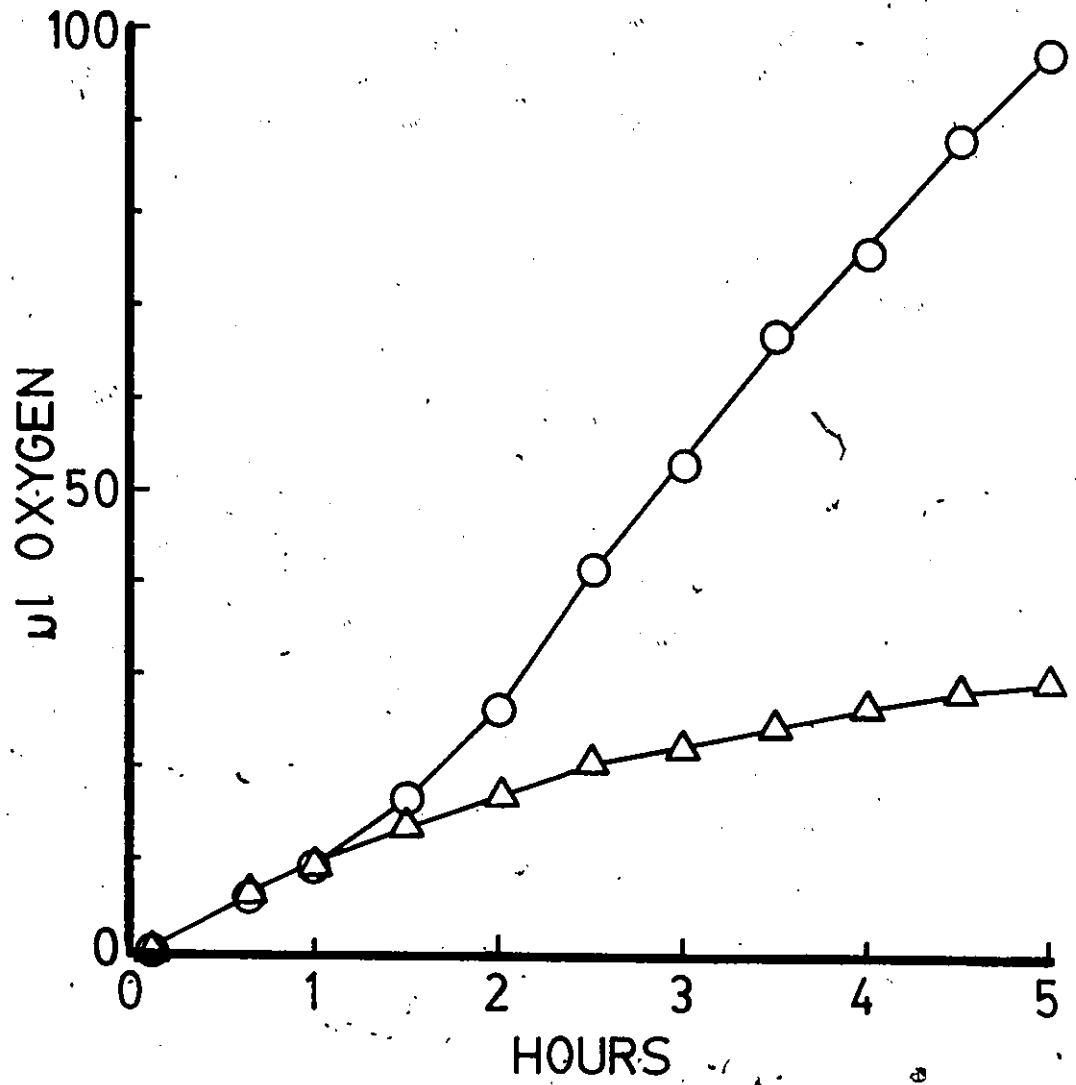
Fig. 17. Effect of azide and cyanide on the respiration of heat acti-

vated spores. A suspension of heat activated spores was divided equal-

ly into 3 aliquots. One aliquot was suspended in buffer while the

other aliquots received buffer containing azide or cyanide to yield

a final concentration of 2 mM. Respiration was monitored for 5 h at

23.5 C. Symbols represent: $\bigcirc$, spores in buffer; $\triangle$, spores in

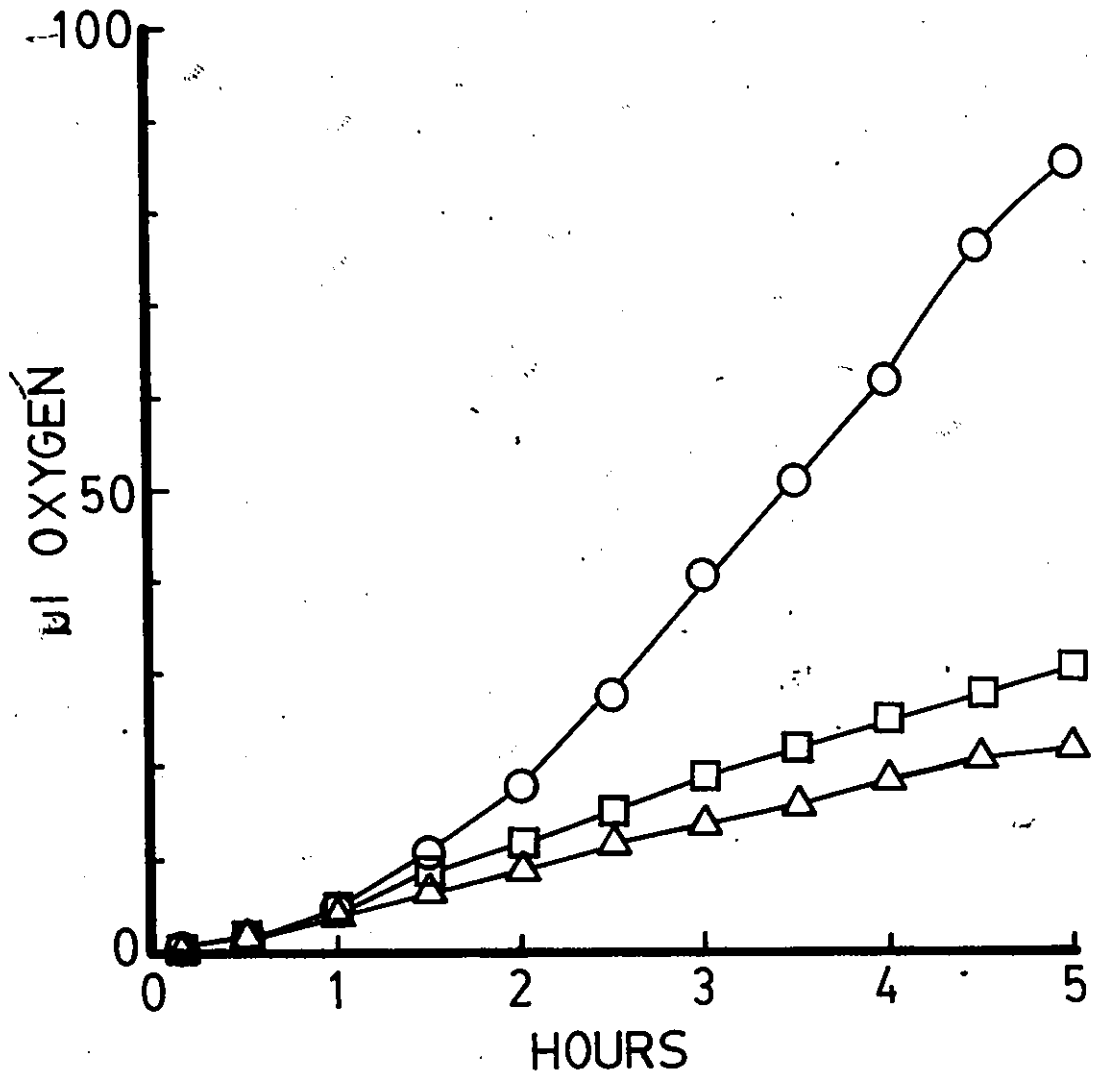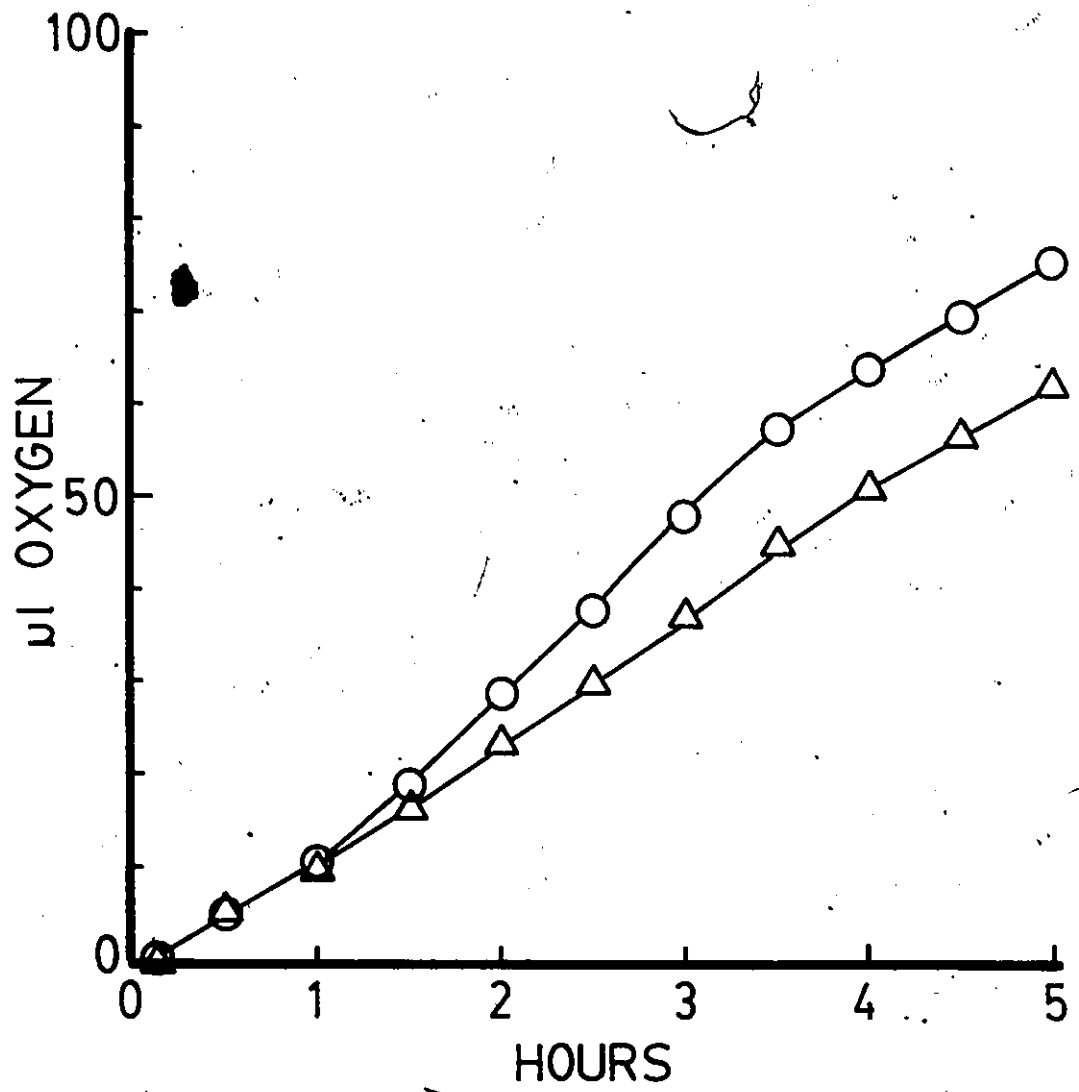2 mM azide and; $\square$, spores in 2 mM cyanide.

Fig. 18. Effect of SHAM on the respiration of activated spores. A sus-

pension of heat activated spores was divided in half; to one half buf-

fer was added and to the other half SHAM was added to yield a final

concentration of 5 mM. Respiration was monitored for 5 h at 23.5 C.

Symbols represent: $\bigcirc$, spores in buffer and; $\triangle$, spores in SHAM.

period (Fig. 19). In contrast, the combination of 2 mM azide and 5 mM SHAM reduced total oxygen usage by 83% over a 5 h period. Even though the combination of cyanide and SHAM was able to virtually eliminate the respiration of heat activated spores, a 5 h treatment period was still insufficient to induce the majority of the treated population to return to the dormant state.

To test if the combination of cyanide and SHAM may have been inhibitory by virtue of increased osmotic pressure, the effect of 2 mM cyanide and 0.25 M sucrose together was investigated (Fig. 20). The 74% reduction in the cumulative amount of oxygen consumed by cyanide + sucrose-treated spores compares to the reduction observed in 0.25 M sucrose-treated spores (eg. 72%). Thus suggesting that the additive effect of cyanide and SHAM was not due to osmotic inhibition but a specific type of inhibition exerted by each of the latter drugs.

As shown previously, dormant spores possess a low level of aerobic respiration. Azide decreased this endogenous level by 39% over a 5 h period (Fig. 21). In contrast, cyanide stimulates this endogenous respiration by 1.8 fold. Although SHAM had little effect upon the respiration of dormant spores (data not shown), the mixture of cyanide + SHAM resulted in a 97% reduction of the total oxygen used by dormant spores over a 5 h period (Fig. 21). The uncoupler of oxidative phosphorylation 2,4-dinitrophenol also stimulated the respiration of dormant spores resulting in a 1.5 fold increase over a 5 h period. The addition of a mixture of 2,4-dinitrophenol + SHAM to dormant spores resulted in a respiratory rate which parallelled that of the non-treated controls (data not shown).

Bacon and Sussman (1973) have previously reported that sodium fluo-

Fig. 19. Effect of the mixtures of azide + SHAM and cyanide + SHAM on the respiration of activated spores. Heat activated spores from the same population were placed into buffer containing mixtures of azide + SHAM, cyanide + SHAM, or buffer. The inhibitors azide and cyanide were used at a final concentration of 2 mM while a 5 mM concentration of SHAM was utilized. Respiration was monitored for 5 h at 23.5 C. Symbols represent: $\bigcirc$, spores in buffer; $\triangle$, spores in cyanide + SHAM and; $\square$, spores in azide + SHAM.
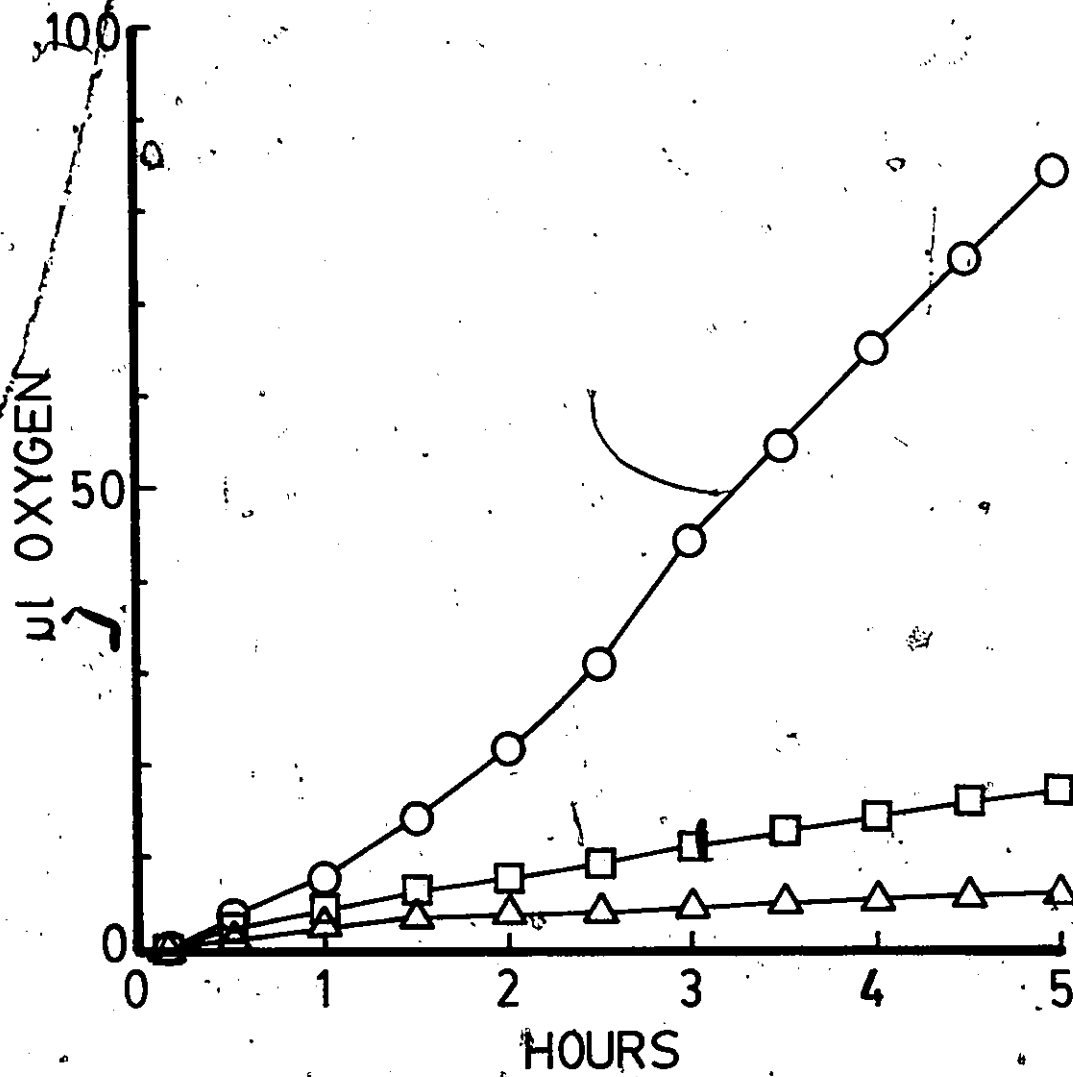
Fig. 20. Comparison of the effects of the mixtures of cyanide + SHAM and cyanide + sucrose on the respiration of activated spores. Heat activated spores from the same population were placed into buffer containing the mixtures of cyanide + SHAM, cyanide + sucrose, or buffer. Final concentrations of the compounds used were: 2 mM cyanide, 5 mM SHAM and 0.25 M sucrose. Respiration was monitored for 5 h at 23.5 C. Symbols represent: ○ , spores in buffer; △ , spores in cyanide + sucrose and; □ , spores in cyanide + SHAM.

Fig. 21. Effect of respiratory inhibitors on the endogenous respiration of dormant spores. Washed dormant spores were suspended in buffer and buffer containing: cyanide, azide, or cyanide + SHAM. Final concentrations of the inhibitors were: 2 mM cyanide, 2 mM azide and 5 mM SHAM. Respiration was monitored for 5 h at 23.5 C. Symbols represent: ◯ , spores in buffer; △ , spores in azide; ☐ , spores in cyanide; ■ , spores in cyanide + SHAM and; ⬡ , buffer without spores.
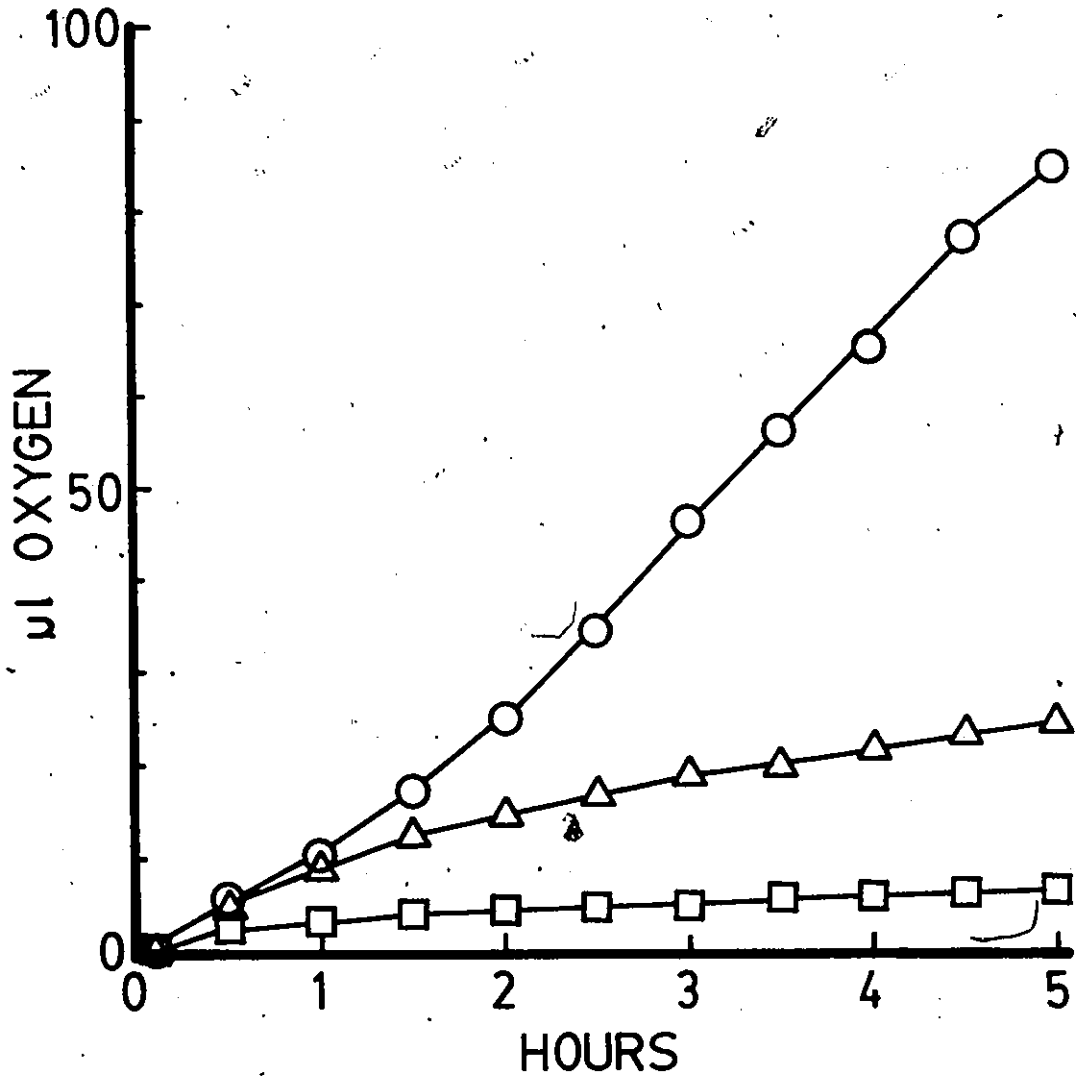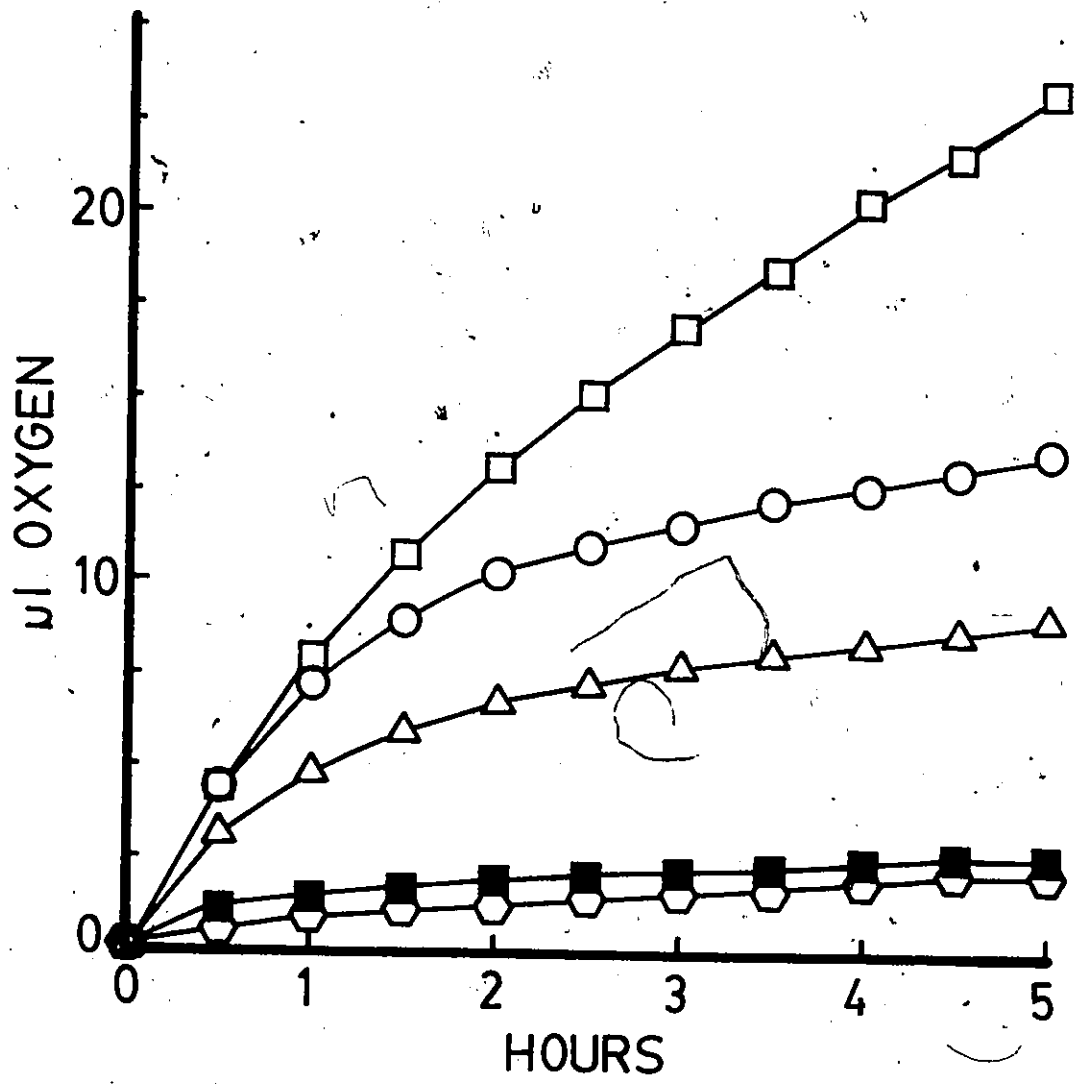
ride, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase of the glycolytic pathway, and malonate, an inhibitor of the citric acid cycle at malate dehydrogenase, result in 99 and 100% inhibition of respiration, respectively. Attempts to duplicate these results failed (Fig. #23). The presence of 0.02 M fluoride resulted in a 46% decrease of cumulative oxygen utilized by heat activated spores with a part (40%) of the population being deactivated after 5 h. Deactivated spores were able to germinate after a second activation treatment. Treatment of heat activated spores with 0.08 M malonate resulted in a 73% reduction in cumulative oxygen use with 90% of the population being deactivated after the 5 h treatment. As before, deactivated spores were able to germinate upon a second activation treatment. Comparing the inhibition elicited by 0.08 M malonate to that of 0.25 M sucrose it was noted that the two values are virtually identical. In fact the ionic strength of 0.08 M malonate is 0.24 M. Further, malonate did not exert any inhibitory effects upon either the respiration or germination of heat activated spores at a concentration of 0.02 M (data not shown). This suggests that the inhibition observed with 0.08 M malonate was in fact an osmotic effect.

Cycloheximide, an inhibitor of cytoplasmic protein synthesis, reduced the cumulative volume of oxygen consumed by 15-20% in a 5 h period of incubation (Fig. 24). As observed previously (Cotter and Raper, 1966, 1968a), activated spores swell normally in the presence of cycloheximide but fail to emerge. Chloramphenicol, a specific inhibitor of mitochondrial protein synthesis, has recently been reported to prevent emergence of myxamoebae from swollen spores of D. discoideum strain

Fig. 22. Effect of fluoride on the respiration of activated spores.
Heat activated spores from the same population were placed into buf-
.fer and buffer containing fluoride yielding a final concentration of
the inhibitor of 0.02 M. Respiration was monitored for 5 h at 23.5 C.
Symbols represent: ◯ , spores in buffer and; △ , spores in fluo-
ride.
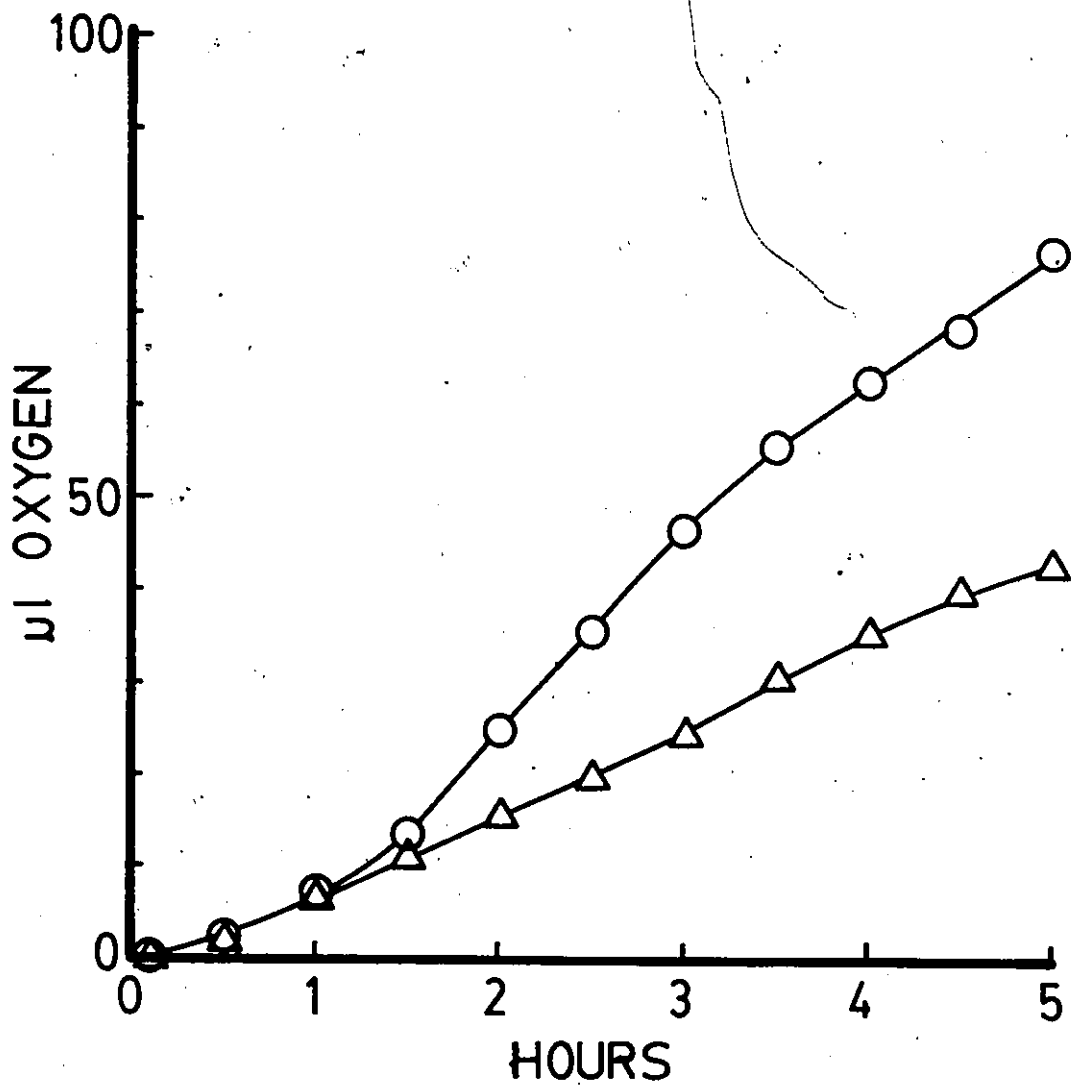
Fig. 23. Effect of malonate on the respiration of activated spores.
Heat activated spores from the same population were placed into buf-
fer and buffer containing malonate yielding a final concentration of
the inhibitor of 0.08 M. Respiration was monitored for 5 h at 23.5 C.
Symbols represent: $\bigcirc$, spores in buffer and; $\triangle$, spores in mal-
onate.
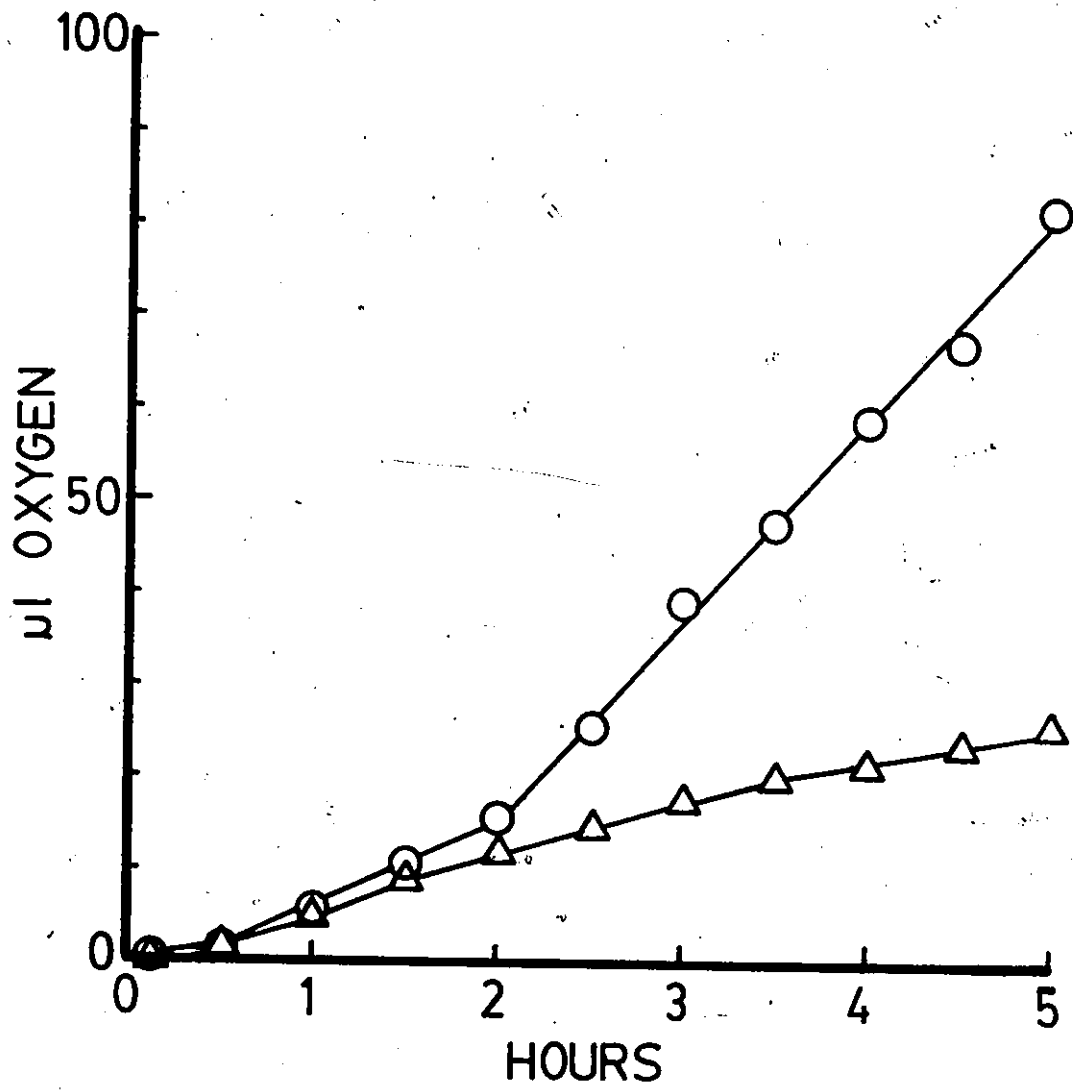
Fig. 24. Effect of protein synthesis inhibitors on the respiration of activated spores. Heat activated spores from the same population were placed into buffer, buffer containing cycloheximide at a final concentration of 200 µg/ml, buffer containing chloramphenicol at a final concentration of 3 mg/ml, or buffer containing a mixture of cycloheximide + chloramphenicol each at the latter concentrations. Respiration was monitored for 5 h at 23.5 C. Symbols represent: ◯ , spores in buffer; △ , spores in cycloheximide; ☐ , spores in chloramphenicol and; ⬡ , spores in cycloheximide + chloramphenicol.
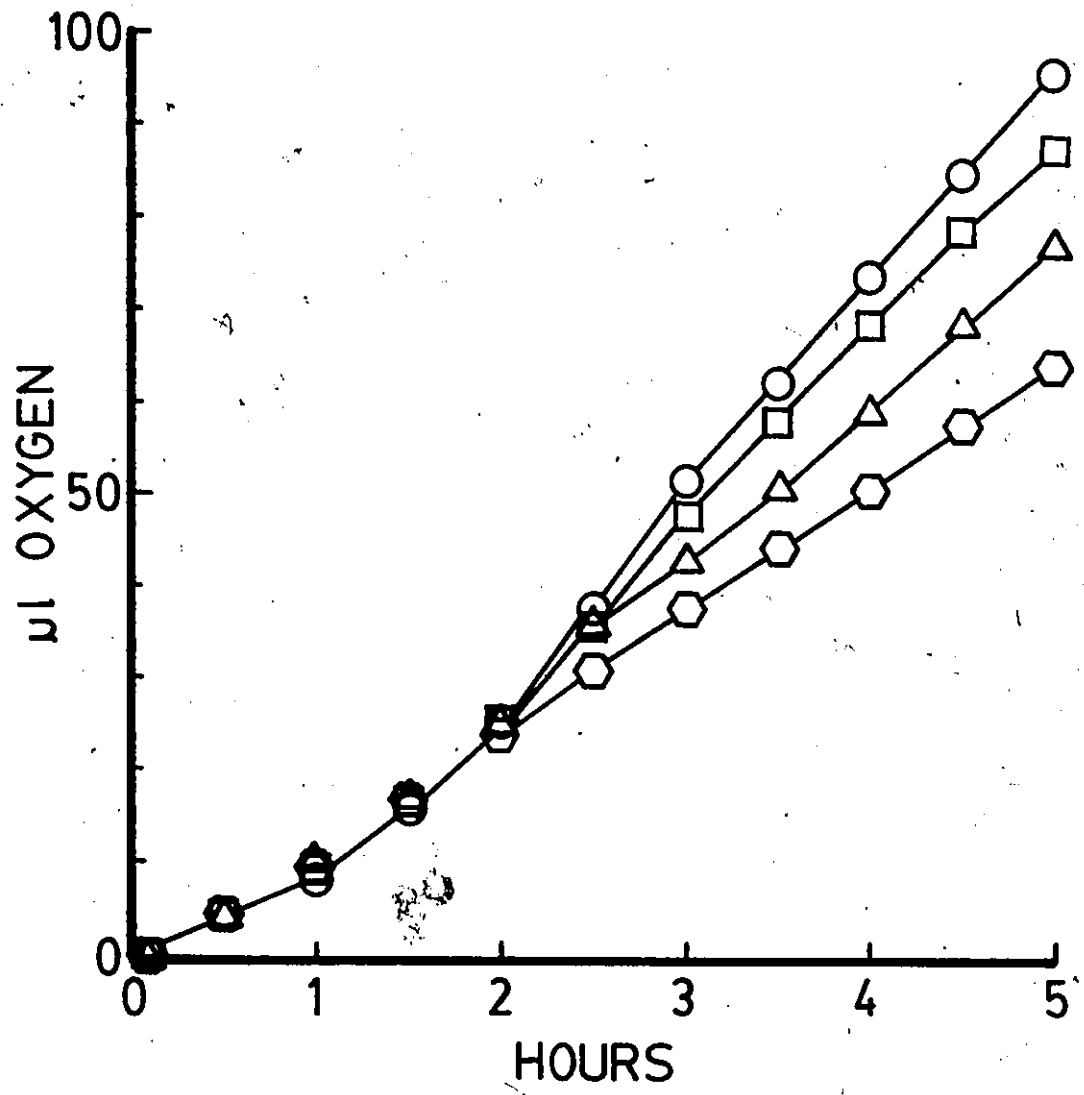
AX3 (Kobilinsky and Beattie, 1977). Chloramphenicol is soluble up to a concentration of 2.5 mg/ml in water at 25 C (Merck Index of Chemicals and Drugs, 8[th]ed.). The 3 mg/ml concentration used in this study was above saturation. Heat activated spores treated at this concentration were observed to swell at a rate virtually identical to that of control cultures. Emergence of these spores was slowed in the presence of the antibiotic but was not completely inhibited as reported by the latter authors. However, differences in the strains used in these two studies may account for the discrepancy. Chloramphenicol reduced total oxygen consumption by 9% after a 5 h period. The data in Fig. 24 also show that the combination of cycloheximide + chloramphenicol elicited an additive reduction in the total volume of oxygen utilized during the 5 h incubation period (eg. 33%). Spores treated with this combination were swollen but failed to emerge until washed free of the drugs.

Radiolabel Experiments

Previous work with radioactively-labelled precursors of protein, RNA and DNA have demonstrated that macromolecular syntheses occurs during the germination of D. discoideum spores (Bacon and Sussman, 1973, Yagura and Iwabuchi, 1976, Giri and Ennis, 1977). The data in Fig. 25 show the uptake kinetics obtained for ^{14}C-amino acids and ^{3}H-uridine in this study. Samples of activated spores incubated with ^{14}C-leucine or ^{3}H-uridine were taken to determine the overall effect of various restrictive environmental conditions upon protein and RNA syntheses.

Dormant spores incubated at 23.5 C for 5 h with ^{14}C-leucine or ^{3}H-uridine were observed to assimilate only a fraction of the precursors taken up by activated spores under the same conditions (Table 3).

Fig. 25. Incorporation of ^{14}C-amino acids and ^{3}H-uridine into TCA-in-soluble materials of whole cells during synchronized germination of D. discoideum spores. Symbols represent: $\bigcirc$, uptake of ^{14}C-amino acids corresponding to the left ordinate and; $\triangle$; uptake of ^{3}H-uridine corresponding to the ordinate on the right.
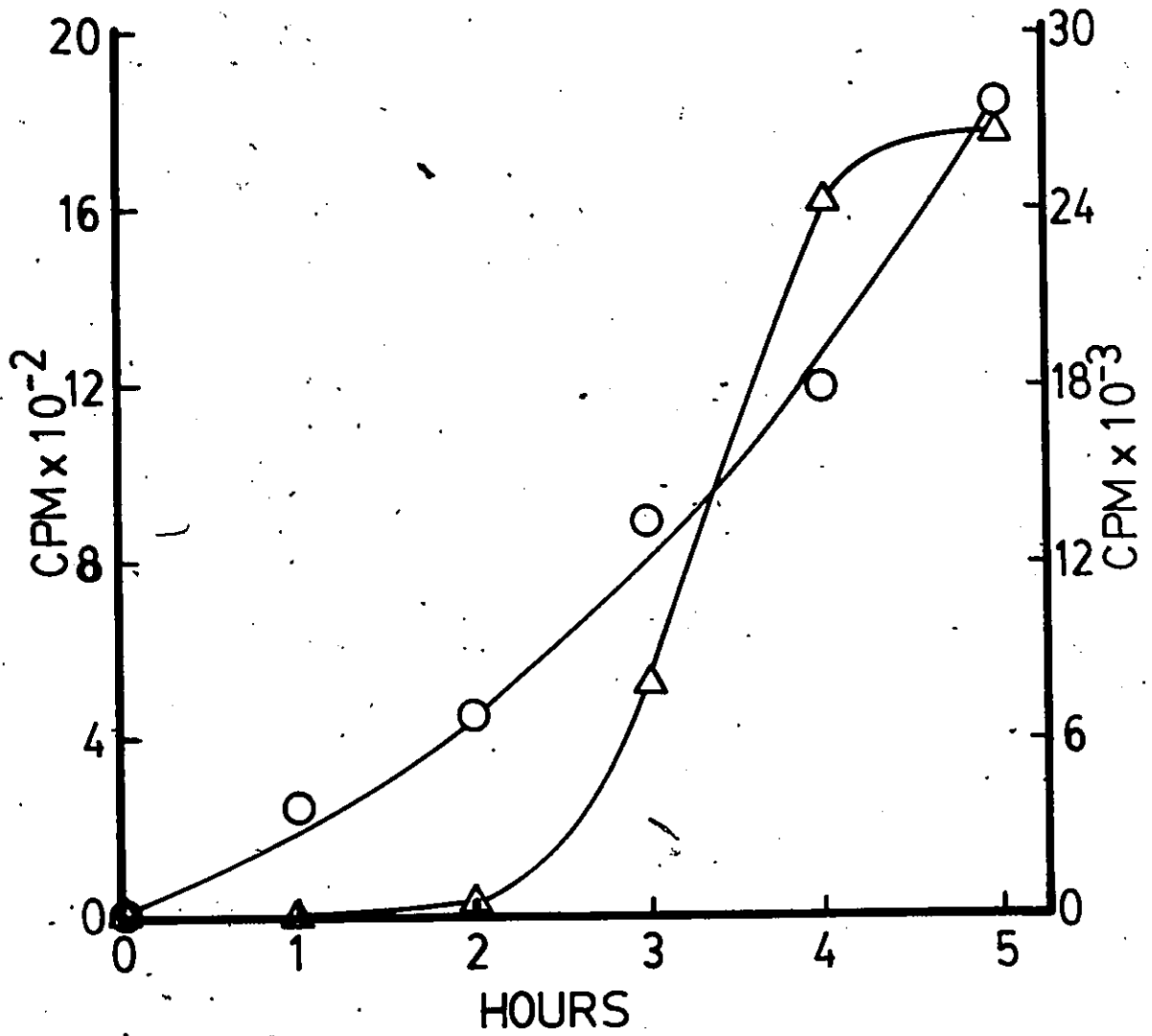
Table 3. Effect of restrictive environmental conditions upon ^{14}C-leu-
cine and ^{3}H-uridine uptake of activated spores. Heat activated spores
were incubated under the various restrictive environmental conditions
listed in the presence of ^{14}C-leucine or ^{3}H-uridine (each at 1 μCi/ml)
for 5 h. Triplicate samples were taken at time zero and at 5 h to be
assessed for radioactively-labelled components. The counts per minute
(CPM) from time zero samples were subtracted from the 5 h samples to
yield the reported values. All values are also displayed as percentages
of the control value for comparison.

^{14}C-leucine Incorporation

Treatment	CPM/10^7spores/ml	Relative percent of control
Control	2578.5	100.0
Dormant	144.4	5.6
0 C	44.6	1.7
37 C	62.6	2.4
pH 2	102.3	4.0
pH 12	0.0	0.0
0.25 M sucrose	309.4	12.0
2 mM azide	110.3	4.3
2 mM cyanide	15.0	0.6

^3H-uridine Incorporation

Treatment	CPM/10^7spores/ml	Relative percent of control
Control	27111.9	100.0
Dormant	1515.1	5.6
0 C	0.0	0.0
37 C	446.7	1.6
pH 2	34.1	0.1
pH 12	388.6	1.4
0.25 M sucrose	996.0	3.7
2 mM azide	831.8	3.1
2 mM cyanide	0.0	0.0

In comparison, activated spores incubated with these labelled precursors at low temperature (0 C), high temperature (37 C), pH 2 or 12, 2 mM azide or 2 mM cyanide all exhibit lower assimilation as shown in Table 3. Spores held in 0.25 M sucrose were the exception in the case of leucine uptake although uridine uptake was decreased in relation to dormant spores. This deviant sample may have been associated with an anomaly caused by the presence of the sugar since sucrose-treated samples yielded inconsistent data with the ^{14}C-leucine incorporation procedure.

Trehalase Assays

Activated spores held under various deactivation conditions exhibited specific activities comparable to or lower than that elicited by dormant spores (Table 4). Protein concentrations were generally 0.20-0.25 mg/ml with a high value of 0.80 mg/ml and a low of 0.06 mg/ml as determined by the protein assay used (Bradford, 1976). The lower value was obtained from pH 12 treated spores. Higher protein concentrations were difficult to achieve in this case which was probably due to protein denaturation and subsequent leakage from the spores during prolonged incubation at the pH extreme. Spores treated at pH 12 also were extremely difficult to break due to their plasmolyzed state subsequent to treatment. Spores treated at pH 2 also tended to yield lower protein concentrations and although mechanical breakage of these spores was not affected, protein denaturation may have been the cause for the lowered protein yield after the 6 h incubation period.

Table 4. Trehalase activity in spores incubated under restrictive
environmental conditions.

Treatment	Specific activity	Ratio of treated to dormant
Dormant spores incubated at 23.5 C	0.067	1.00
Activated spores incubated at 23.5 C	0.414	6.12
Activated spores incubated at 0 C for 48 h	0.077	1.15
Activated spores incubated at 37 C	0.058	0.87
Activated spores incubated at: pH 2	0.084	1.25
pH 12	0.082	1.22
Activated spores incubated in 0.25 M sucrose	0.086	1.28
Activated spores incubated under anaerobic conditions	0.073	1.09
Activated spores incubated in: 2 mM azide	0.069	1.03
2 mM cyanide	0.065	0.97

Spores were heat activated and placed under the various treatment
conditions outlined above for a period of 6 h (except where noted other-
wise). Controls included non-treated activated spores and dormant
spores incubated at 23.5 C for 6 h in buffer. After treatment the
spores were disrupted and assayed for trehalase activity as described
in Materials and Methods. The values for specific activity generated
were divided by the specific activity obtained from dormant spores to
yield a ratio for comparison.

The specific activity levels obtained for dormant and non-treat-ed activated spores compare closely to the data of earlier experiments on D. discoideum concerning trehalase (Ceccarini, 1966, Cotter and Raper, 1970). The fact that the specific activity of trehalase remained near the dormant level in activated spores which were prevented from germinating under restrictive environmental conditions suggests that protein synthesis, which would normally occur during the later stages of germination, was prevented.

DISCUSSION

Deactivation may be defined as the reversible, environmentally induced return of activated spores to the dormant state. All of the restrictive environmental conditions surveyed; high (37 C) or low (0 C) temperature, extreme pH (2 and 12), high osmotic pressure (0.25 M su-rose), respiratory inhibitors and anaerobic conditions cause deactivation of spores given a sufficient period of time. Spores closest to the period 7-10 minutes prior to the initiation of swelling are most sensitive to environmental insults as demonstrated by their maximized efficiency of deactivation. At this point in the temporal sequence of development, a large proportion of the activated population is able to achieve a similar "metabolic state" which enables them to "read" the signal imposed by the unfavorable environment. These observations conform with that predicted by the "multistate model of activation" (see Fig. 26) concerning the events occuring in the activated spore system during the activation and postactivation lag stages (Cotter, 1973, Cotter et al., 1976). In relation to this model, the majority of spores in the late postactivation lag phase have reached the "relaxed" conforma-
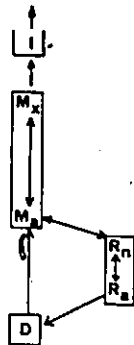
Fig. 26. The "multistate model for spore activation" (Cotter et al.,
1976). According to the model there is a single dormant conformation
(D) of the regulatory proteins in the inner mitochondrial membrane
which partially restricts oxidative phosphorylation in dormant spores.
Activation results in the production of one of a series of reversible
metastable (M) states which allow some oxygen uptake. The respiration
at this particular point is attributed to slight uncoupling of electron
transport from oxidative phosphorylation. Under certain conditions the
metastable states decay towards a lower energized conformation and
reach the relaxed (R) states. The relaxed states, which consist of a
small set of major conformations of the regulatory proteins, produce a
stable coupling of oxygen uptake and phosphorylation. The resultant
coupling allows ATP formation, which is required for spore swelling.
Inhibition of the relaxed state may result in further decay back to
the dormant conformation. The "I" states represent irreversibly de-
natured conformations which presumably result in the death of the
spore.

tion under restrictive conditions and, due to the negative signal received, return to dormancy. The dormant state is reinstituted through the transition of the presumptive regulatory protein from the relaxed to the dormant conformation. Activated spores in early lag phase require a longer period of time in order to revert to dormancy since they initially possess a higher metastable state which must decay to the relaxed conformation before the system is able to discriminate between favorable and restrictive environmental conditions. On the other hand, spores within 7-10 minutes of swelling or that have begun to swell are committed to the continuation of the process due to their hypothetically tightly coupled constitution.

Reversible activation is a temperature-dependent phenomenon. This was clearly demonstrated by the observation that DMSO activated spores incubated at 37 C require only 1 h in which to deactivate whereas DMSO activated spores incubated at 0 C required at least 24 h to achieve the same percentage of deactivation. A similar observation was made in the case of bacterial spores. Keynan et al. (1964) reported that the germination rate of activated spores of Bacillus cereus incubated at 28 C for 72 h was reduced to that of dormant spores, while activated spores incubated at 4 C for 72 h yielded a germination rate which was 40% of that exhibited by activated spores which had been placed directly into germination medium. As in the case of D. discoideum spores, deactivated bacterial spores were able to germinate after a second heat activation treatment when incubated in L-alanine- or adenosine-containing media (Keynan et al., 1964).

Activated spores are able to germinate from pH 3 to pH 11 and apparently survive for a short period of time under the more extreme

ends of the pH range. Whether or not these germinated individuals are able to successfully grow and divide at the far ends of the pH spectrum (eg. pH 3-4, pH 10-11) is doubtful but would require further experimentation for verification. It is apparent however, that these pH regimes are out of the normal physiological range encountered by these organisms since their natural habitat, forest soil, certainly never reaches such pH extremes. The marked decrease in viability of spores incubated at the extreme pH values of 2 and 12 is undoubtably due to the departure from the pH regimens encountered in nature. However, the observation that 20% of activated spores were able to survive via deactivation under the severe stress exerted by pH 2 and 12 demonstrates the remarkable survival capacity of the spores. The survivors also show that the regulatory mechanisms of the spores are effective even under the most severe environmental conditions.

Increased osmotic pressure prevents the germination of activated spores and causes the treated spores to return to the dormant state. The effects of osmotic pressure on spore germination have recently been examined by Cotter (1977). The deactivation caused by osmotic pressure was suggested to be induced through decreased water potential. That is, increased osmotic pressure functionally acts to reduce water activity which creates a pseudo-dehydration, thereby preventing development. In fact, actual air-drying of activated spores results in deactivation of the population (Cotter, 1977), adding feasiblity to such an argument.

The respiratory inhibitors azide and cyanide as well as anaerobic conditions deactivate spores. Since D. discoideum is an obligate aerobe, the dependence upon respiration during development is not surprising. However, the ability of the organism to return to dormancy without a

loss in viability in the treated population is less expected. Reversible activation was first observed by Goddard (1935) who found that heat activated Neurospora tetrasperma ascospores were not only unable to germinate under anaerobic conditions but were deactivated after a period of oxygen deprivation. Goddard also noted that the cycle of activation-deactivation could be repeated several times without loss of viability in the spore population. These results were later corroborated by Sussman et al.(1956) who also used Neurospora as a model system.

Activation of D. discoideum spores is apparently independent of the presence of oxygen, unlike the later stages of germination. Similarly, spores of Neurospora, Mucor rouxiana and Mucor hiemalis, for example, may also be activated in the absence of oxygen (Sussman et al., 1956, Sussman and Halvorson, 1966); as in the slime mold, the later stages of germination in these organisms are dependent upon aerobic conditions. These observations tend to strengthen the idea that a reaction such as the proposed partial helix-coil transition of a regulatory protein may be responsible for activation, since such a reaction would not require oxygen.

The loss of resistance to harsh environmental conditions during germination is well known for bacterial and fungal spores. Once D. discoideum spores are swollen, their resistance to severe environmental conditions is partially lost regardless of the phase of swelling. Low temperature or increased osmotic pressure as environmental shifts are not as deleterious to the survival of swollen spores as the other conditions tested. In addition, there was virtually no loss in viability detected even in the populations of emerged amoebae incubated under

these environmental conditions. However, if ice crystals had formed under low temperature incubation death of both swollen spores and e-merged amoebae would have ensued as previously shown by Cotter and Raper (1968b). The survival of vegetative amoebae is, as expected, severely affected by conditions other than low temperature or high osmotic pressure.

The incubation of activated spores under restrictive environmental conditions prevents the marked increase of respiratory activity observed in spores incubated under a permissive milieu. After prolonged incubation under most restrictive conditions, the respiratory rates of activated spores declines to a level comparable to the endogenous respiratory rate of dormant spores. Such a relationship was first observed by Goddard (1935) who reported that deactivated $\underline{N}$. tetrasperma ascospores respired at a rate slightly higher than dormant spores. Thus, spores induced to respire at lower rates due to environmental factors are able to revert back to the dormant respiratory constitution and maintain the lower rate. This ability demonstrates a crucial survival mechanism possessed by these spores. The particularly drastic reduction in oxygen consumption observed in $\underline{D}$. discoideum spores treated at the pH extremes of 2 and 12 may be attributed to both loss of viability and deactivation within the populations. The reduction in respiration of activated spores incubated in 0.25 M sucrose may be correlated with the effect upon the mitochondria since changes in tonicity are known to affect mitochondrial function. For example, the conidia of Aspergillus niger possess a respiratory quotient (QO_2) of greater than 5 at 100% relative humidity whereas at 60% relative humidity the QO_2 of these spores is diminished to a value of 1 (Terui and Machizuki in Sussman

and Douthit, 1973). In addition, various enzyme systems may be affected similarly due to conformational shifts under reduced water potential.

D. discoideum spores possess a cyanide-resistant respiratory pathway. Cyanide and 2,4-dinitrophenol (2,4-DNP) stimulate oxygen uptake of dormant spores. Both compounds apparently stimulate the alternate electron transport system. This conclusion is based on the observations that dormant spores treated with the combination of 2,4-DNP + SHAM yields a respiratory rate virtually equal to that of nontreated dormant spores. The combination of cyanide + SHAM results in the elimination of oxygen uptake of dormant spores. Likewise, heat activated spores treat-with the combination of cyanide + SHAM are prevented from respiring, although SHAM by itself has little effect upon the respiration of either dormant or activated spores. The inhibition of respiratory metabolism shown by these inhibitors apparently does not constitute a more efficient means to deactivate heat activated spores since a 5 h treatment is still ineffective in causing the treated spores to return to dormancy. This observation suggests that the deactivation process must certainly involve other, as yet undetermined mechanisms along with the regulation of respiration as part of the organism's control system.

The alternate electron transport system of these spores is not required for germination since SHAM (5 mM) does not deactivate or significantly inhibit respiration of heat activated spores. The effect of SHAM seems to be specific because the combination of cyanide + 0.25 M sucrose failed to reduce the respiration of heat activated spores to the extent of cyanide + SHAM. The mixture of azide + SHAM did not reduce oxygen uptake to the extent of cyanide + SHAM. Evidently, azide exerts an

unexpected interaction which reduces the effectiveness of SHAM. Other organisms, such as higher plants, bacteria and fungi also have been found to possess a cyanide-resistant respiratory system (Bendall and Bonner, 1971, Weber and Hess, 1976). Since this system has recently been characterized in conidiospores of N. crassa, it shall serve as a model system for further discussion here. N. crassa possesses a cyanide-resistant respiration which normally constitutes 10% of its total respiration (Lambowitz and Slayman, 1971). This alternate electron transport pathway may be induced to a rate 20 fold the normal rate in the presence of cyanide, antimycin A, or chloramphenicol (Lambowitz and Slayman, 1971). Although oxygen is consumed through this non-cytochrome system (Lambowitz et al., 1972a), oxidative phosphorylation (eg. the production of ATP) does not occur (Lambowitz et al., 1972b). The decreased efficiency of oxidative phosphorylation in the so-called "poky" strains of Neurospora is offset by increased respiratory levels via the alternate electron transport system which functions to maintain a low level of reduced pyridine nucleotides and other metabolites. This mechanism allows the glycolytic and citric acid cycles to operate at increased rates in order to compensate for the cell's reduced potential for ATP production (Lambowitz et al., 1972b). Thus Lambowitz et al. (1972b) concluded that under normal conditions, respiration in the wild type organism may be partitioned so that the cytochrome system operates at full capacity, whereas the alternate system handles the excess electron flux. This mechanism may well be functioning similarly in D. discoideum spores since the pathway seems not to be essential for germination.

Sodium fluoride and sodium malonate partially inhibit the respiration of activated spores. The fact that fluoride, an inhibitor of the glycolytic enzyme enolase, prevented 40% of an activated spore population from swelling seems to suggest that glycolysis may at least play a supportive role during germination. Malonate, an inhibitor of succinic dehydrogenase in the citric acid cycle, at a concentration of 0.08 M markedly affects respiration of activated spores. However, taking into account the ionic strength imposed by this concentration of malonate (eg. 0.24 M) it is likely that this compound never entered the spores and asserted a nonspecific osmotic pressure inhibition similar to that of 0.25 M sucrose. The fact that a lower concentration (eg. 0.02 M) did not affect spore germination supports this hypothesis. The role of the major metabolic pathways during D. discoideum spore germination has been neglected in the past. Recently, dormant spores were found to contain malate, glutamate and the NAD-linked isocitrate dehydrogenases of the citric acid cycle with specific activity levels equal to or higher than that found in vegetative amoebae (Kobilinsky and Beattie, 1977). Therefore it would seem that enzyme levels are not a major regulatory facet in the maintenance of dormancy. However, drawing such a conclusion from the results of one study would be presumptuous. Thus, the presence and function of the major metabolic pathways during D. discoideum spore germination is a worthy topic for future study.

The protein synthesis inhibitors cycloheximide and chloramphenicol fail to inhibit oxygen consumption of activated spores to a significant degree. Though cycloheximide-treated spores were prevented from entering the emergence stage of germination, chloramphenicol had no effect upon the rate of swelling and only slightly lowered the rate at which

emergence took place. It is certain that some de novo protein synthesis, which is directed by cytoplasmic ribosomes, is required for emergence. On the other hand, protein synthesis directed by mitochondrial ribosomes would not seem to be required for spore germination. It must be noted that due to possible permeability problems in the nonswollen spore, such interpretations may be subject to error. However, supportive evidence for such conjecture has recently been presented by Kobilinsky and Beattie (1977) who found that dormant spores of D. discoideum contained a structurally complete mitochondrial respiratory system. These authors also presented evidence that chloramphenicol (4 mg/ml) prevented the development of activated spores. Unfortunately with such a high concentration of the inhibitor it is difficult to rule out non-specific effects upon the spore system. Thus, their conclusion that mitochondrial protein synthesis is required for germination becomes questionable. Examining other model spore systems, for instance, the ascospores of Botryodiplodia theobromae, which possess lowered levels of cytochromes b and c with cytochrome a missing (Brambl and Josephson, 1977), are completely inhibited from germinating and exhibiting increased respiratory activity in the presence of cycloheximide (Brambl, 1975). Chloramphenicol partially inhibits a secondary increase of respiratory activity in the biphasic respiration profile of these spores (Brambl, 1975). On the other extreme, although a rise in oxygen consumption along with increased cytochrome oxidase activity are concomitant with the germination of Saccharomyces cereviseae ascospores (Rousseau and Halvorson, 1973) neither event is required for germination or outgrowth (Tingle et al., 1974). Thus, although there may be parallels in different germinating systems the absolute requirements of each may ultimately be unique.

The initiation of protein and RNA syntheses, which usually occur during the germination of activated D. discoideum spores, are inhibited when spores are incubated under restrictive environmental conditions. This observation suggests that protein and RNA syntheses are prevented in activated spores which are returning to dormancy. The increase in trehalase activity during the late swelling phase of germination is cycloheximide- sensitive (Cotter and Raper, 1970). The fact that deactivating spores failed to yield an increased trehalase activity demonstrates that de novo protein synthesis, which normally occurs during the later phases of germination, is prevented. However, further experimentation is required to determine whether protein synthesis occurs in the early phases of deactivation. There is a possibility that membrane associated transport systems were shut down as a result of the incubation of activated spores in unfavorable conditions, thereby preventing the uptake of the labelled precursors used as indicators of macromolecular syntheses. For instance, azide and cyanide are known to inhibit active transport systems in a number of different cellular systems (Lehninger, 1973). It is doubtful, however, that all the restrictive environmental conditions used in this study acted to specifically inhibit such transport systems. Since these spores require no exogenous metabolites except for water and oxygen for the entire process of germination, it is physiologically insignificant that transport mechanisms for displacing exogenous materials into the spores were inactivated. On the other hand, the possibilty exists that the inhibition of internal transport mechanisms may be a factor through which these unfavorable environmental conditions facilitate the deactivation process.

Trehalose is a common storage product in many fungal spores. For

instance, spores of <u>Neurospora</u> and <u>Phycomyces</u> <u>blakesleeanus</u> utilize this disaccharide during germination (Sussman in Sussman and Halvorson, 1966, Van Assche <u>et al.</u>, 1972). Although heat shock activates trehalase activity in <u>P.</u> <u>blakesleeanus</u> (Van·Assche <u>et al.</u>, 1972), this activity is not required for the activation stage of germination, but is utilized later in germination, as in <u>D.</u> <u>discoideum</u>. The events occuring during the postactivation lag and early swelling in <u>D.</u> <u>discoideum</u> spores probably utilize endogenous reserves such as free glucose, glutamate and/or amino acids rather than trehalose as an energy source. The fact that spores placed in environmental conditions which cause deactivation do not produce trehalase and possibly other new proteins demonstrates the conservatism built into this system.

SUMMARY

The major physiological effects of restrictive environmental changes on activated <u>D.</u> <u>discoideum</u> spores were examined. The major points observed were as follows; i) activated spores may be deactivated by a diverse range of environmental shifts and spores which are closest to the period 7-10 minutes prior to the initiation of swelling are most sensitive to such shifts, ii) the survival of swollen spores under certain restrictive environments is independent of the relative phase of swelling, iii) respiration of activated spores is severely inhibited by restrictive environmental conditions and given time respiration declines to a rate comparable to the endogenous respiratory rate of dormant spores. This decline marks the deactivation of the treated spores, iv) elimination of respiratory processes (eg. by cyanide + SHAM) apparently does not constitute a more efficient means to induce deactivation, v)

amino acid and uridine uptake is inhibited in activated spores which were incubated under restrictive conditions suggesting an inhibition of de/novo protein and RNA sytheses, vi) trehalase activity fails to increase from dormant levels when activated spores are placed under conditions which cause deactivation, indicating that protein synthesis, at least for late events during germination is prevented in deactivat-ing spores. It is concluded from these studies that the function of the mitochondrial respiratory system is required for every aspect of ger-mination except activation. Thus, a major regulatory position in both the maintenance and breaking of dormancy may be held by the mitochon-dria in these spores. Since all the structural components for mitochon-drial respiration along with components of the citric acid cycle are present in dormant spores (Kobilinsky and Beattie, 1977), a mechanism regulating the function of the organism's respiratory metabolism rather than the biosynthesis of respiratory elements may control spore dormancy. Furthermore, the fact that the elimination of respiratory metabolism does not cause immediate deactivation suggests the possibility that a cascade of events are required in spores which are induced to return to the dormant state by the imposition of restrictive environmental conditions.

Currently, aspects of protein synthesis which include the effects of cycloheximide and chloramphenicol during D. discoideum spore germin-ation are being investigated in our laboratory. The outcome of these studies should prove to be enlightening. Future work concerning the process of germination include; i) investigation of the pyridine nucleo-tide levels, ii) cytochrome levels and iii) the activity levels of the enzymes of the major metabolic pathways.

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