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THE ROLE AND EXPRESSION OF TREHALASE AND β -GLUCOSIDASE
DURING SPORE GERMINATION OF DICTYOSTELIUM DISCOIDEUM

By



Agnes How-Ching Chan

A thesis
submitted to the Faculty of Graduate Studies
through the Department of
Biology in Partial Fulfillment
of the Requirements for the Degree
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ABSTRACT

The final stage in the developmental life cycle of Dictyostelium discoideum is the formation of a fruiting body. This structure consists of a basal disk, a column of dead stalk cells, and a sorus containing dormant spores in a viscous matrix of extracellular material. Analysis of crude extracts of the sorus demonstrates that there is a higher specific activity of β -glucosidase and trehalase in the extracellular matrix than in the dormant spores. These extracellular enzymes, which were synthesized by stalk cells, degrade or denature upon aging of intact sorocarps. This may indicate that the enzymes are probably not involved in the autoactivation phenomenon.

Electrophoresis of matrix material from the sori reveals the presence of β -glucosidase 2 with a trace amount of β -glucosidase 1. Both forms of β -glucosidase are present during spore germination of D. discoideum. In dormant spores only β -glucosidase 2 is detected. Upon heat-activation of the spores, this form of β -glucosidase is reduced to an undetectable level. During emergence of myxamoebae, β -glucosidase 2 is replaced by a newly-synthesized enzyme, β -glucosidase 1. The formation of the latter form of β -glucosidase requires both RNA and protein syntheses.

The β -glucosidase 2 enzyme that is found in dormant spores appears to have a surface location since trypsin

treatment will cause total degradation of the enzyme. The normal germination of these trypsin treated spores suggests that β -glucosidase 2 is not required for the germination process.

The complete germination of a β -glucosidase 1 minus mutant also casts doubt on the necessity for β -glucosidase 1 activity during emergence. Spores (which have been blocked from emerging by cycloheximide treatment) will release spheroplasts when treated with pronase and D. discoideum cellulase. However, addition of β -glucosidase 1 to the cellulase-pronase treated spores does accelerate the rate of spheroplast formation. Thus, it is concluded that β -glucosidase 2 is not required for spore germination but β -glucosidase 1 plays a minor but dispensable role in D. discoideum spore germination.

Trehalase activity, unlike β -glucosidase 2, is not denaturated upon heat-induced activation of spores. The trehalase specific activity remains at a basal level in the early stages of germination and by late spore swelling the enzyme activity begins to increase. The latter increase in trehalase activity requires both RNA and protein syntheses. This newly-synthesized trehalase is released into the extracellular medium during spore germination.

Trehalose, the substrate for the enzyme trehalase, is hydrolyzed before late spore swelling. This indicates that trehalase which increases during maximum spore swelling is not involved in trehalose degradation. Therefore the

trehalase synthesized during late spore swelling may not have a critical role in spore germination. The above hypothesis is supported by the normal utilization of trehalose in UV irradiated and cycloheximide treated spores which swell but do not synthesize trehalase or β -glucosidase 1. Thus, it is concluded that the newly-formed trehalase, like β -glucosidase 1, does not play a critical role in the spore germination process.

Temporal as well as quantitative shifts in expression of trehalase and β -glucosidase 1 are observed when spores are subjected to chemical activation and autoactivation. These shifts are more pronounced in the expression of trehalase than in β -glucosidase 1. The shifts in enzyme expression without concomitant shifts in the time of emergence lend additional support to the hypothesis that these enzymes are not critical to the germination process.

To my Parents

ACKNOWLEDGEMENTS

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INTRODUCTION

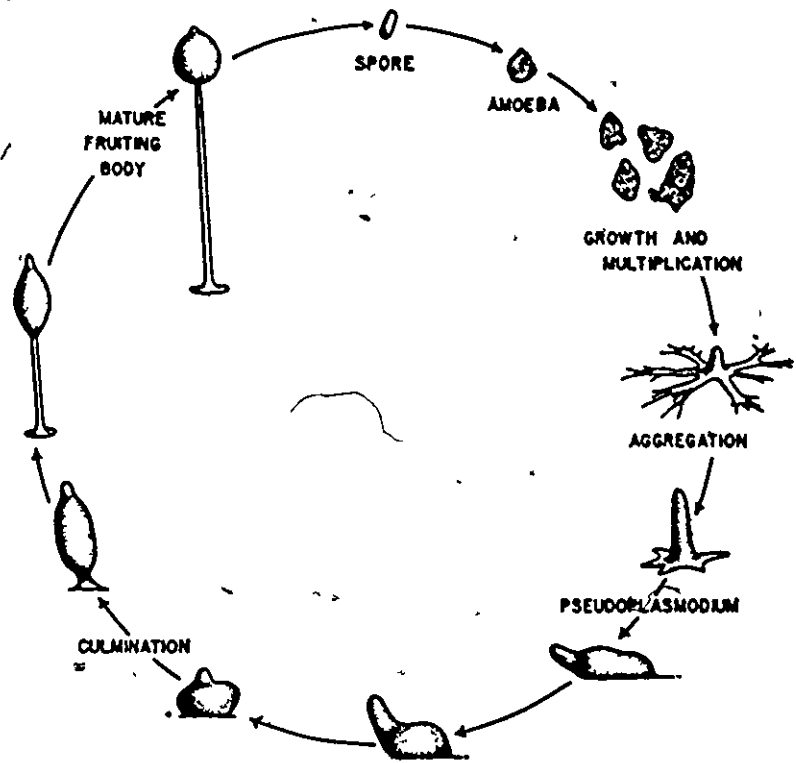
A. General overview of the life history of the cellular slime mold, Dictyostellium discoideum.

The cellular slime mold, Dictyostellium discoideum NC4H, was first isolated by K.B. Raper (1935) from forest soil in North Carolina. It is not, by any means, the most common species of cellular slime mold, but is certainly the one most intensively studied in all research laboratories.

The life cycle of D. discoideum is shown in Figure 1 (Wright, 1963). The amoebae are found in bacteria-rich soils where they are indistinguishable from other soil amoebae. They feed on the bacteria of decaying fallen leaves and will continue to grow and undergo binary fission as long as food is available. However, upon depletion of the food source, a unique and characteristic series of developmental events occur which distinguish D. discoideum from other soil amoebae. These developmental events consist of the following three stages: aggregation, pseudoplasmodial formation and culmination.

The amoebae will first aggregate into a mound containing approximately 10^5 cells. The aggregation is initiated by a substance originally referred to as 'acrasin' which is first secreted by the centrally located amoeba. The presence of this chemical in the environment will then stimulate the neighbouring amoebae to secrete their own 'acrasins' (Bonner, 1967). This chemical was later

FIGURE 1. The life cycle of Dictyostelium discoideum
(after Wright, 1963).



identified to be the nucleotide adenosine 3',5'-cyclic monophosphate (cAMP) (Barkley, 1969).

The aggregate becomes integrated by deposition of a surface sheath covering the whole mound. This aggregate topples over onto the surface and migrates horizontally as a pseudoplasmodium (a grex or a slug). The pseudoplasmodium is phototactic and can move towards a unidirectional light source of extremely low density. Once the migration phase has ended, the pseudoplasmodium rears back upon itself and extends its diameter to form a flattened disc-like object. At this point the cells at the anterior end differentiate into pre-stalk cells, whereas cells at the posterior end differentiate into pre-spore cells.

The pre-stalk cells ultimately develop into large, vacuolate stalk cells with thick cellulosic cell walls. The stalk cells extend lengthwise causing a rapid elongation of the stalk and form the fruiting body or sorocarp with the spore cells at the top. This marks the end of the culmination stage. To complete the life cycle, the spores are released from their container, the sorus, and upon favourable conditions germinate into vegetative amoebae (see Newell, 1971, 1978; Loomis, 1975 for review).

Cellular slime molds are haploid throughout their usual growth and developmental stages, and mutations are therefore much more readily detected than in most eukaryotes which are diploid. Several mutants are used in

this study. They are the axenic mutant, AX3, which unlike the wild type strain NC4H can grow axenically without the presence of bacteria (Loomis, 1971); the β -glucosidase 1 minus mutant, G1, which is a mutant of AX3 and is unable to synthesize the β -glucosidase enzyme (Diamond and Loomis, 1976); and the spontaneous germination mutant, SG1; is a mutant of NC4H which can germinate in the absence of any activation treatments (Cotter and Dahlberg, 1977).

B. Spores and spore germination in *D. discoideum*.

Dormant spores of *D. discoideum* are contained in a viscous matrix of extracellular material at the top of the sorocarp (see Murata and Ohnishi, 1980). The extracellular matrix consists mainly of enzymes (Chan et al., 1981b). Other components of the matrix such as complex carbohydrates (Ceccarini and Filosa, 1965) and discadenine (a spore germination inhibitor) are also found (Abe et al., 1976). The dormant spores of wild type strain NC4H are capsule shaped and vary in size; normally, the spores are approximately 6 to 9 μ m long and 2.5 to 3.5 μ m in diameter (Raper, 1935; Bonner, 1967).

Ultrastructural and enzymatic analysis by Hemmes et al. (1972) demonstrate that the dormant spore of *D. discoideum* consists of the following three layers: the outermost layer which is mainly made up of mucopolysaccharide; the middle layer which is actually constructed of two distinct cellulosic layers. The two sub-layers differ only in the orientation of their fibrils.

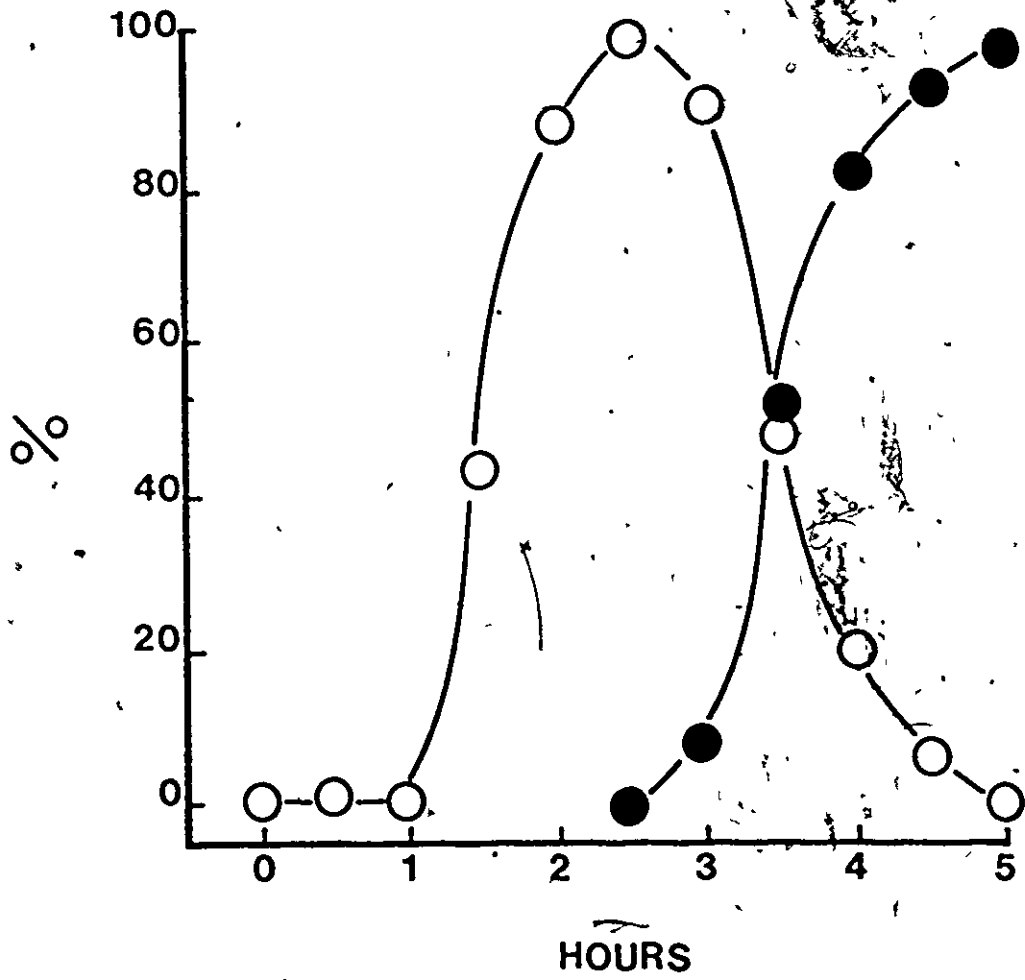
However, it is still not certain if cellulose is the only component present in these sub-layers; the third layer is made up of at least two components, cellulose and protein.

Young D. discoideum spores are constitutively dormant and will not germinate into myxamoebae without an exogenous activation treatment. The conversion of dormant spores to nascent myxamoebae may occur in the following four stages: activation of dormant spores, postactivation lag, swelling of activated spores and the release of a single myxamoeba from each swollen spore (Cotter, 1975) (Figure 2).

Several activation methods, both physical and chemical, have been developed by Cotter and co-workers (see Cotter, 1981 for review). Before activation, the spores have to be washed once in double distilled water and twice in 0.1 M phosphate buffer (pH 6.5) to remove all inhibitor(s). The activation techniques include the following: 1% peptone for 30 min (Cotter and Raper, 1966); heat at 45 C for 30 min (Cotter and Raper, 1968ab); 20% dimethyl sulfoxide (DMSO) for 30 min (Cotter et al., 1976); 8 M urea for 30 min (Cotter and O'Connell, 1976); 3 M ethylene glycol for 60 min (Cotter, 1977); 6 M guanidine HCl for 60 min, 2 M dimethylurea for 60 min, and 2 M tetramethyl urea for 30 min (Cotter, 1979).

The time between the removal of the activating stimulus and the first sign of spore swelling is called the postactivation lag, and can be divided into two almost equal phases: early and late lags (Cotter, 1975). The

FIGURE 2. The germination sequence of heat-activated Dictyostellium discoideum strain NC4H spores (Cotter and Raper, 1968b). Spores were heat-activated at 45 C for 30 min and incubated at 23.5 C for 5 hr. ○, percent of swollen spores; ●, percent of emerged myxamoebae. The semidiagrammatic depiction of spore germination below the abscissa includes late lag, early swelling, mid swelling, late swelling, emerged myxamoebae, and post-emerged myxamoebae.



postactivation lag as a whole lasts from 0.5 to 2 hours depending on the method of activation employed.

The swelling stage of germination begins with the appearance of a lateral protuberance leading to the loss of the first two layers of the spore wall (Cotter et al., 1969; Hemmes et al., 1972). The protuberance enlarges until the entire spore is swollen. This is accompanied by loss of refractility, so that the swollen spore appears phase dark instead of phase bright under a phase contrast microscope. The later part of this stage is characterized by the appearance of granules and one or more contractile vacuoles (Cotter and Raper, 1966, 1968a,b; Cotter, 1975). Splitting of the outer two wall layers begins during spore swelling (Cotter et al., 1969; Hemmes et al., 1972).

The last stage of spore germination, the emergence of myxamoebae, is defined as a splitting of the innermost wall layer (Cotter et al., 1969; Hemmes et al., 1972). The myxamoeba possesses pseudopodia and is phase dark. In this final stage, the lipidial bodies as well as proteinaceous crystals are degraded so that the true vegetative amoeba is devoid of these structures (Cotter et al., 1969; Gregg and Badman, 1970).

Wild type spores, NC4H, will germinate to form more than 95% myxamoebae by the end of 5 hr after activation when incubated at 10^6 - 10^7 spores/ml at 23.5 C in 0.01 M phosphate buffer (pH 6.5) with continuous shaking (Cotter and Raper, 1968a,b). However, when mutants such as, B, AX3

and G1, are germinated, less than 80% of the spores will transform into myxamoebae. In the case of heat-activation, the 80% germination will not be seen until 10 hrs after heat-induced activation (Cotter and Raper, 1968c).

The spontaneous germination mutant, SG1, will germinate to more than 95% at a concentration of 10^7 spores/ml in the absence of any exogenous activation treatments (Cotter and Dahlberg, 1977). Furthermore, the germination process is unchanged even if additional activation treatment is applied to the dormant spores.

As mentioned above, freshly formed spores of NC4H are constitutively dormant and do not germinate without an activation treatment. However, upon aging of the spores in the intact sorocarp for more than 7 days, autoactivation will occur upon suspension of washed spores in phosphate buffer. Spores that are aged for 7 days will show about 1% swelling at 6 hr and 17% at 8 hr, while those that are aged for 10 days show 17% swelling at 6 hr and 86% at 8 hr. If the spores are allowed to age for 13 days in the sorus, washed spores will germinate to at least 95% without any activation treatments (Dahlberg and Cotter, 1978).

The process of spore germination in D. discoideum can be inhibited by exposing spores to one of the following four environmental factors: reduced oxygen tension, high or low pH, high osmotic pressure and high or low temperature. If spores in the lag phase of germination are exposed to any one of the above environmental extremes, then the

spores will not swell but they will return to the dormant stage, i.e., they will deactivate. However, if the same condition is applied to spores in the swelling or emergence stage, then the spores will not return to the dormant state but will die (Cotter and Raper, 1968a,b; Cotter, 1975; Cotter et al., 1979).

C. Expression of β -glucosidase during the multicellular developmental stages of *D. discoideum*.

A series of enzymes have been found to accumulate during discrete stages of the multicellular development of the cellular slime mold, *Dictyostelium discoideum* (see Rosness, 1968; Loomis, 1975). One such enzyme is the presumably lysosomal enzyme, β -glucosidase (Rosness, 1968; Coston and Loomis, 1969). The specific activity of β -glucosidase increases during the first 4 hr after removal of the food source (aggregation stage) and then decreases until culmination begins about 14 hr later. At this time, a second period of increase occurs, and this increase reaches a maximum at the end of the culmination period. Electrophoresis of these cell preparations on 5% continuous polyacrylamide gels shows that two forms of enzyme are present during the developmental stages of *D. discoideum*. The enzyme that forms during aggregation has a faster mobility on the gel than the one formed at the end of culmination. Coston and Loomis (1969) refer to the faster moving enzyme as β -glucosidase 1 and the slower moving one as β -glucosidase 2. Occasionally, a third component which

migrates faster than either of the two enzymes will be seen in both early and late samples, but when observed it is always found to represent less than 5% of the total activity.

The accumulation of β -glucosidase 1 occurs independently of either RNA or protein synthesis. Whereas, increase of β -glucosidase 2 seems to require both types of synthesis. Actinomycin D (an inhibitor of RNA synthesis) as well as cycloheximide (an inhibitor of protein synthesis) are found to prevent the formation of β -glucosidase 2. Because of its late accumulation, Coston and Loomis (1969) have proposed that β -glucosidase 2 might have a function in the spores and is probably involved in the splitting of the spore wall during germination.

Besides their difference in mobilities on 5% polyacrylamide gels, the two enzymes are also found to differ in their respective K_m values for p-nitrophenyl- β -D-glucoside substrate. The K_m value of β -glucosidase 1 is 1.28×10^{-3} M and that for β -glucosidase 2 is 0.83×10^{-3} M. However, both forms of β -glucosidase exhibit optimum activities at pH 4.5 - 5.5 (Coston and Loomis, 1969).

When β -glucosidase 1 is purified to electrophoretic homogeneity from vegetative cells of strain M3 (a mutant of AX3 that lacks α -mannosidase activity), then the enzyme is found to consist of two subunits with apparent molecular weights of 68,000 and 88,000 daltons (Diamond and Loomis, 1976). The enzymatic activity of β -glucosidase 1 is found

to sediment as a molecule of 8S and has an apparent molecular weight of 160,000 (Coston and Loomis, 1969; Every and Ashworth, 1973). Thus, the active enzyme appears to contain one copy of each of the subunits.

Several mutants that are deficient in β -glucosidase 1 activity have been isolated (Diamond and Loomis, 1976). These mutants are derived from the axenic strain AX3 which, unlike the wild type NC4, excretes β -glucosidase 2 enzyme into the medium during growth on bacteria (Ashworth and Quance, 1972). The release of β -glucosidase 2 enzyme during development thus aids in the procedure used to screen for mutants. One of the mutants, G1, is found to contain only about 2.5% of its parental (AX3) β -glucosidase 1 activity, and most of this remaining activity can be accounted for by the activity of a β -galactosidase enzyme that can nonspecifically hydrolyze the β -glucoside substrate (Diamond and Loomis, 1976; Loomis, 1980).

Besides G1, six other mutants lacking measurable β -glucosidase 1 activity have been isolated. All these mutants which lack β -glucosidase 1 activity are also found to be unable to accumulate β -glucosidase 2 activity (Diamond and Loomis, 1976). All seven mutations affecting β -glucosidase activity in D. discoideum are found to be non-complementing, recessive to the wild-type allele, and to occur in the gene locus, glu A (Loomis, 1980).

To explain why there are two polypeptides in purified β -glucosidase 1 and yet only one recognized gene

affecting the activity, Loomis (1980) proposed the following explanations: 1) one of the subunits might not be required for the activity measured; 2) the polypeptides might be derived from cleavage of a larger precursor; or 3) the polypeptides might be derived from the same primary translation product but be differentially modified at a later time. The probability that one of the polypeptides is a contaminating protein is eliminated since the polypeptides tested are in equimolar proportions after greater than 2,000 fold purification, and the enzyme activity comigrates with 90% of the total protein following electrophoresis at three different pH conditions (Loomis, 1980).

Unlike β -glucosidase 1 which is rather stable at low temperature, β -glucosidase 2 is very unstable and demonstrated a consistent loss of about 15% of its activity per day during the purification process (Diamond and Loomis, 1976).

When the immunological relationship between the two forms of enzyme was studied (Diamond and Loomis, 1976). It is found that both β -glucosidases as well as several hydrolases share a common immunological determinant. The antigen is unaffected by boiling or mild NaOH treatment. However, it is totally destroyed by proteinase K digestion and partially destroyed by periodate oxidation. Thus it has been suggested that the antigenic determinant requires intact protein and probably represents a glycosylation of

that protein. Loomis (1980) later showed that both polypeptides of β -glucosidase 1 are glycosylated.

Loomis (1980) further postulates that both enzymes, β -glucosidase 1 and β -glucosidase 2, are controlled by a single gene and that their difference in mobility and other factors are contributed to by a post-translational modification process. This modification is restricted to the modA gene (Free et al., 1978a; Free and Schimke, 1978b).

D. Expression of β -glucosidase during spore germination of *D. discoideum*.

Dormant spores of *D. discoideum* contain a low amount of β -glucosidase. This enzyme decreases in specific activity during heat activation. However, upon emergence of myxamoebae, the enzyme undergoes at least a ten fold increase in specific activity when compared to post activated spores at zero time (Tisa and Cotter, 1980). The increase of this enzyme requires protein and RNA syntheses since spores that are subjected to UV irradiation or germinated in the presence of 200 μ g/ml of cycloheximide fail to express any β -glucosidase activity (Tisa and Cotter, 1980; Demsar, personal communication). The enzyme unlike other enzymes such as, cellulase, are not released into the medium during spore germination (Jones et al., 1979; Tisa and Cotter, 1980).

Decryptification experiments performed by Tisa and Cotter (1980) suggested that the β -glucosidase enzyme is

probably located near, or bound to, the external surface of the plasma membrane of dormant spores since similar enzyme activity was found in dormant spores that had undergone breakage and those that have not been subjected to any treatments. However, the above observation is not true for emerged myxamoebae. A higher enzyme activity is found in those amoebae that have undergone at least some type of decryptification treatments than in those that had not been treated (Tisa and Cotter, 1980).

Deactivating conditions such as, incubating spores in the presence of sodium azide (2 mM) or potassium cyanide (2 mM), or at 0 C for 48 hr, block the expression of the increase in β -glucosidase activity (Tisa and Cotter, 1980).

E. Relationship of trehalase and trehalose during the multicellular developmental stages of D. discoideum.

The relationship between trehalase and trehalose was first studied by Ceccarini (1967). Trehalase enzyme activity was found to be present in almost all development stages of D. discoideum. The activity was found maximally in the vegetative amoebae and was preferentially released into the external medium during aggregation according to Ceccarini (1967). The activity of the enzyme remained low for the rest of the developmental cycle. Upon germination of the mature spores, the enzyme reappeared in the germinating amoebae (Ceccarini, 1967). The release of trehalase during aggregation was independent of either RNA

or protein synthesis. Once the enzyme had been released to the external medium it did not reappear until the cells were fed with E. coli (Ceccarini, 1967).

The results of Roth and Sussman (1966) and Killick and Wright (1972) on the activity of trehalase during differentiation differ from those of Ceccarini (1967). Instead of having a low level of trehalase enzyme activity in the spores. These authors claimed that the enzyme reappeared in the terminal stage of sorocarp formation, and thus trehalase was present in a fair amount in the spores.

By means of an ultra-microtechnique, Jefferson and Rutherford (1976) showed that most, if not all, of the activity present in the sorocarp stage was stalk specific. Thus the high trehalase activity found in the sorocarp by Killick and Wright (1972) was actually that of the stalk cells and not the spore cells.

The level of carbohydrate, trehalose, varies considerably throughout the life cycle of D. discoideum (Ceccarini and Filosa, 1965). In the vegetative amoebae and migrating slugs, the level of trehalose is very low and occupies less than 0.5% of the total dry weight. However, upon culmination, the level of trehalose increases to 1.5% of the dry weight and reaches a maximum of 5% in mature spores. Trehalose and glucose are found to be the only water-soluble carbohydrates in the mature spores. Indirect evidence obtained from Muller and Hohl (1975) suggested that trehalose is localized in vesicles within the spores.

F. Expression of trehalase during spore germination of D. discoideum.

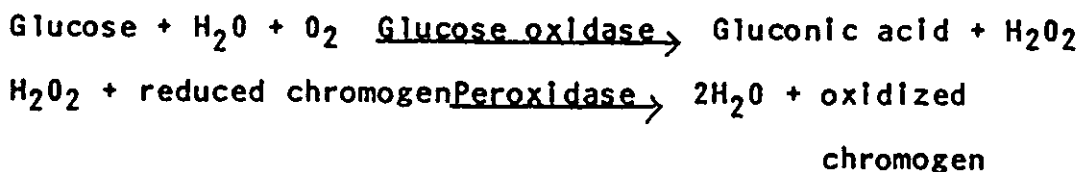
Very low trehalase activity is found in the post-activation lag and early swelling stages of heat activated D. discoideum NC4H spores (Cotter and Raper, 1970; Cotter et al., 1979; Tisa and Cotter, 1979b). Unlike β -glucosidase, this low level of trehalase is unaffected by heat treatment. By late swelling, about 15-30 min before the emergence of myxamoebae, the enzyme increases dramatically in activity. The increase in activity of trehalase requires both RNA and protein syntheses. UV irradiation of spores or addition of 200 ug/ml of cycloheximide at the beginning of germination blocks the expression of trehalase activity (Cotter and Raper, 1970; Tisa and Cotter, 1980; Demsar, personal communication). However, little is known on the utilization of trehalose during the germination process (Ceccarini, 1967). Whether the enzyme will be released to the external medium during the above process is also unknown.

G. Requirement for an improved coupled assay for the detection of glucose released by acid glucosidases.

The requirement for assay of acid glucosidase activities is frequently encountered in biochemical research involving both prokaryotic and eukaryotic organisms. Acid glucosidases such as, trehalase and β -glucosidase, release glucose as one of their endproducts after hydrolysis of their natural substrates. For β -

glucosidase, the enzyme activity can be detected by using a synthetic compound (p-nitrophenyl- β -D-glucoside) as substrate. However, trehalase which is a very substrate specific enzyme cannot hydrolyze such a synthetic compound. Thus a rapid and sensitive method for determining the activities of acid glucosidases, especially that of trehalase, is required. The existing methods employed are either complicated (Ballario et al., 1978; Killick, 1979), are expensive (Ballario et al., 1978), are of low sensitivity or work only at neutral pH (Ceccarini, 1965; Deshpande et al., 1978).

In this study, an improved reagent for detecting glucose which is released by acid glucosidases at low pH is introduced (Chan and Cotter, 1980). The reagent was first utilized by Becker (1974) to detect free glucose in blood plasma at pH 7.0. The principle of this method is outlined below:



The chromogen employed in this study is 2,2'-Azino-di-(3-ethyl-benzthiazolin)-6-sulphonate (ABTS).

Unfortunately, the enzymes used by Becker had neutral pH optima and thus were unsuitable for measuring glucose at acidic pH values.

H. The purpose of this study.

The purpose of this study was to investigate the role

of two enzymes, β -glucosidase and trehalase, in the germination process.

The expression of different forms of β -glucosidase during spore germination was also studied.

An improved coupled assay for detecting glucose released by acid glucosidases was introduced for the detailed study of trehalase activity during the germination process. The utilization of trehalose was investigated using the above assay.

Finally, the relationship between methods of activation and the expression of enzymes was also studied.

MATERIALS AND METHODS

A. Media Used.

Several strains of Dictyostelium discoideum were used in this study. All strains (NC4H, SG1, AX3, and G1) were grown in conjunction with Escherichia coli B/r on glucose-salt agar (Adams, 1959). This medium consists of the following components: 1.0 g of NH_4Cl , 0.13 g of MgSO_4 , 3.0 g of KH_2PO_4 , 6.0 g of Na_2HPO_4 and 20.0 g of Bacto agar (Difco) in 1 liter of double distilled water. After autoclaving, 10 ml of 0.4 g/ml of sterile glucose (Fisher) was added to the above medium. After complete mixing, the medium was cooled to 50 C and dispensed into 100 X 15 mm plastic petri dishes.

Spores of D. discoideum were germinated in phosphate buffer consisting of 1.04 g of KH_2PO_4 in 1 liter of double distilled water; the pH of the 0.01 M solution was adjusted to 6.5 with concentrated KOH prior to autoclaving.

B. Formation of D. discoideum sorocarps.

Spores of D. discoideum were transferred aseptically from a stock plate to 20-80 ml of sterile double distilled water yielding a spore density of 10^7 spores/ml. A loopful of E. coli B/r was then transferred to the suspension and mixed thoroughly; 2 ml of the mixture was transferred onto the glucose-salt plates and incubated at 23.5 C. The culture was shaken at 24 hr and 48 hr after plating to ensure even growth and synchronous formation of sorocarps.

Myxamoebae will usually be found on the second day after plating and by the fourth day young sorocarps bearing spores at the top will be seen. For germination experiments, the sorocarps were allowed to age from 1-3 days before the spores were harvested in order to ensure that all spores used in experimental work had entered dormancy.

C. Collection of matrix enzymes.

Dormant spores of D. discoideum are contained in a viscous matrix of extracellular material at the top of the sorocarp. This extracellular material is composed mainly of enzymes. To collect these enzymes, 1-13 days old sori were removed from the stalk cells by passing a microscopic slide several millimeters above the agar surface. The sori which consisted of spores and extracellular material were suspended into 5-15 ml of sterile double distilled water or acetate buffer at 0 C. The spores were then removed from the extracellular material by low speed centrifugation at setting 6 in an IEC clinical centrifuge. The supernatant containing the yellowish-green matrix material was stored at -21 C until use.

D. Germination of D. discoideum spores.

Spores which have been washed once in distilled water were washed twice again in 0.01 M phosphate buffer (pH 6.5) to ensure that all spores were free of extracellular material.

Both physical and chemical activation methods were

used in this study. If heat activation was used, spores were suspended in 5 ml of 0.01 M phosphate buffer (pH 6.5) and placed in a 45 C waterbath for 30 min. At the end of heat activation, the spores were diluted out to a concentration of approximately 1×10^7 spores/ml in phosphate buffer and incubated at 23.5 C with gentle shaking to allow germination to occur. The number of spores/ml was calculated with a hemacytometer (Cotter and Raper, 1966, 1968a,b).

Spores were also activated in 5 ml of phosphate buffer with the following chemicals: 20% dimethyl sulfoxide (DMSO) for 30 min (Cotter *et al.*, 1976) and 8 M urea for 30 min (Cotter and O'Connell, 1976). In both cases, chemicals were removed by filtration through a 1.2 um Millipore filter at the end of activation. The spores were then washed twice in phosphate buffer.

In addition to using both physical and chemical activation treatments, the spontaneous germination mutant, SG1, was also allowed to undergo autoactivation (Cotter and Dalhberg, 1977). The spores of SG1 were harvested as indicated above and washed twice in phosphate buffer. The spores were then diluted to a concentration of approximately 10^7 spores/ml in phosphate buffer and incubated at 23.5 C with continuous shaking.

Spores were germinated after activation in one of two ways depending on the volume needed. In the case of small volumes, activated spores were placed in 10 X 1 cm test

tubes and incubated at 23.5 C in a Braun Thermomix 1420 waterbath. The spores were stirred with magnetic stirring bars propelled by a submersible stirring unit (Tri-R micro-submersible magnetic stirrer). In the case of large volumes, a liter batch of activated spores was introduced into a 2.8 liter fernback flask shaken gently with a Gyrotory shaker (Model G2) at 200 oscillations/min.

The percentage of swollen spores and released myxamoebae was monitored by placing approximately 0.03 ml of the suspension on a slide and counting the first 200 objects with a Zeiss phase contrast microscope at a magnification of 320 X. The objects were placed into three groups: unswollen spores, swollen spores, and emerged myxamoebae.

E. Production of vegetative myxamoebae.

Vegetative amoebae were prepared by inoculating glucose-salt plates with D. discoideum spores and E. coli B/r together with 10 ml of sterile double distilled water. The plates were shaken continuously in a Gyrotory shaker (Model G2) at 100 oscillations/min. After several days of growth the vegetative amoebae were harvested and washed free of any bacteria by repeated centrifugation at 1,000 x g in a Beckman J 21-C centrifuge.

F. The study of β -glucosidase enzymes.

1. Preparation of β -glucosidase enzymes.

Spores were activated as described in section D in order to analyze the expression of β -glucosidase activity

during spore germination of D. discoideum. At half hour intervals during the germination process, 10 ml samples were removed, washed free of phosphate buffer by low speed centrifugation at setting 6 in an IEC centrifuge, and resuspended in 5 ml of 0.05 M acetate buffer (pH 5.0). The samples were then passed through a French pressure cell three times at 20,000 PSI and stored immediately at -21 C for 24 hours. After a single freeze and thaw, the spore extracts were centrifuged at 8,200 x g for 15 min at 4 C in a refrigerated Beckman J-21 C centrifuge. The supernatants after centrifugation served as crude β -glucosidase preparations. The above procedure was also used for the collection of β -glucosidase preparations obtained from vegetative myxamoebae.

To obtain β -glucosidase samples from the extracellular matrix, spori were first centrifuged at 1000 x g to separate spores from the crude matrix material. Matrix material that was free of spores was again centrifuged at 8,200 x g for 15 min in a Beckman J 21-C centrifuge at 4 C; the supernatant after this second centrifugation served as a β -glucosidase matrix preparation.

2. Assay of β -glucosidase specific activity during spore germination of D. discoideum.

A modification of the procedure of Tisa and Cotter (1980) was used to assay for the expression of β -glucosidase activity during spore germination. A volume of

0.25 ml containing the substrate p-nitrophenyl- β -D-glucoside (0.01 M in 0.05 M acetate buffer, pH 5.0) was added to 0.25 ml of the enzyme extract and the reaction mixtures were shaken gently at 23.5 C in a GCA/precision scientific waterbath for 50 min. The reaction was terminated by the addition of 1 ml of 1 M sodium carbonate and the absorbance was measured at 420 nm with a Spectro-Plus (MSE) spectrophotometer against a blank containing 0.5 ml of 0.05 M acetate buffer and 1 ml of sodium carbonate.

In order to correct for the apparent substrate degradation that occurs in the absence of the enzyme (due to the long incubation time) and to critically examine the decrease in the specific activity following heat-activation, a substrate control was utilized. The control was prepared by adding 0.25 ml of substrate and incubated as described above. After the incubation period 0.25 ml of enzyme extract together with 1.0 ml of sodium carbonate was added simultaneously to each substrate control tube and the absorbance was measured as described above.

One unit of enzyme activity was defined as 1 nmole of p-nitrophenol released per min, at 23.5 C under the assay conditions as described above. Protein was measured by the method of Bradford (1976) using γ -globulin as a standard; units of specific activity were defined as units of enzyme activity per mg of protein.

3. Enrichment of β -glucosidase.

Spores or amoebae were harvested by centrifugation and resuspended in 0.01 M tris-HCl (pH 7.5), 0.01 M mercaptoethanol, 0.01 M $MgCl_2$, hereafter referred to as buffer A, and were broken in a French pressure cell as described in section F.1. The extract was immediately frozen at -21 C and stored overnight. After thawing at 23.5 C the preparation was centrifuged at 37,000 x g for 30 min. at 4 C. The supernatant was withdrawn and centrifuged at 105,000 x g for 90 min in a Beckman ultracentrifuge using a 60 Ti fixed angle rotor at 4 C. The supernatant was made to 0.2 M KCl by adding one volume of 1.0 M KCl in buffer A to four volumes of supernatant. The extract was then passed down a DEAE-52 column (Whatman) which had previously been equilibrated with 0.2 M KCl in buffer A at 4 C. The enzyme was eluted from the column directly (in the void volume) using the equilibration buffer. Fractions of 1.0 ml each were collected with a flow rate of approximately 12 ml/hr; a total of thirty fractions was collected. The first few fractions with an A_{280} greater than 1.0 were pooled for vacuum dialysis. Vacuum dialysis was performed using a colloidion bag with a molecular weight exclusion limit of 75,000 (Schleider and Schuell Co.); dialysis was performed against two changes of buffer A containing 10% glycerol v/v, and a final change of 50% v/v glycerol. Vacuum dialysis was routinely performed to a final volume of 0.5 ml using a colloidion bag accelerator

(Schleicher and Schuell Co.) and required between 1.5 and 2 hours depending on the sample size. All samples were stored at -21 C until use.

4. Polyacrylamide gel electrophoresis.

Electrophoresis of enriched β -glucosidase samples was performed using 5% continuous non-denaturing gels as described by Coston and Loomis (1969). Gels were prerun in 0.1 M phosphate buffer (pH 7.1) for 30 min at 5-6 mA/gel and were focused for 30 min at 0.5 mA/gel after sample application. The gels were then run at 5-6 mA/gel for 4 hr at 4 C. After electrophoresis, the gels were removed and sliced into 2 mm fractions and the enzyme was eluted at 23.5 C for 30 min in 0.25 ml of 0.15 M acetate buffer (pH 5.0). Para-nitrophenyl- β -D-glucoside (0.01 M in 0.05 M acetate buffer, pH 5.0) was added in an equal volume and the samples were incubated at 23.5 C for 50 min. The reaction was terminated by the addition of 1.0 ml of 1.0 M sodium carbonate and the absorbance was measured at 420 nm in a Spectro-Plus (MSE) spectrophotometer. One unit of enzyme activity was defined as 1 nmole of p-nitrophenol released per min at 23.5 C under the assay conditions as described above.

5. Trypsin treatment of dormant spores.

Dormant spores of D. discoideum NC4H were suspended in 1 mg/ml of trypsin (in 0.01 M phosphate buffer, pH 6.5) for 2 hr to degrade all the β -glucosidase activity that was present. The spores were centrifuged at low speed, washed

once with phosphate buffer and resuspended in 1 mg/ml of trypsin-inhibitor for 1 hr to neutralize all residual trypsin activity. After the one hour incubation, the trypsin-inhibitor was washed from the spores by low speed centrifugation and the spores were then heat shocked at 45 C for 30 min in phosphate buffer. Spore germination was monitored as described in section D.

6. Preparation of cellulase.

Cellulase released from spores was prepared by allowing NC4H spores to germinate in the presence of 200 ug/ml of cycloheximide. Spores under the above conditions will swell and release cellulase into the medium without the formation of β -glucosidase. At the end of the 5 hr germination period, the extracellular medium was collected by passing the spore suspension through a 0.45 um Millipore filter. The cellulase in the medium which passed the filter was concentrated by vacuum dialysis against sterile distilled water using a collodion bag with a molecular weight exclusion limit of 10,000. The concentrated cellulase was lyophilized and stored at -21 C until use.

7. Formation of spheroplasts.

The method of Hemmes et al. (1972) was used to study the effect of β -glucosidase 1 on the splitting of the inner spore coat. Spores of NC4H were heat-shocked and germinated to late swelling in the presence of 200 ug/ml of cycloheximide as previously described by Tisa and Cotter (1980). A mixture containing 1 mg/ml of commercial

cellulase (Worthington Chemicals) in 0.05 M acetate buffer was added to the spores in the presence of 200 ug/ml of cycloheximide. The spore suspension was then allowed to incubate at 23.5 C for 1 hr. At the end of the incubation period, the spores were again centrifuged at low speed and resuspended in 1 or 5 mg/ml of Pronase (Calbiochem) (in 0.01 M phosphate buffer, pH 6.5) containing 200 ug/ml of cycloheximide. The above procedure was repeated again by substituting commercial cellulase with 1 mg/ml of spore cellulase or a mixture of 1000 U/ml of enriched β -glucosidase 1 and 1 mg/ml of spore cellulase in 0.05 M acetate buffer.

The percent spheroplast formation was monitored by placing 0.03 ml of the suspension after pronase treatment on a slide and counting the first 200 objects with a Zeiss phase contrast microscope at a magnification of 320 X. The objects were divided into two groups: swollen spores and spheroplasts.

G. The development of an improved coupled assay for detecting glucose released by acid glucosidases.

1. Preparation of enzymes and enzyme extracts.

Crude trehalase extracts were prepared from spores and emerged myxamoebae of D. discoideum NC4H. Spores were harvested and germinated to myxamoebae as described in section D. Trehalase preparations from both spores and myxamoebae were obtained by French pressing the cells three times in 0.1 M acetate buffer (pH 5.5) at 20,000 PSI. The

cellular extracts were centrifuged at 27,000 x g at 4 C for 30 min in a Beckman J 21-C centrifuge. Supernatants after centrifugation were collected and used as a crude preparation of trehalase.

Enzymes with β -1,6 hydrolase activity were obtained from yeast cells (Fleishmann Co.) since D. discoideum spores and myxamoebae contained very little of these enzymes. Dry living yeast cells were allowed to swell and starve overnight in 0.1 M phosphate buffer (pH 6.5) at room temperature. The cells were washed several times in acetate buffer (pH 5.5) and were then broken by French pressing three times at 20,000 PSI. The cells were centrifuged at 27,000 x g for 30 min; the resultant supernatant served as a crude preparation of β -1,6 hydrolases.

The commercially available pure enzymes, β -glucosidase (Grade B, from almond emulsion; Calbiochem) and invertase (Grade VI, from Baker's yeast; Sigma), were dissolved in 0.1 M acetate buffer (pH 5.5).

2. Preparation of carbohydrate substrates.

Trehalose dihydrate (BDH Chemicals), amygdalin (Sigma), sucrose (Fisher), and cellobiose (BDH Chemicals) were dissolved respectively in 0.1 M acetate buffer to a final concentration of 50 μ mole/ml.

3. Preparation of glucose reagent.

The glucose oxidase (from Aspergillus niger; Miles Biochemicals) chosen for this modified reagent had an

acidic pH optimum which facilitated coupling to acid glucosidases. The glucose reagent was prepared by dissolving 45 U/ml of glucose oxidase, 7.5 U/ml of peroxidase (from horseradish roots; Miles Biochemicals) and 2.3 mM of 2,2'-Azino-di-(3-ethyl-benzthiazolin)-6-sulphonate (ABTS; Boehringer Mannheim) in 0.1 M acetate buffer (pH 5.5). The reagent was stored at 4 C in a brown bottle before use (Chan and Cotter, 1980).

4. Glucose assay (Standard curve).

Solutions containing 10 nmole to 100 nmole of glucose (Fisher) in a volume up to 1.0 ml were pipetted into 12 X 100 mm test tubes. Glucose reagent of 1.0 ml volume was then added to each tube. The contents were mixed well, and allowed to incubate in a 23.5 C GCA/precision scientific waterbath with continuous shaking at 60 oscillations/min for 60 min. Absorbances at 420 nm were measured with a Spectro-Plus (MSE) spectrophotometer against a blank containing 1.0 ml of glucose reagent and 1.0 ml of buffer. The amount of glucose in the standard tubes was plotted against the appropriate absorbance; this resulted in a standard curve which was used to determine the amount of glucose in unknown samples (Chan and Cotter, 1980).

5. Continuous coupled glucosidase assay.

In all experiments, activities of the acid glucosidases were assayed in 0.1 M acetate buffer (pH 5.5). Pure enzymes or enzyme extracts of 0.1 ml to 0.5 ml volume

were added to 0.5 ml of 50 umole/ml of the appropriate substrates. The total volume of each tube was then brought to 1.0 ml by the addition of acetate buffer; 1.0 ml of glucose reagent was introduced into the tubes and the contents were mixed with gentle shaking at 23.5 C. In each coupled assay the following two sets of controls were used: an enzyme control containing the same volume of pure enzymes or enzyme extracts as in the above sample but without the substrate, and a substrate control containing only 0.5 ml of the appropriate substrate. The total volume of both tubes was brought to 1.0 ml with acetate buffer and 1.0 ml of glucose reagent was then added. All three tubes were allowed to incubate in a 23.5 C GCA/precision scientific waterbath with continuous shaking at 60 oscillations/min for 1 hr. At the end of the incubation period, absorbances at 420 nm were measured against a blank containing 1.0 ml of buffer and 1.0 ml of glucose reagent. In the case of high substrate background due to glucose contamination in commercial carbohydrates, the amount of glucose released by acid glucosidases at the end of the 60 min incubation was measured by diluting the contents with 2 ml of acetate buffer. Absorbances of these diluted samples and control tubes were measured against a blank containing 3 ml of acetate buffer and 1 ml of glucose reagent. In all cases, enzyme activity was expressed as nmole of glucose released in the sample tube minus the amount of glucose detected in the enzyme and substrate controls (Chan and

Cotter, 1980).

6. Discontinuous glucosidase assay.

In these experiments, pure enzymes or enzyme extracts were allowed to incubate with their respective substrates for 1 hr at 23.5 C to release glucose. With this method, the enzyme reactions were stopped after the 1 hr incubation period by boiling the contents in a waterbath for 10 min. A 0 time control containing the same amount of enzyme and substrate was prepared by introducing both components simultaneously into a pre-heated test tube. This mixture was also boiled for 10 min in a waterbath. After boiling, the tubes were removed from the waterbath and immediately cooled in ice. Precipitated proteins were removed by centrifuging at setting 7 in an IEC clinical centrifuge for 2 min. A 1.0 ml aliquot of the supernatants containing glucose was removed from each tube and introduced into a clean test tube. To each tube 1.0 ml of glucose reagent was added and the mixture were allowed to incubate at 23.5 C for 45 min. Absorbances at 420 nm were measured using a blank containing 1.0 ml of buffer and 1.0 ml of glucose reagent. Again, in the presence of high substrate background, the mixtures in both the 0 time controls and the sample tubes were diluted with 2 ml of acetate buffer after the 45 min incubation. Absorbances at 420 nm were measured against a blank containing 3.0 ml of buffer and 1.0 ml of glucose reagent (Chan and Cotter, 1980).

H. The study of trehalase enzyme activity.

1. Preparation of endogenous and exogenous trehalase samples.

Spores of D. discoideum were activated and germinated as described in section D. At half hour intervals, 10 ml samples were collected and centrifuged at setting 6 in an IEC clinical centrifuge to remove spores and/or myxamoebae. The supernatants after centrifugation were collected and centrifuged again at 27,000 x g in a Beckman J 21-C at 4 C for 30 min. The supernatants after this centrifugation were collected and stored at -21 C for 24 hours. These samples were used as exogenous trehalase preparations.

Spores or myxamoebae that were pelleted after the low speed centrifugation were resuspended in 5 ml of 0.1 M acetate buffer (pH 5.5). The suspensions were French pressed three times at 20,000 PSI and stored at -21 C for 24 hr. After a single freeze and thaw, the spore or myxamoeba extracts were centrifuged at 27,000 x g for 30 min in a Beckman J 21-C centrifuge at 4 C. These supernatants, in turn, served as endogenous trehalase preparations.

To obtain trehalase samples from the extracellular matrix, sori were first centrifuged at 1000 x g to separate spores from the crude matrix material. The matrix material that was free of spores was again centrifuged at 27,000 x g for 30 min in a Beckman J 21-C centrifuge at 4 C; the supernatant after this second centrifugation served as a trehalase matrix preparation.

2. Assay of trehalase activity.

The continuous coupled glucosidase assay as described in Section G.4 was used to detect the activities of both endogenous and exogenous trehalase. Before performing the assay, the pH of the exogenous preparation was lowered to 5.5 by the addition of 1-2 ul of concentrated glacial acetic acid to a 2 ml aliquot of the exogenous trehalase sample. A total volume of 0.5 ml of either endogenous or exogenous trehalase sample was added to 0.5 ml of 50 umole/ml of trehalose. An enzyme control containing 0.5 ml of the trehalase sample and 0.5 ml of acetate buffer (pH 5.5); and a substrate control containing 0.5 ml of trehalose and 0.5 ml of acetate buffer were also used. A volume of 1 ml of glucose reagent was added respectively to the above tubes and the mixture was incubated at 23.5 C for 1 hr with continuous shaking in a GCA/precision scientific waterbath at 60 oscillations/min. Absorbances were taken at 420 nm against a blank containing 1.0 ml of acetate buffer and 1.0 ml of glucose reagent.

3. Partial purification of trehalase samples.

Trehalase was purified from matrix material since its enzymatic activity is much higher in the matrix than in the dormant spores or myxamoebae. To purify the enzyme, the matrix preparation was centrifuged at 27,000 x g for 30 min. to remove all dormant spores. The supernatant was first brought to 40% (v/v) alcohol by addition of 100% (v/v) ethanol at 0 C. After two hours of precipitation, the

sample was centrifuged again at 27,000 x g for 30 min. The precipitate was discarded and the 40% (v/v) alcohol supernatant was then brought to 60% (v/v) alcohol. The sample was again allowed to sit in the ice for another two hours. At the end of the precipitation period, the sample was centrifuged at 27,000 xg for 30 min and the precipitate was dried under vacuum at 4 C. After the ethanol had completely evaporated, the precipitate was dissolved in an minimum volume of 0.1 M acetate buffer (pH 5.5) (Ceccarini, 1966; Killick, 1979, 1980).

4. Preparation of trehalose samples.

The carbohydrate, trehalose, was prepared by means of ethanol extraction (Cotter and Niederpruem; 1971). The spores were allowed to germinate as previously described. At half-hour intervals, 10 ml samples were removed and centrifuged at low speed to pellet the spores or myxamoebae. After one wash with 0.01 M phosphate buffer the samples were extracted with two portions of ethanol (80% v/v) at 80 C for 30 min. The ethanol extracts were then air dried at 45 C. The dry extracts were redissolved in small quantities of 0.1 M acetate buffer (pH 5.5).

5. Measurement of trehalose utilization during germination of UV or cycloheximide treated spores.

The continuous coupled glucosidase assay as described in Section G.4 was modified to detect the amount of

trehalose present during germination of UV or cycloheximide treated spores. Instead of using a fixed concentration of trehalose, a constant amount of trehalase was used in the assay to convert the extracted trehalose into glucose. A volume of 0.5 ml of the trehalose extract was added to 0.5 ml of 95 U/ml of partial purified trehalase enzyme. To the above mixture 1.0 ml of glucose reagent was added and the tube was incubated at 23.5 C for 1 hr. An enzyme control containing 0.5 ml of 95 U/ml of partial purified trehalase enzyme and 0.5 ml of acetate buffer, and a substrate control containing 0.5 ml of the trehalose extract and 0.5 ml of acetate buffer were also used. A volume of 1 ml of glucose reagent was added respectively to the above tubes before incubation. Absorbances were taken at 420 nm against a blank containing 1.0 ml of acetate buffer and 1.0 ml of glucose reagent.

1. Measurement of glucose released during spore germination of *D. discoideum*.

Glucose released into the medium during spore germination was measured during the coupled assay as previously described. Spores of *D. discoideum* were allowed to germinate either in the presence or absence of cycloheximide. At half hour intervals during spore germination, 5 ml samples were collected and centrifuged at 27,000 x g in a Beckman J 21-C centrifuge to remove all spores or myxamoebae. The supernatants that were free of

spores or myxamoebae were used to determine the amount of glucose in the external medium.

RESULTS

A. Expression of β -glucosidase specific activity in matrix material and in dormant spores of *D. discoideum* strain NC4H.

For a constant number of sorocarps, a higher specific activity was observed in extracellular matrix material than in dormant spores (Table 1). This high specific activity was contributed to by both high enzymatic activity and low protein concentration in the matrix material.

It had been shown that when spores of NC4H were allowed to age in the intact sorocarp for more than 7 days, autoactivation would occur upon suspension of washed spores in phosphate buffer (Dahlberg and Cotter, 1978). When β -glucosidase activity was analyzed in 1, 7, and 13 day old sorocarps, the enzymatic activity was found to decrease in both matrix material and dormant spores undergoing aging (Figure 3). This decrease was probably due to degradation or denaturation of the enzyme and therefore β -glucosidase may not play an essential role during autoactivation. This observation was in contrast to that of the other hydrolases such as, β -galactosidase and N-acetyl- β -D-glucosaminidase, which were released from the dormant spores to the extracellular matrix upon aging of the sorocarps (Chan et al., 1981).

B. The enzyme pattern of β -glucosidase in matrix material.

TABLE 1. The specific activity of β -glucosidase in 2 day old matrix material and in dormant spores.

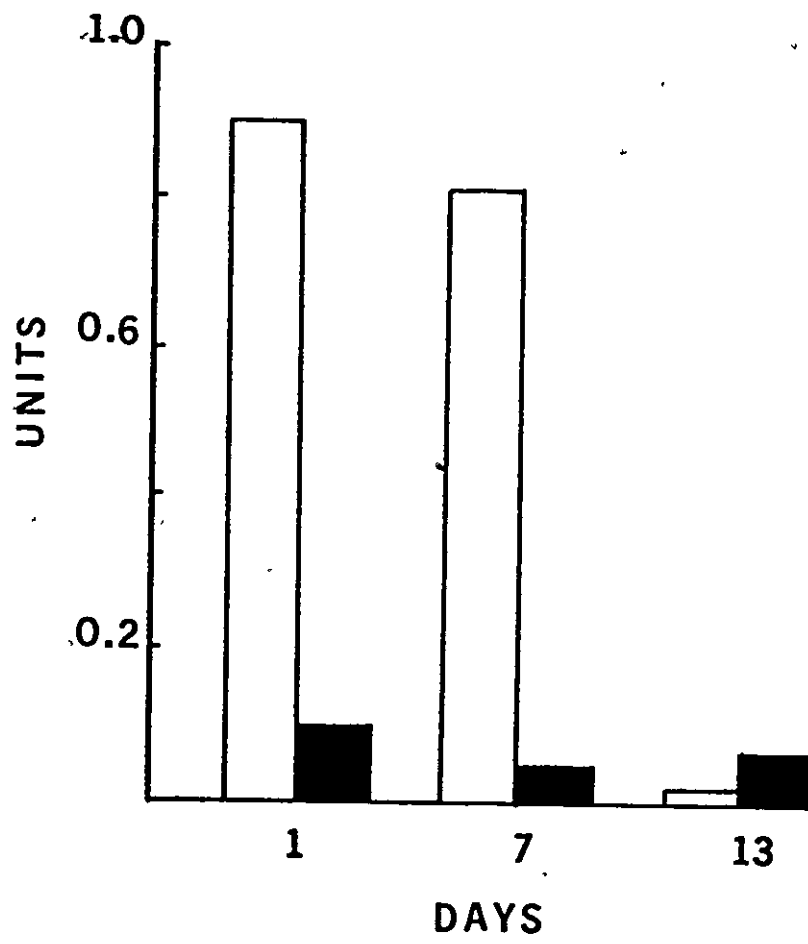
Preparation ^a	Enzyme activity ^b (Units/ml)	Protein (mg/ml)	Specific activity ^c	Fold
Dormant spores	2.08	0.40	5.2	1
Matrix material	48.40	0.07	691.4	133

^aSpore were placed in 5 ml of acetate buffer (0.05 M, pH 5.0) and suspended in a matrix fraction and a spore fraction by centrifugation. After two washes in acetate buffer, the spores were resuspended in 5 ml of fresh acetate buffer. The matrix material and spore suspension were processed and assayed as described in the Materials and Methods.

^bUnits of enzyme activity are equivalent to nmoles of p-nitrophenol released per min at 23.5 C.

^cUnits of specific activity are equivalent to units of enzyme activity per mg of protein.

FIGURE 3. The activity of β -glucosidase in the matrix material and in the dormant spores. -Units of enzyme activity versus days of aging in the intact sorocarp. Units of enzyme activity are equivalent to nmoles of p-nitrophenol released per min at 23.5 C per 10^7 spores. \square , enzyme activity in matrix material; \blacksquare , enzyme activity in dormant spores.



When the matrix material of the 2 day old sorl was electrophoresed, β -glucosidase 2 was found to be present with only trace amounts of β -glucosidase 1 (Figure 4). Protein from vegetative amoebae which contained only β -glucosidase 1 activity was also electrophoresed to indicate the exact position of this form of enzyme on the gels (Coston and Lodms, 1969). Protein from vegetative amoebae served routinely as a marker for the β -glucosidase I position throughout the study.

C. Expression of crude β -glucosidase activity during heat-induced spore germination of *D. discoideum* strain NC4H.

The change in specific activity of β -glucosidase during spore germination of *D. discoideum* NC4H is shown in Figure 5. This enzyme decreased in specific activity during heat activation. (Tisa and Cotter, 1980). However, upon emergence of myxamoebae, the enzyme underwent at least a tenfold increase in specific activity when compared to post-activated spores at zero times. With the modified technique used in this work, a larger decrease in specific activity was observed after heat activation than previously shown (Tisa and Cotter, 1980; Figure 5). The specific activity was about 5 to 7.5 in dormant spores and decreased to an almost undetectable amount in the post activated spores.

The latter increase in specific activity of β -glucosidase coincided with the rise in the number of

FIGURE 4. Polyacrylamide gel electrophoresis patterns of β -glucosidase in matrix material and in vegetative amoebae. Each gel received 0.4 ug of protein and the gels were electrophoresed as described in the Materials and Methods section. ●, β -glucosidase pattern in matrix material; Δ , β -glucosidase pattern in vegetative amoebae.

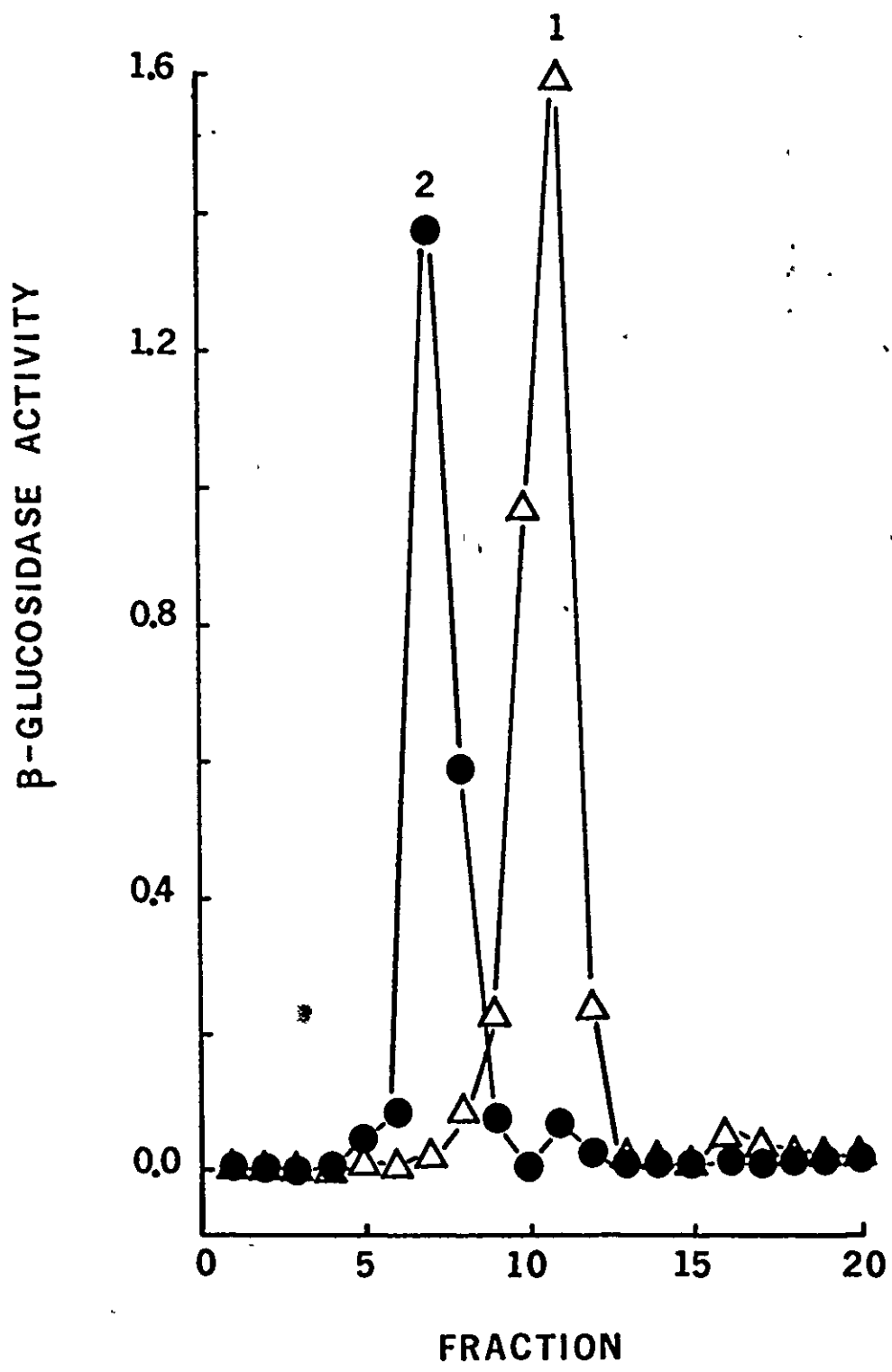
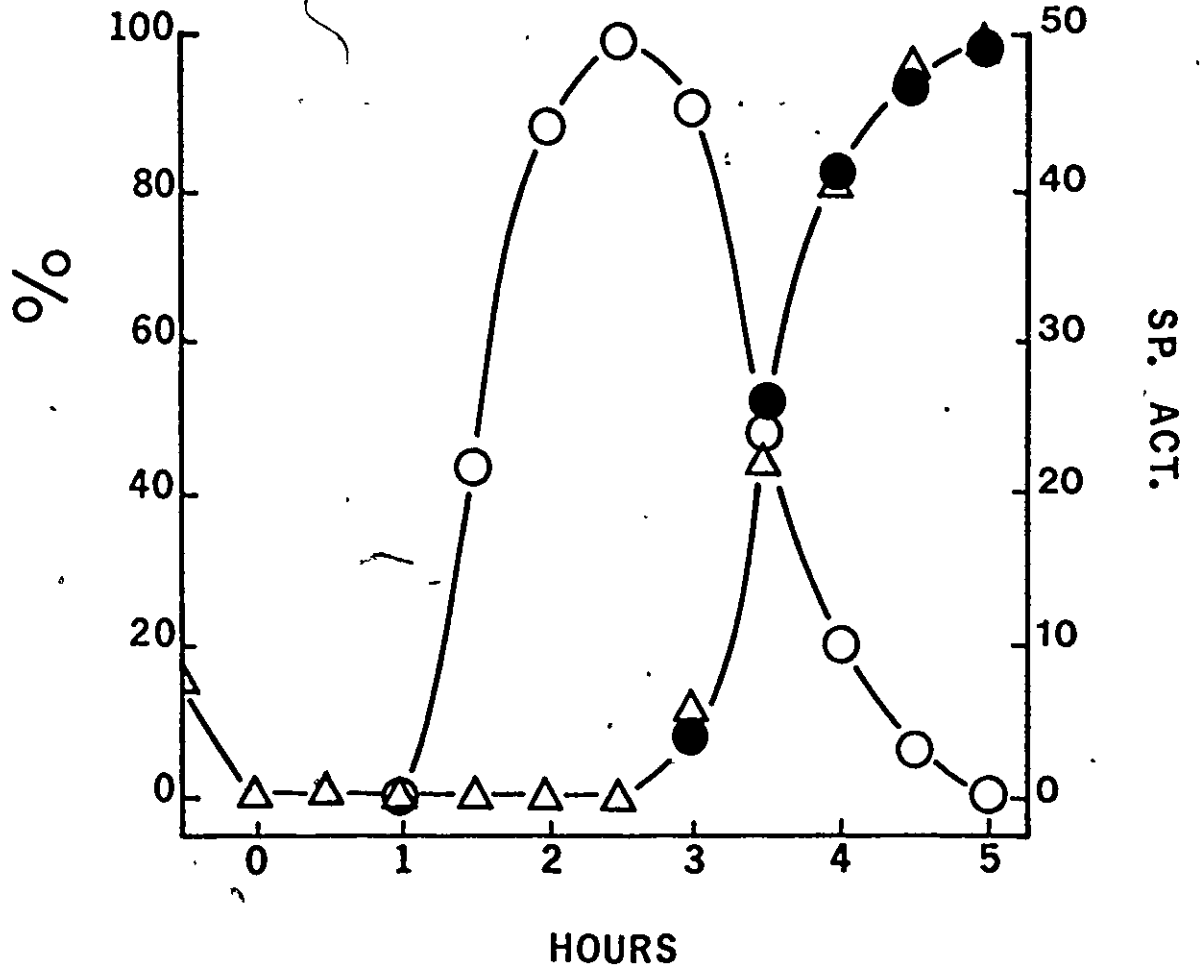


FIGURE 5. The specific activity of β -glucosidase during spore germination of D. discoideum strain NC4H. Spores were heated at 45 C for 30 min and incubated at 23.5 C for 5 hr. \circ , percent of swollen spores; \bullet , percent of emerged myxamoebae; Δ , β -glucosidase specific activity. The semidiagrammatic depiction of spore germination below the abscissa includes late lag, early swelling, mid swelling, late swelling, emerged myxamoebae, and post-emerged myxamoebae.



myxamoebae during emergence (Figure 5).

D. Enzyme patterns of β -glucosidase during heat-induced spore germination of *D. discoideum* strain NC4H.

Different forms of β -glucosidase were found in the spores than in myxamoebae, as indicated by polyacrylamide gel electrophoresis (Figure 6). The predominant form of β -glucosidase in the dormant spore was β -glucosidase 2. Upon heat activation, this form of enzyme decreased to less than 25% of that found in the dormant spores. During emergence of myxamoebae, a new form of β -glucosidase was synthesized. The expression of this newly-formed enzyme, β -glucosidase 1, required both RNA and protein syntheses since UV irradiation as well as cycloheximide (Tisa and Cotter, 1980; Demsar, personal communication) would block the increase in specific activity of this enzyme.

E. Expression of crude β -glucosidase activity during spore germination of *D. discoideum* strain SG1.

The spontaneous germination mutant, SG1, was used to study the expression of β -glucosidase during chemical activation and autoactivation of *D. discoideum* spores. Strain SG1 was selected over strain NC4H because a higher percentage of myxamoebae emergence could be achieved upon chemical activation of the former spores. As shown in Figures 7-10, the β -glucosidase activity of *D. discoideum* strain SG1 exhibited both temporal and quantitative shifts in activity, which were dependent on the methods used for spore activation. It was important to note that although

FIGURE 6. Polyacrylamide gel electrophoresis patterns of different forms of β -glucosidase during spore germination. The gels containing 400 ug of protein were electrophoresed as described in the Materials and Methods. ○ , dormant spores; □ , heat-activated spores at zero times; ▲ , fully emerged myxamoebae.

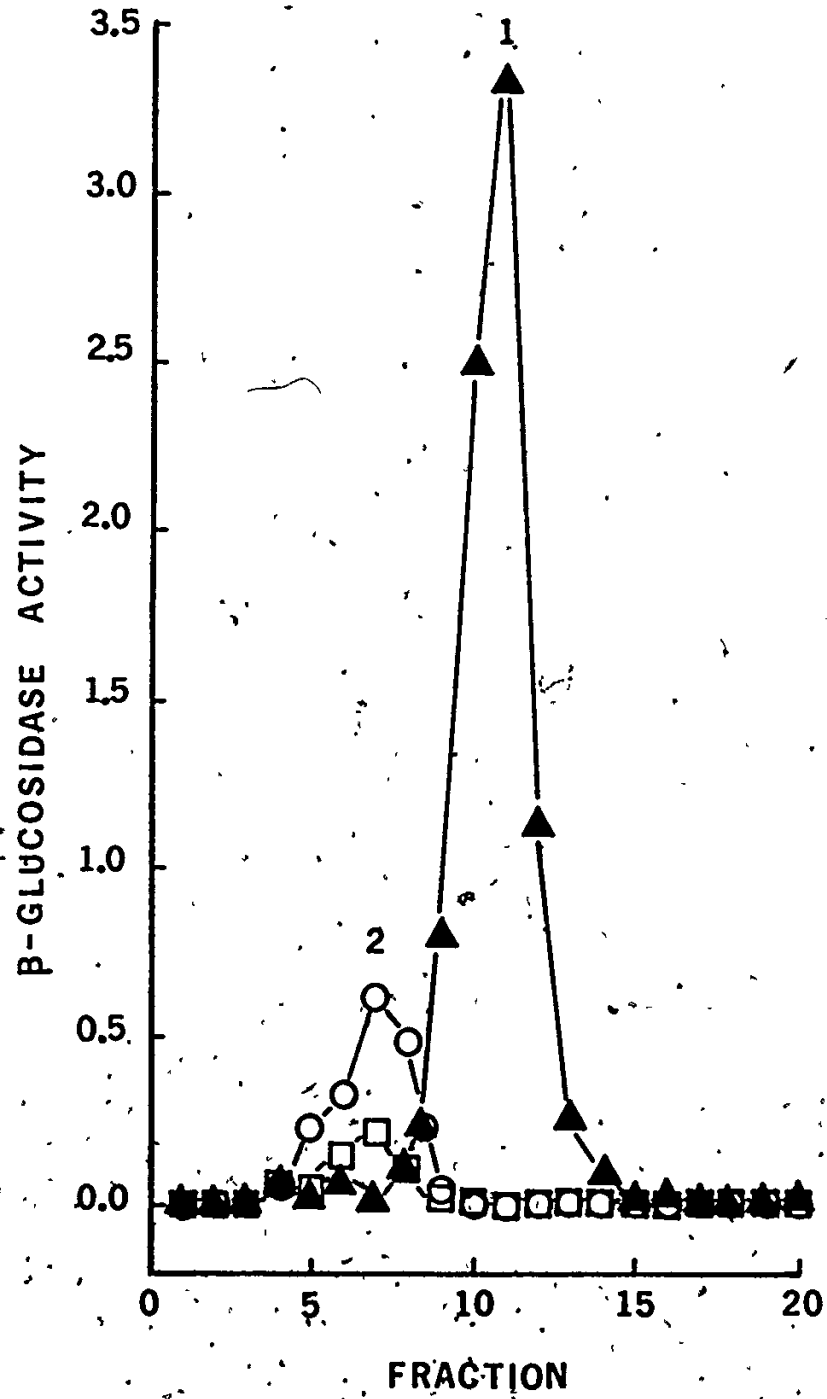


FIGURE 7. The specific activity of β -glucosidase during heat-induced germination of D. discoideum strain SG1 spores. Time zero denotes the time directly after heat activation (45 C for 30 min).

○, percent of swollen spores; ●, percent of emerged myxamoebae; Δ , β -glucosidase specific activity.

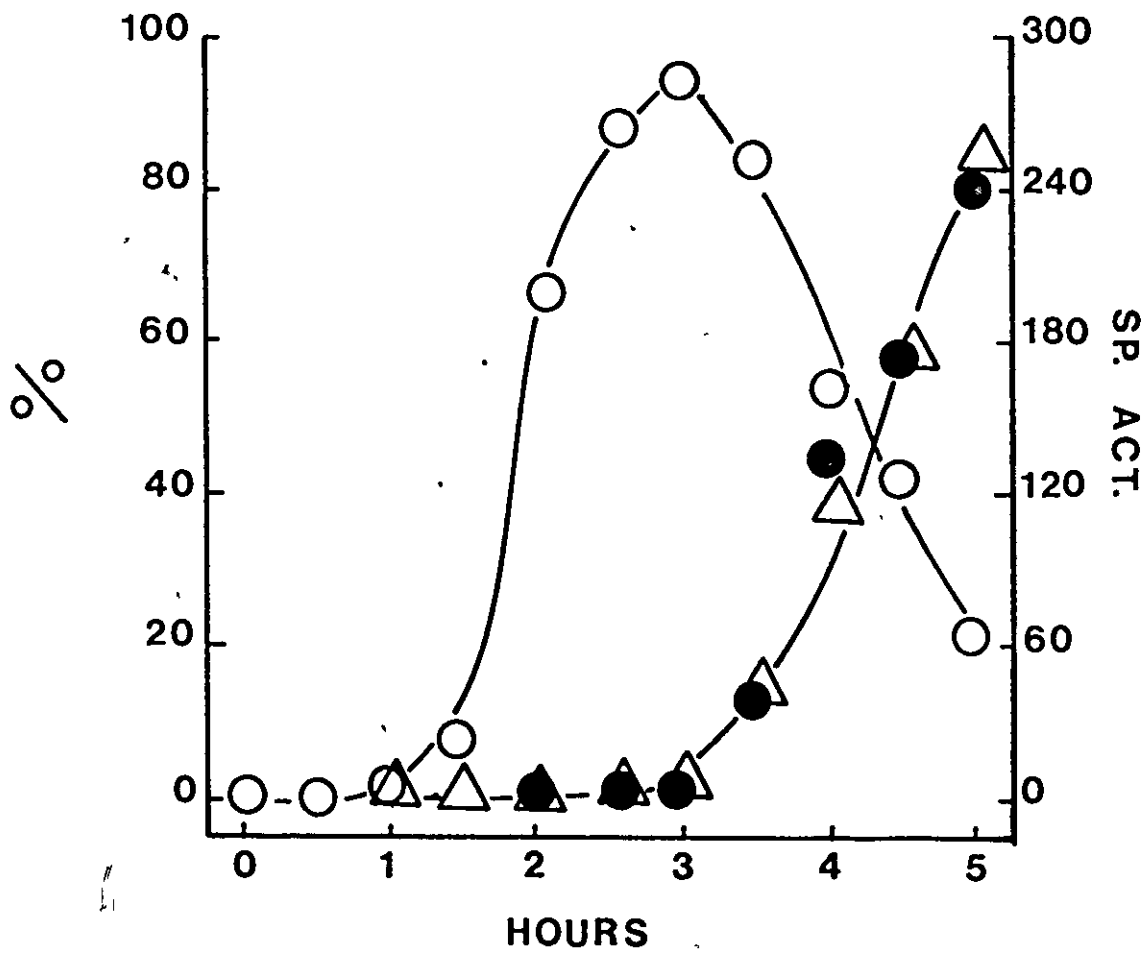


FIGURE 8. Specific activity of β -glucosidase during germination of autoactivated D. discoideum strain SG1 spores. Spores at a concentration of 10^7 /ml were incubated in 0.01 M phosphate buffer at 23.5 C. Time zero denotes the time when spores were diluted at the required concentration in phosphate buffer. ○, percent of swollen spores; ●, percent of emerged myxamoebae; Δ , specific activity of β -glucosidase.

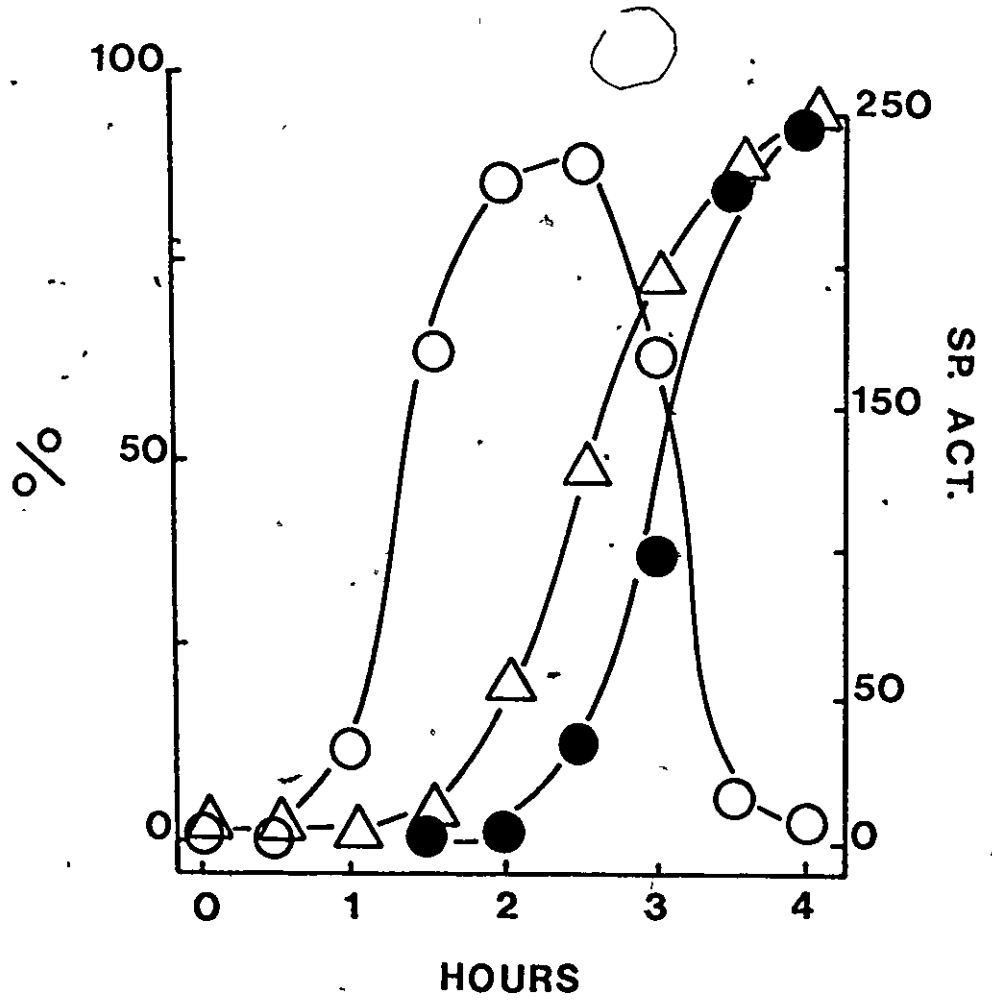


FIGURE 9. Specific activity of β -glucosidase during germination of DMSO activated D. discoideum strain SG1 spores. Spores were activated by incubation in 20% DMSO for 30 min. Time zero denotes the time when spores were released from the chemical.

○, percent of swollen spores; ●, percent of emerged myxamoebae; Δ , specific activity of β -glucosidase.

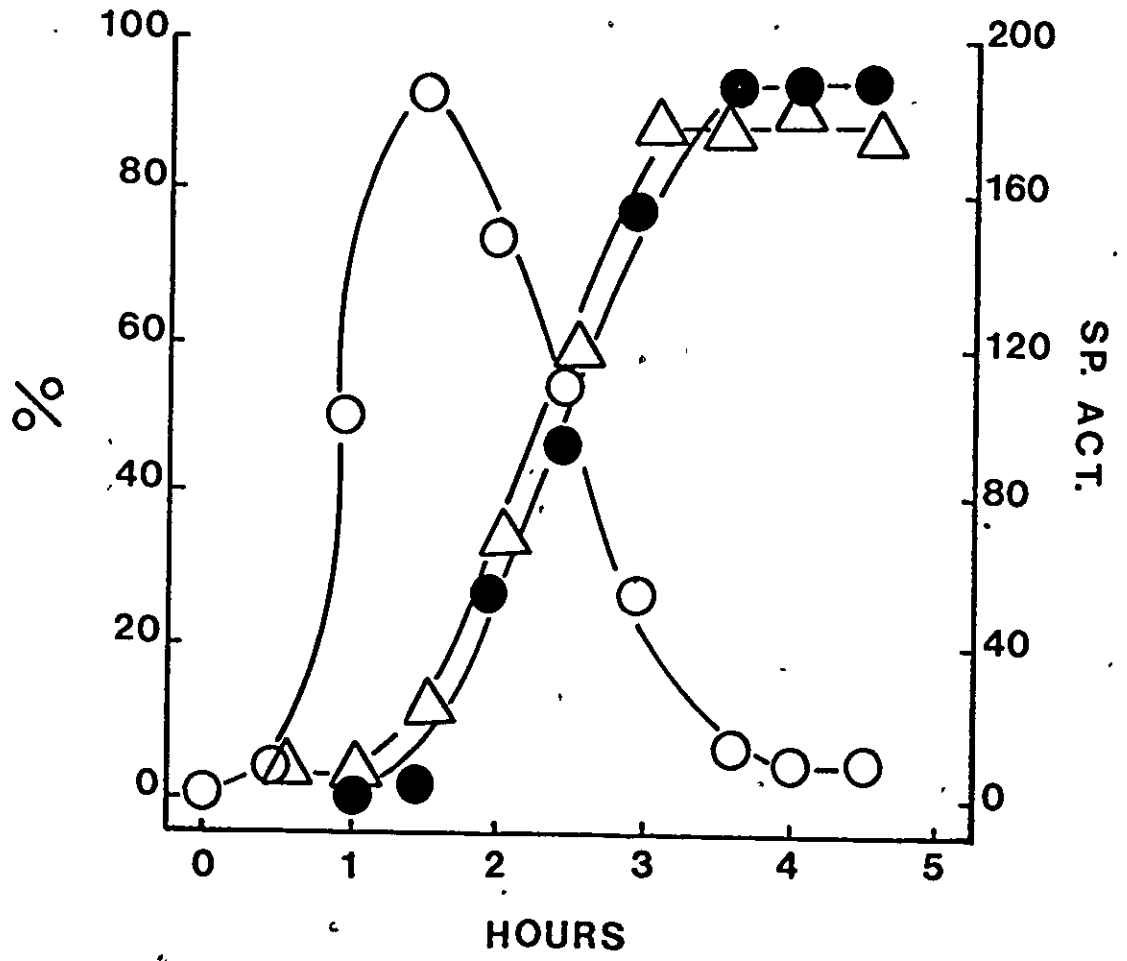
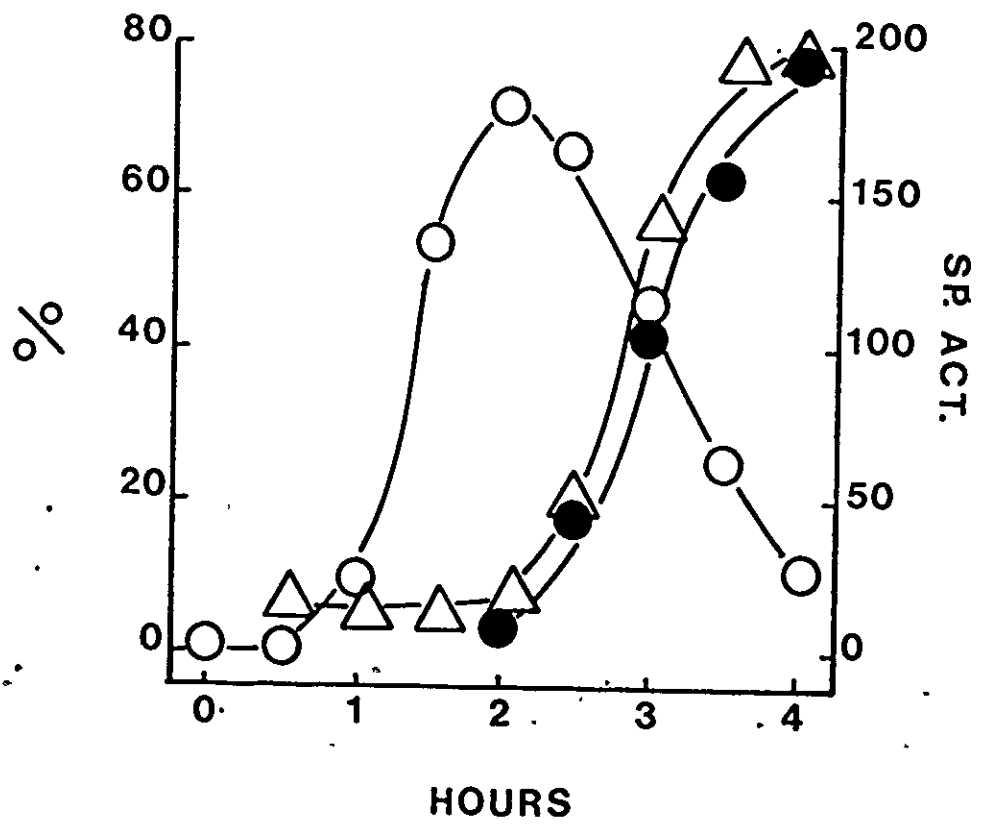


FIGURE 10. Specific activity of β -glucosidase during germination of urea-activated D. discoideum strain SG1 spores. Spores were activated by incubation in 8 M urea for 30 min. \circ , percent of swollen spores; \bullet , percent of emerged myxamoebae; \triangle , specific activity of β -glucosidase.



there was a difference in specific activity in NC4H and SG1 spores after heat shock, both strains showed an increase in β -glucosidase specific activity concurrently with the emergence of myxamoebae (Figure 8).

Among the different types of activation, auto-activation caused the most drastic temporal shift in β -glucosidase formation. The enzyme was formed almost 30 min before the emergence of myxamoebae. In the case of chemical activation, both DMSO and urea caused an early synthesis of β -glucosidase by about 15 min when compared to heat shock treatment.

The quantitative shifts in β -glucosidase formation varied from 180 specific activity with DMSO treatment to 250 with autoactivation. In all cases, except that of heat activation, the spores showed a basal level of β -glucosidase specific activity in the pre-emergent stages of germination.

The β -glucosidase enzyme seemed to be retained in the myxamoebae once it was synthesized. As shown in Figure 9, both the number of myxamoebae and β -glucosidase specific activity remained constant for at least 2 hr. This was not surprising since β -glucosidase was not normally released into the medium during germination of D. discoideum spores (Jones et al., 1979; Tisa and Cotter, 1980). Also, if myxamoebae were UV irradiated to prevent RNA synthesis at any given time, the level of β -glucosidase that was already present in the pre-irradiated myxamoebae remained

constant over a long incubation period (Demsar, personal communication). This indicated that β -glucosidase 1 which was synthesized during emergence, was well protected inside the myxamoebae

F. The role of β -glucosidase during spore germination of *D. discoideum* strain NC4H.

1. Trypsin treatment of dormant spores.

In order to test the requirement for β -glucosidase 2 during germination of *D. discoideum*, the enzyme was inactivated by exposing dormant spores to trypsin treatment. As shown in Table II, no β -glucosidase 2 activity could be detected in dormant spores which had been treated with trypsin for 2 hours. When these spores were then heat activated in the absence of trypsin, normal germination occurred with more than 95% emergence at the end of 5 hr (Data same as in Figure 5). When the β -glucosidase activity was measured at this time, the enzymatic activity of the newly-formed β -glucosidase 1 was found to be similar to that of non-trypsin treated myxamoebae. However, the total protein in the trypsin treated preparation was about 6 fold lower (Table II).

2. Expression of β -glucosidase activity during heat-induced spore germination of *D. discoideum* strains G1 and AX3.

When the β -glucosidase 1 minus mutant, G1, was heat shocked at 45 C for 30 min, emergence began at 5 hr and reached a maximum of 80% at the end of 10 hr (Figure 11).

TABLE 11. Effect of trypsin on the expression of β -glucosidase in dormant spores and in fully emerged myxamoebae.

Preparation ^a	Enzyme activity ^b (Units/ml)	Protein (mg/ml)	Specific Activity ^c
Dormant spores	0.00	0.020	0.00
Emerged amoebae	0.49	0.006	327

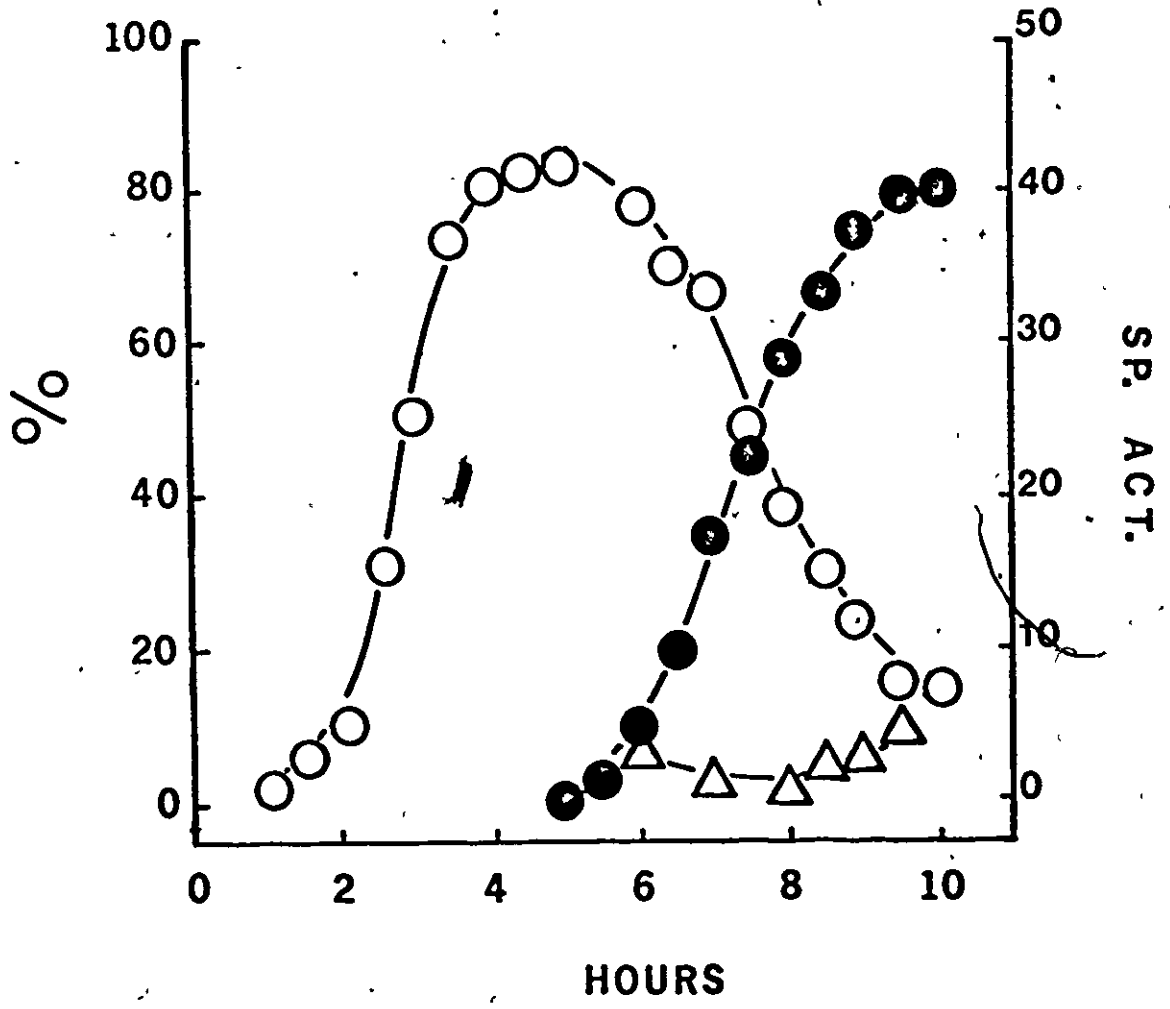
^aDormant spores were suspended in 1 mg/ml of trypsin (in 0.01 M phosphate buffer, pH 6.5) for 2 hr to degrade all the β -glucosidase 2 activity that was present. The spores were centrifuged at low speed, washed once with phosphate buffer and resuspended in 1 mg/ml of trypsin-inhibitor for 1 hr to neutralize all residual trypsin activity. After the 1 hr incubation period, the inhibitor was removed by low speed centrifugation, and spores were heat-shocked at 45 C for 30 min. Fully emerged amoebae were collected at the end of 5 hr as described in the Materials and Methods.

^bUnits of enzyme activity are equivalent to nmoles of p-nitrophenol released, per min at 23.5 C.

^cUnits of specific activity are equivalent to units of enzyme activity per mg of protein.

FIGURE 11. Specific activity of β -glucosidase during germination of D. discoidium strain G1 spores. Spores of G1 were heat-activated at 45 C for 30 min and incubated at 23.5 C for 10 hr.

○, percent of swollen spores; ●, percent of emerged myxamoebae; Δ , apparent β -glucosidase specific activity.



The germination pattern in this strain was very similar to that of strain AX3 which was the parental strain of the mutant (Figure 12). However, only very low β -glucosidase activity was found during germination of this mutant when compared to that of AX3 (Figures 11, 12), and most of the activity in G1 might be accounted for by the activity of a β -galactosidase enzyme which can hydrolyze the β -glucoside substrate (Diamond and Loomis, 1976; Loomis, 1980).

3. Formation of spheroplasts.

Spheroplasts could be formed in cycloheximide treated spores by incubating swollen spores with a mixture of cellulase and Pronase (Hemmes *et al.*, 1972). The production of spheroplasts is believed to stimulate relief from cycloheximide inhibition by partially replacing missing enzyme functions (Hemmes *et al.*, 1972). A set of enzymes required for emergence are inner wall hydrolyzing enzymes (Cotter *et al.*, 1969; Hemmes *et al.*, 1972). Using a different combination of cellulase and pronase, maximum spheroplast formation was found to occur when swollen spores were incubated with 1 mg/ml of cellulase followed by 5 mg/ml of pronase at 23.5 C (Figure 13). However, commercial cellulase usually contained a high concentration of β -glucosidase (Data not shown). In order to demonstrate the requirement for β -glucosidase activity in the formation of spheroplasts, cellulase that was free of any β -glucosidase was used. The cellulase was obtained by

FIGURE 12. Specific activity of β -glucosidase during germination of D. discoideum strain AX3 spores. Spores were heat-shocked at 45 C for 30 min and incubated at 23.5 C for 10 hr. \bigcirc , percent swollen spores; \bullet , percent emerged myxamoebae; Δ , β -glucosidase specific activity.

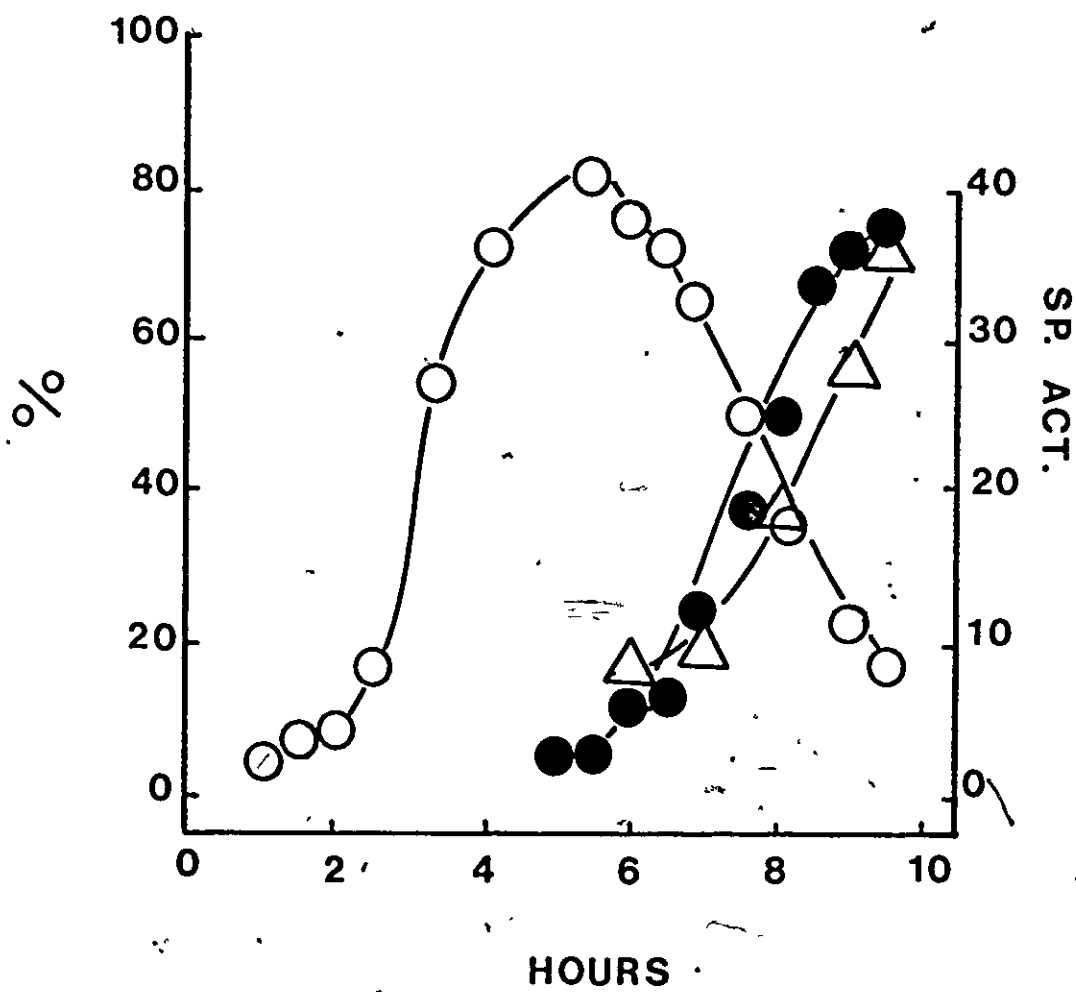
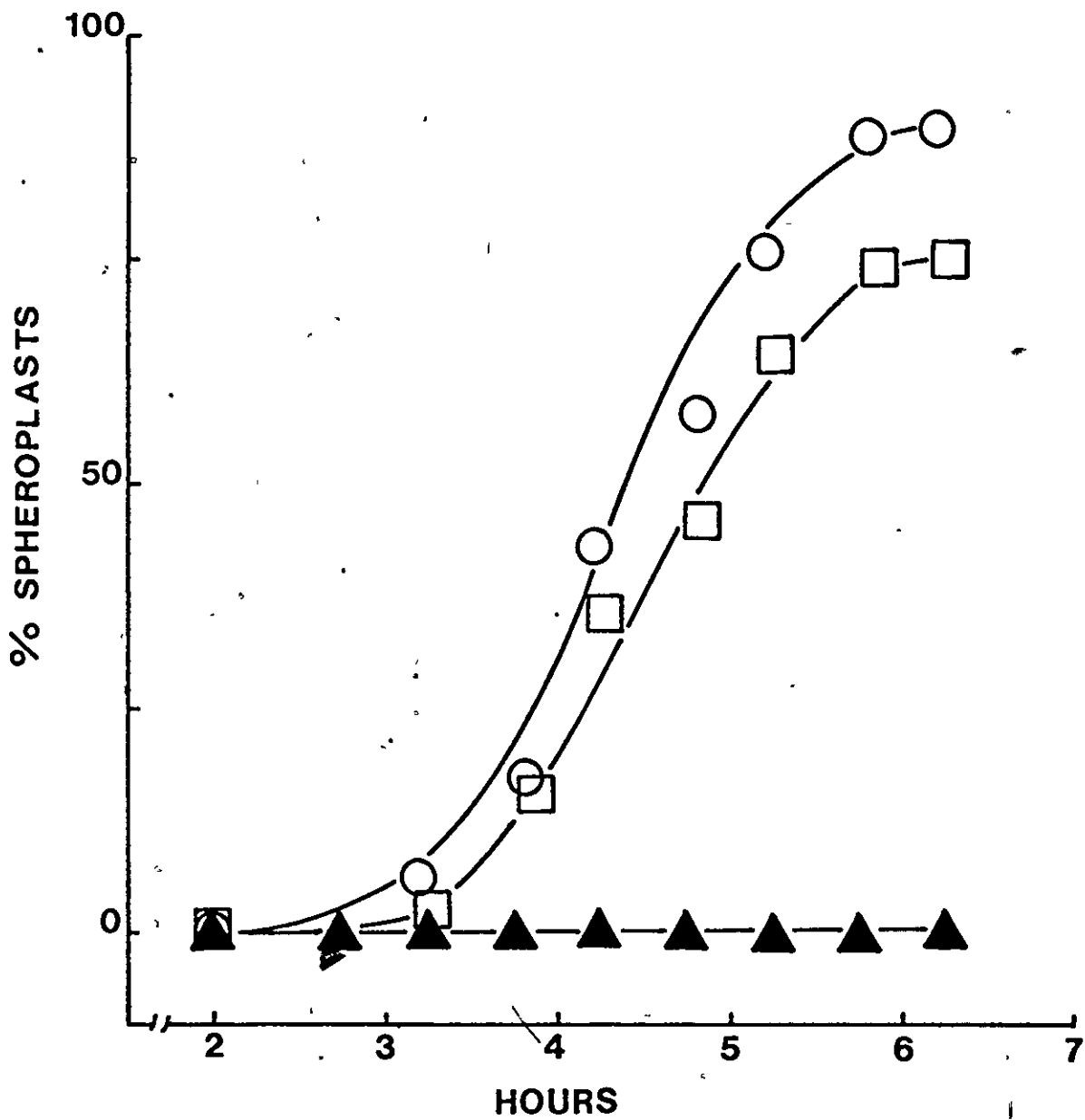


FIGURE 13. Spheroplast formation using commercial cellulase and pronase. Time zero denotes the time immediately after the addition of pronase to heat-activated D. discoideum strain NC4H spores (see Materials and Methods for detail). ▲, 200 ug/ml of cycloheximide without enzyme addition; □, 1 mg/ml of cellulase and 1 mg/ml of pronase in the presence of 200 ug/ml of cycloheximide; ○, 1 mg/ml of cellulase and 5 mg/ml of pronase in the presence of 200 ug/ml of cycloheximide.



-70-

germinating spores in the presence of cycloheximide. Spores germinating under the above condition would swell and release cellulase into the extracellular medium without the formation of β -glucosidase (Jones *et al.*, 1979). The release of spheroplasts in the absence of β -glucosidase was thus evidence that spheroplast formation did not require β -glucosidase activity (Figure 14). However, when β -glucosidase 1 was added to the cellulase-pronase treated spores, then spheroplasts were formed at a slightly higher rate and reached a 70% maximum 2 hours after addition of pronase (Figure 14).

G. The development of an improved coupled assay for detecting glucose released by acid glucosidases.

1. Sensitivity, stability and linearity of the discontinuous assay.

A linear relationship was obtained between the absorbance and the amount of glucose in the mixture up to 100 nmole per tube (Figure 15). This linear relationship remained for up to 200 nmole of glucose per tube when the contents were diluted with 2 ml of buffer (Figure 16). With proper dilution, the assay can detect a glucose concentration as high as 400 nmole per tube (Data not shown). The rate of oxidized chromogen formation in a 10 nmole solution of glucose is presented in Figure 17. The absorbance was monitored at 1 min intervals for the first 5 min and then at 5 min intervals for up to a period of 1 hr. As seen from the graph, the colour development was essentially complete at 10 min and remained relatively

FIGURE 14. Spheroplast formation using spore cellulase and commercial pronase with addition of β -glucosidase 1. The conditions of enzymatic treatment of D. discoideum strain NC4H spores are as described in the Materials and Methods section. Δ , 200 ug/ml of cycloheximide without enzyme addition; \square , 1 mg/ml of cellulase and 5 mg/ml of pronase in the presence of 200 ug/ml of cycloheximide; \circ , 1000 U/ml of β -glucosidase 1, 1 mg/ml of cellulase and 5 mg/ml of pronase in the presence of 200 ug/ml of cycloheximide.

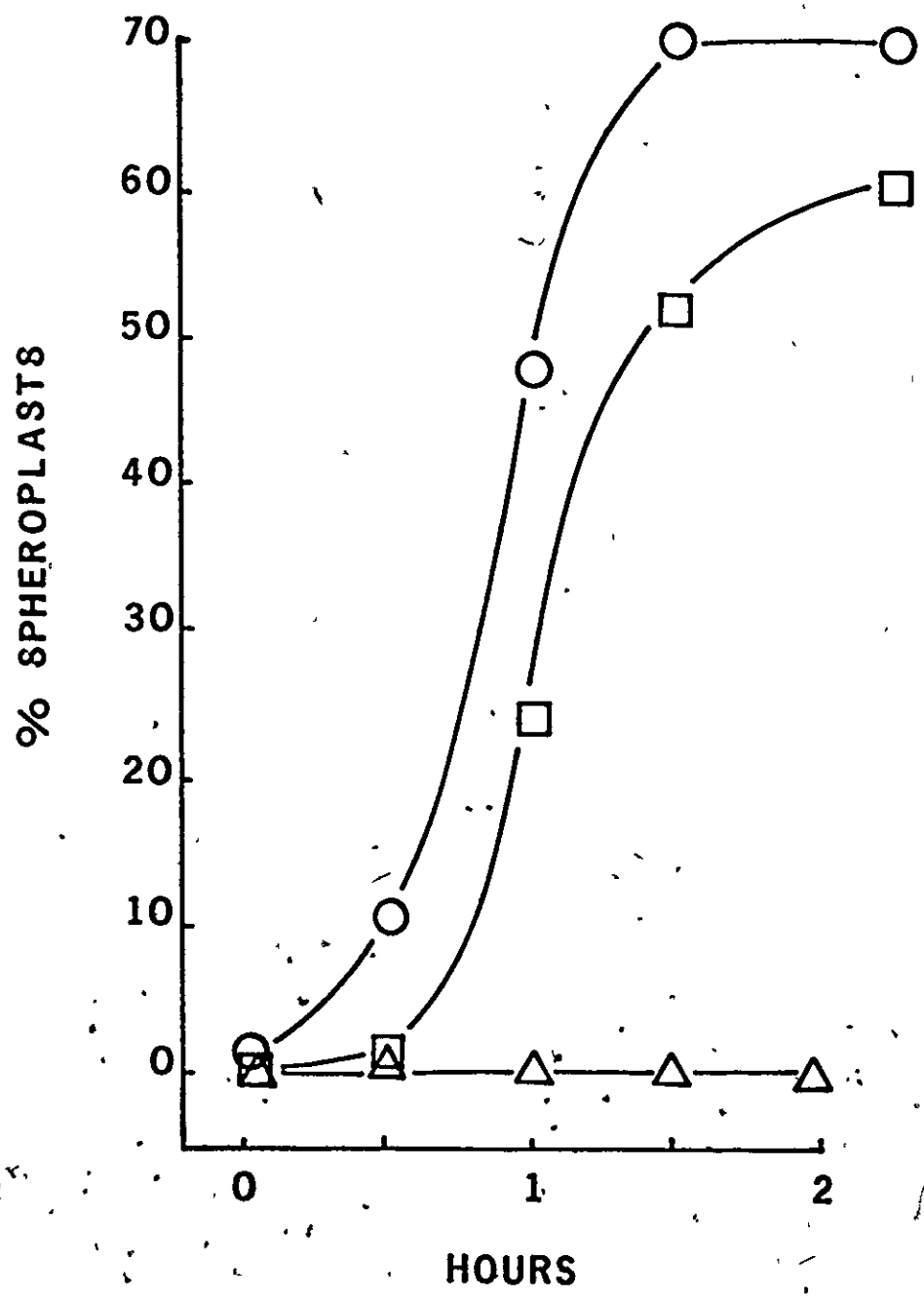


FIGURE 15. Formation of oxidized chromogen upon addition of various amounts of glucose. A 1.0 ml volume of glucose reagent was added into tubes containing 1.0 ml of between 10 nmole to 100 nmole of glucose. The mixtures were incubated at 23.5 C with shaking for 45 min. Absorbances at 420 nm were taken as described in the Materials and Methods section. ♦

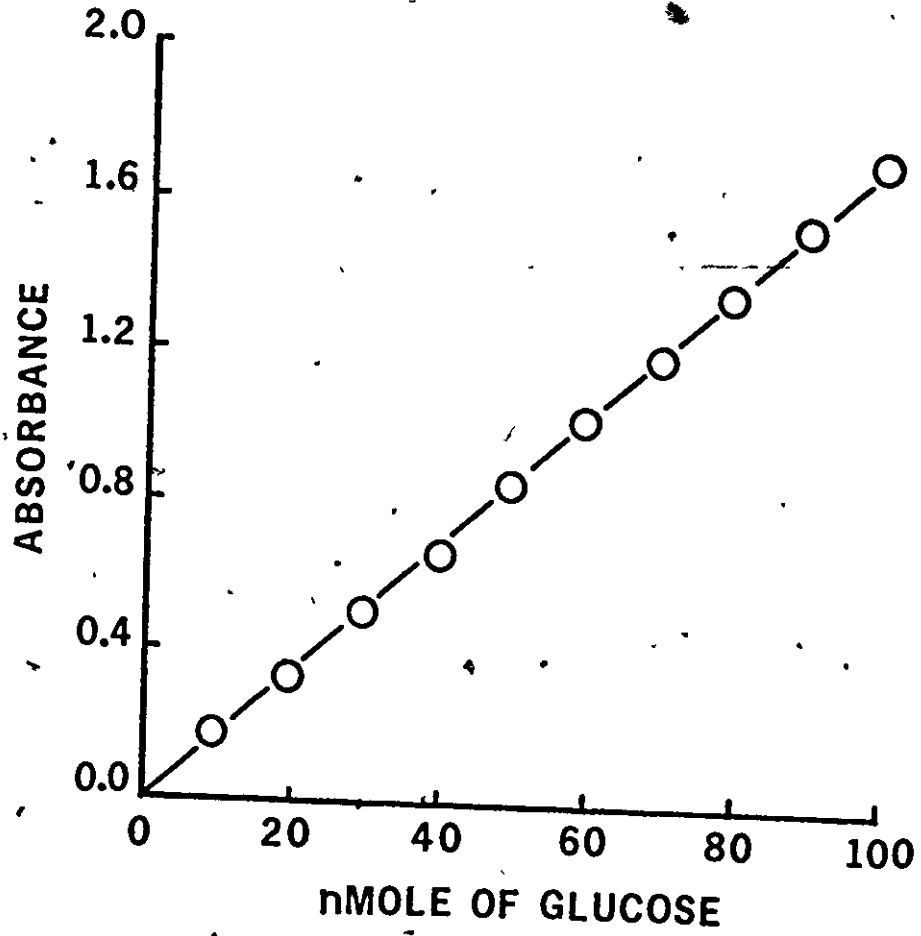


FIGURE 16. Oxidized chromogen formation upon dilution as a function of the amount of glucose added. A 1.0 ml volume of glucose was added into tubes containing 1.0 ml of between 20 nmole to 200 nmole of glucose. The mixture was allowed to incubate at 23.5 C for 60 min. At the end of the incubation period, the mixture in each tube was diluted with 2 ml of acetate buffer. Absorbances at 420 nm were taken as described in the Materials and Methods section.

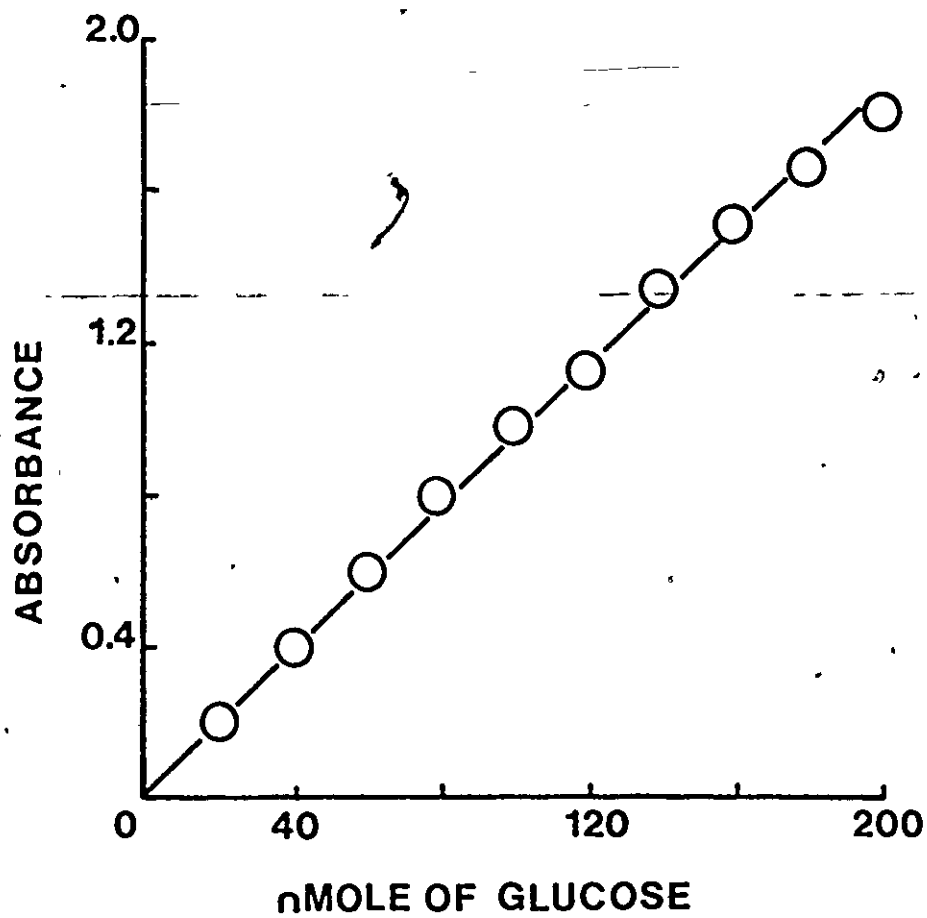
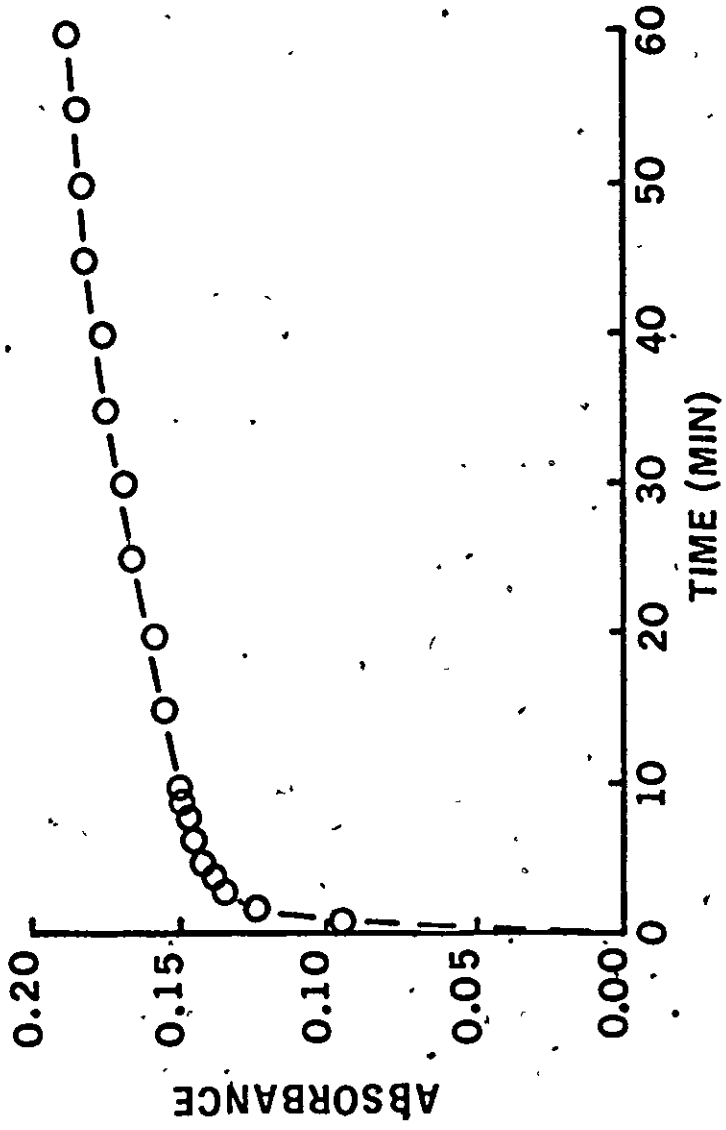


FIGURE 17. The effect of time on the formation of oxidized chromogen. To 1 ml of buffer, 10 nmole of glucose was added together with 1 ml of reagent. Conditions for assay were as described in the Materials and Methods section. Absorbance at 420 nm was monitored at 1 min intervals for the first 5 min and then at 5 min intervals for up to a period of 1 hr.



stable for the rest of the hour.

2. Linearity of the continuous coupled assay.

A linear relationship was obtained between absorbance and time when a constant amount of trehalase extract was incubated with 0.5 ml of 50 μ mole/ml of trehalose (Figure 18). This increase in absorbance was found to be linear with time for, at least 100 min at 23.5 C. When different volumes of pure enzymes or enzyme extracts were added to 0.5 ml of their appropriate substrates, a linear relationship was again observed for all enzymes tested (Figure 19).

3. Comparison of continuous and discontinuous assays.

Both the continuous coupled and discontinuous assays yielded a linear relationship between the absorbance after 60 min of incubation and the volume of crude trehalase extract added (Figure 20). The absorbance was found to be higher in the discontinuous assay with the same amount of trehalase extract used. This is expected since in the discontinuous assay, one is measuring the amount of glucose already present in the tube; whereas in the continuous assay, one is measuring the glucose as it is being released by the acid glucosidases.

H. Activity of trehalase in matrix material and in dormant spores during aging of D. discoideum strain NC4H.

Similar results as that observed for β -glucosidase activity, were found for trehalase activity. A higher specific activity of trehalase was found in the matrix

FIGURE 18. The effect of time on the formation of oxidized chromogen using the continuous assay. A volume of 0.5 ml of crude trehalase extracts from *D. discoideum* spores was introduced into 0.5 ml of 50 μ mole/ml of trehalose. The mixture was allowed to incubate at 23.5 C. At various time intervals, the absorbance was taken as described in the Materials and Methods section. The plotted values represent absorbances at various times after subtracting the absorbances of enzyme and substrate controls.

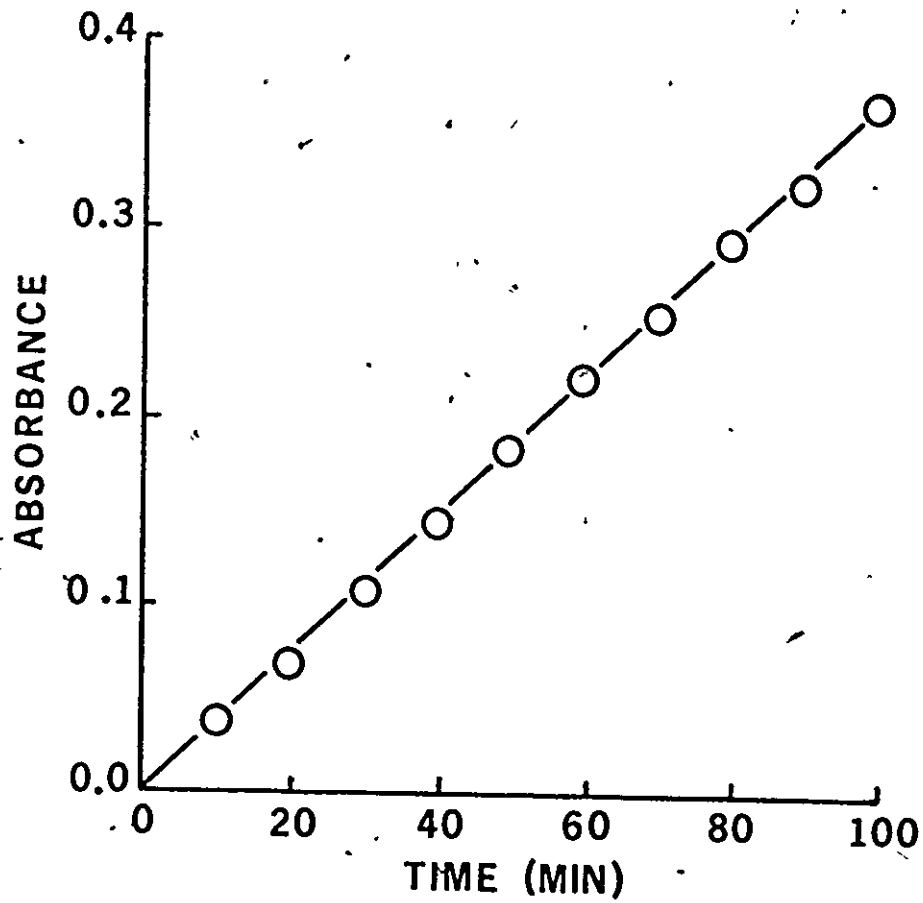


FIGURE 19. Oxidized chromogen formation as a function of the amount of added pure enzymes or enzyme extracts using the continuous coupled assay. A 0.1 ml to 0.5 ml volume of enzyme was added to 0.5 ml of the appropriate substrates. All assays, except β -glucosidase on cellobiose, were performed with a total volume of 2 ml as described in the Materials and Methods section. With cellobiose, the high substrate background was corrected by diluting the final product with 2 ml of buffer to make a total volume of 4 ml. The plotted values represent absorbances of sample tubes after subtraction of enzyme and substrate controls. \circ , crude yeast β -1,6 hydrolases on amygdalin; \triangle , 0.15 U/ml of commercially pure invertase on sucrose; \square , crude *D. discoideum* myxamoebae trehalase extract on trehalose; \hexagon , 6.9 U/ml of commercially pure β -glucosidase on ~~cellobiose~~.

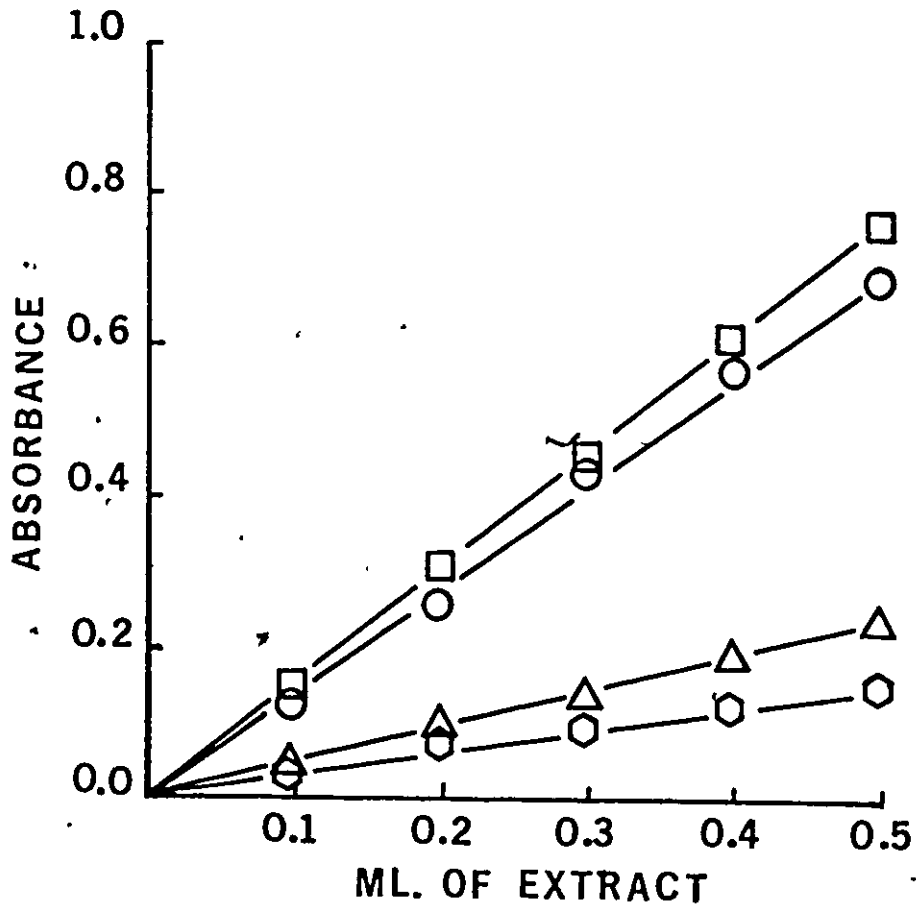
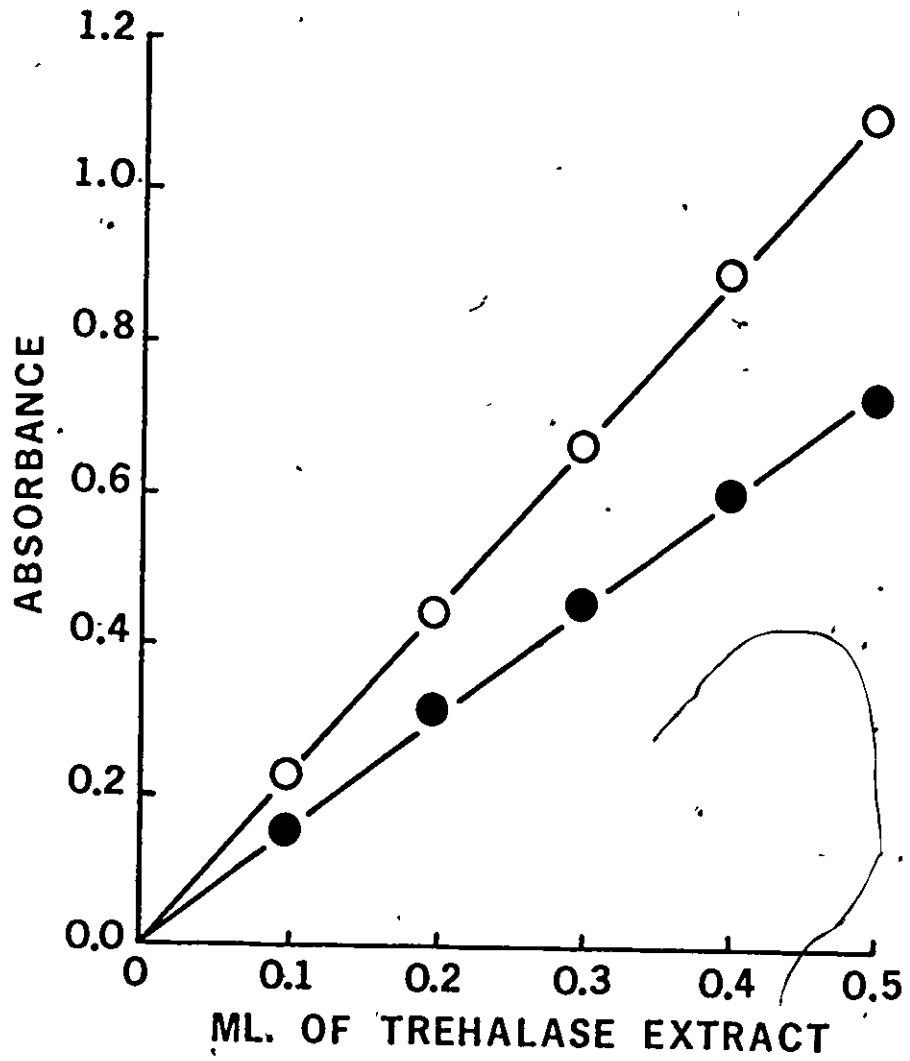


FIGURE 20. Oxidized chromogen formation as a function of the amount of crude D. discoideum myxamoebae trehalase extract. The experimental conditions for both continuous coupled and discontinuous assays were as described in the Materials and Methods section. The plotted values represent the absorbances of mixtures after correction for enzyme and substrate controls.

●, corrected absorbance obtained in a continuous coupled assay; ○, corrected absorbance obtained in a discontinuous assay.



material than in dormant spores (Table III). When the enzyme activity was analyzed during aging of spores in the sorocarps, trehalase in both dormant spores and matrix material was found to decrease (Figure 21).

1. Expression of crude trehalase specific activity during heat-induced germination of *D. discoideum* strain NC4H spores.

The change in specific activity of trehalase during germination is plotted in Figure 22. Dormant spores contained a low level of trehalase, this amount of enzyme remained until late swelling. At this time, about 30 min before the first sign of emergence, the specific activity of trehalase began to increase. This drastic increase in specific activity reached a maximum at 5 hr which coincided with the maximum emergence of myxamoebae. The latter increase in specific activity could be blocked by both UV irradiation (Demsar, personal communication) and addition of protein synthesis inhibitors (Cotter and Raper, 1970; Cotter et al., 1979; Tisa and Cotter, 1979). After the trehalase had reached a maximum level, the specific activity of the enzyme inside the amoebae began to decrease. This decrease in activity could be due to denaturation or release of trehalase into the extracellular medium.

When trehalase activity was studied in detail, it was discovered that the enzyme was released into the medium at late spore swelling, this coincided with the time of increase in

TABLE III. The specific activity of trehalase in 1 day old matrix material and in dormant spores.

Preparation ^a	Enzyme activity ^b (Units/ml)	Protein (mg/ml)	Specific activity ^c Fold
Dormant spores	0.74	0.43	1.72
Matrix material	96.42	0.04	2410.50

^a Spores were placed in 5 ml of acetate buffer (0.1 M, pH 5.5) and separated into a matrix fraction and a spore fraction by centrifugation. After two washes in acetate buffer, the spores were resuspended in 5 ml of acetate buffer. The matrix material and spore suspension were processed as described in the Materials and Methods.

^b Units of enzyme activity are equivalent to nmoles of glucose per min at 23.5 C under the conditions described in the Materials and Methods.

^c Units of specific activity are equivalent to units of enzyme activity per mg of protein.

FIGURE 21. The enzyme activity of trehalase in matrix material and in dormant spores of D. discoideum strain NC4H. Log units of enzymatic activity are plotted versus the days of aging in the intact sorocarp. Units of enzyme activity are expressed as nmole of glucose released per min at 23.5 C per 10^7 spores. □, enzyme activity in matrix material; ■, enzyme activity in dormant spores.

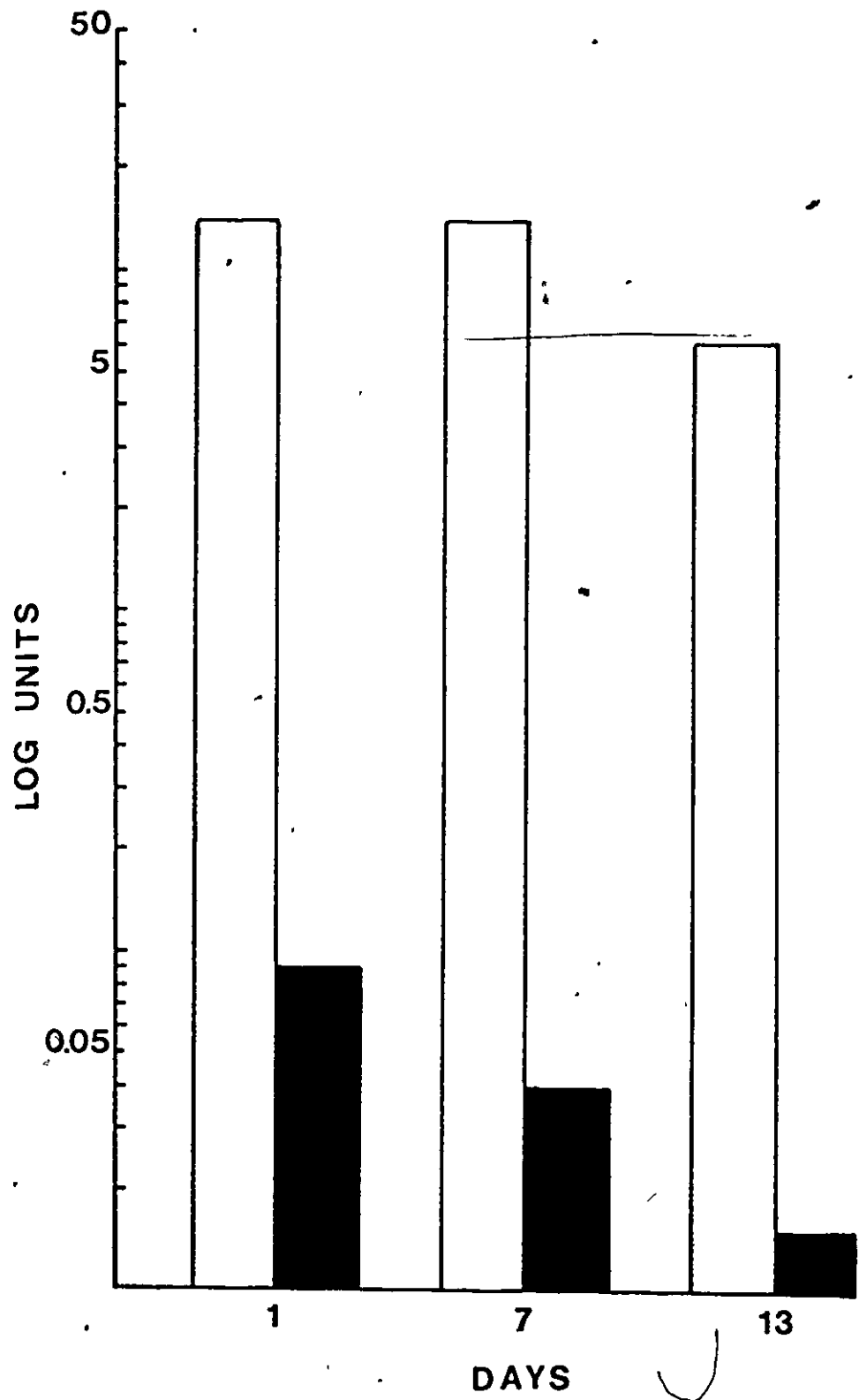
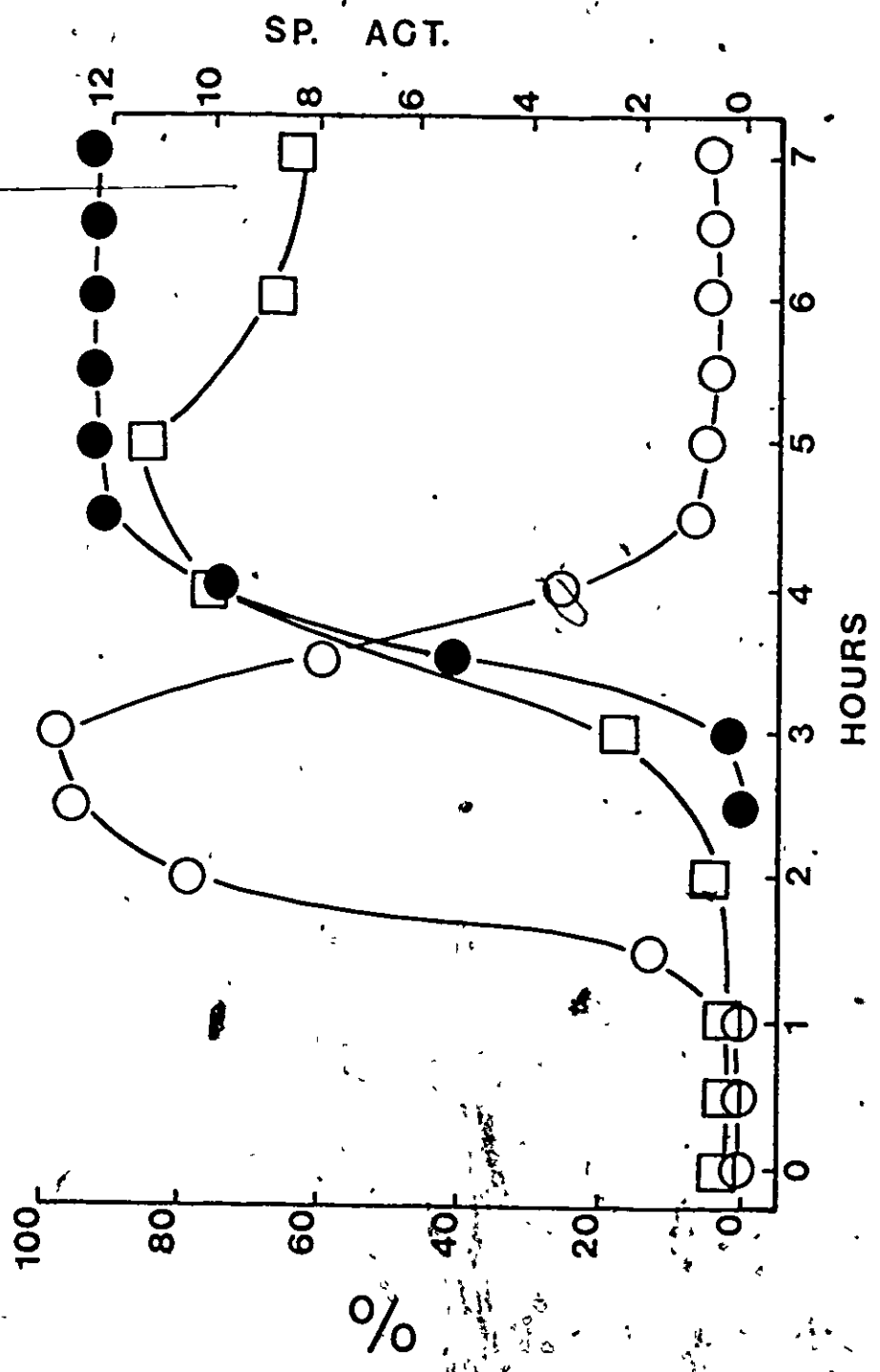


FIGURE 22. Specific activity of endogenous trehalase during heat-induced spore germination of D. discoideum strain NC4H. Spores were heat-activated at 45 C for 30 min.

○ , percent swollen spores; ● , percent emerged myxamoebae; □ , specific activity of endogenous trehalase.



specific activity of endogenous trehalase (Figure 23). The rate of enzyme release appeared to decrease after one hour. This might be due to denaturation of enzyme in the high pH extracellular medium. When total (endogenous + exogenous) enzyme activity, per 10^7 spores was plotted against time of germination (Figure 23), trehalase was found to increase in enzymatic activity from 2 to 5 hr. At the end of 5 hr, which was the time of maximum emergence, total enzyme activity was found to decrease. This decrease was probably due to denaturation of trehalase enzyme outside the starving amoebae.

To further prove that trehalase enzyme was undergoing both release and denaturation during germination, spores were allowed to germinate in phosphate buffer. At 65% emergence, the population of myxamoebae and swollen spores was UV irradiated to prevent RNA synthesis with a fluence of $1.7 \text{ J/m}^2/\text{sec}$ for 2:5 min (total fluence 250 J/m^2). When the endogenous trehalase was monitored, its enzyme activity was found to decrease. The exogenous trehalase, on the other hand, showed a slight increase in activity a few minutes after UV irradiation and then decreased (Figure 24). These data indicate that trehalase was preferentially released into the medium during germination. Once the enzyme was in the extracellular medium, the pH and/or temperature outside caused its denaturation.

J. Expression of crude trehalase specific activity during spore germination of *D. discoideum* strain SG1.

FIGURE 23. Enzyme activity of endogenous and exogenous trehalase during heat-induced germination of *D. discoideum* strain NC4H spores. \square , enzymatic activity of endogenous trehalase per 10^7 spores; \triangle , enzymatic activity of exogenous trehalase per 10^7 spores; \circ , total enzymatic activity per 10^7 spores.

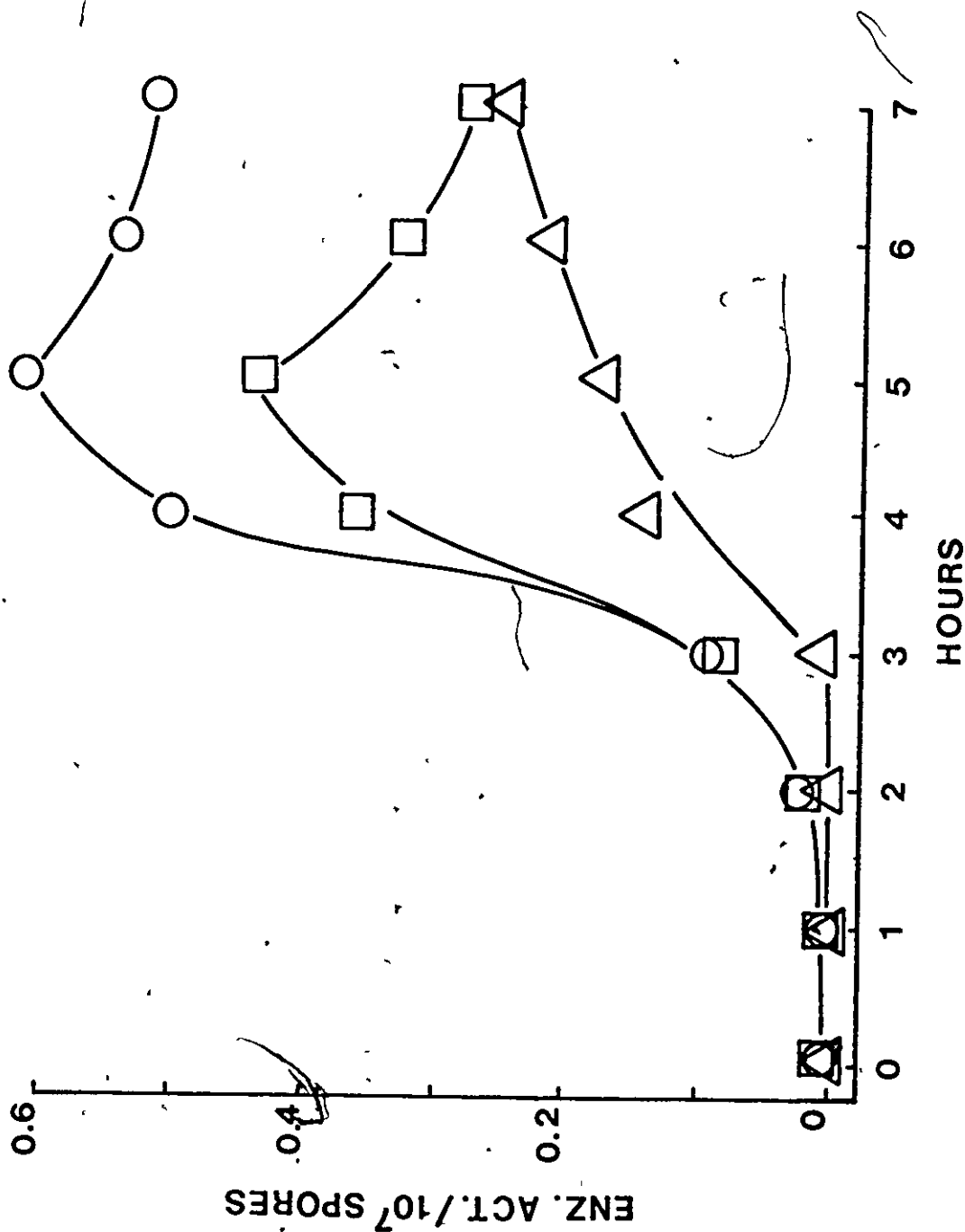
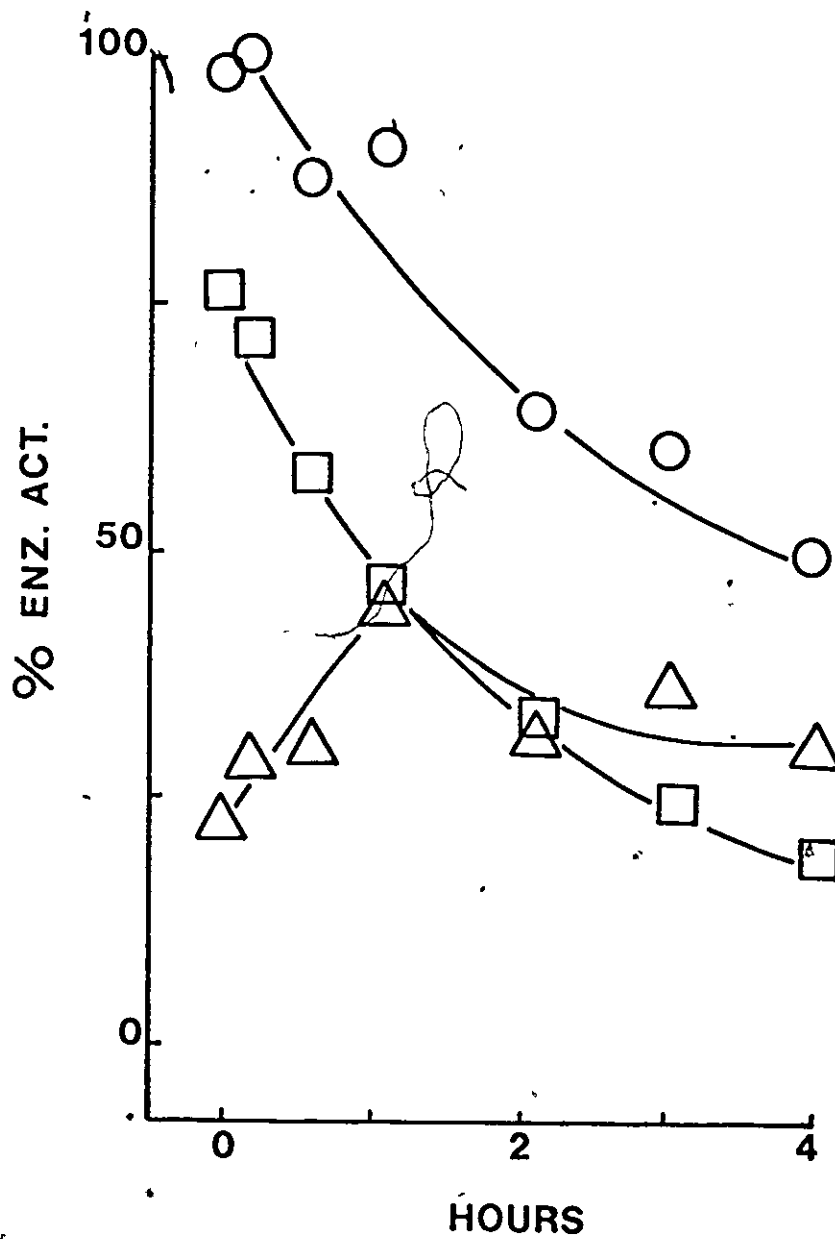


FIGURE 24. Expression of trehalase after UV irradiation. Spores of D. discoideum strain NC4H, at 65% emergence, were UV irradiated with a total fluence of 250 J/m². Endogenous and exogenous trehalase were monitored as described in the Materials and Methods section.

□, enzymatic activity of endogenous trehalase per 10⁷ spores; △, enzymatic activity of exogenous trehalase per 10⁷ spores; ○, total activity of 10⁷ spores.



S.C.

The spontaneous germination mutant, SG1, was again used to study the expression of trehalase during chemical activation and autoactivation of D. discoideum spores. Similar patterns as observed for β -glucosidase activity were found for trehalase, except the temporal shift in trehalase expression were more drastic (Figures 25-32).

Autoactivation was again found to be the treatment which produced the most drastic temporal shift in enzyme expression. The increase in trehalase shifted from late swelling in heat-activated spores to early swelling in autoactivated spores (Figure 27). In chemical activated spores, the increase in trehalase activity occurred at mid swelling (Figures 29,31).

The quantitative shift in trehalase expression was also more drastic than the shift in β -glucosidase expression. Autoactivation, which resulted in the most drastic temporal shift, was found to yield the lowest maximum specific activity. The activity was 5 fold less than that in heat-activated spores. The specific activity of trehalase in DMSO and urea treated spores was also lowered. In all cases, the low specific activity of endogenous trehalase was not due to the over release of the enzyme, since the total enzyme activity was also lower compared with that of heat activated spores (Figures 26,28,30,32).

K. Trehalose utilization during germination of UV and cycloheximide treated D. discoideum strain NC4H spores.

FIGURE 25. Specific activity of endogenous trehalase during heat-induced germination of D. discoideum strain SGI spores. Spores were heat-activated at 45 C for 30 min. ○, percent swollen spores; ●, percent emerged myxamoebae; □, specific activity of trehalase.

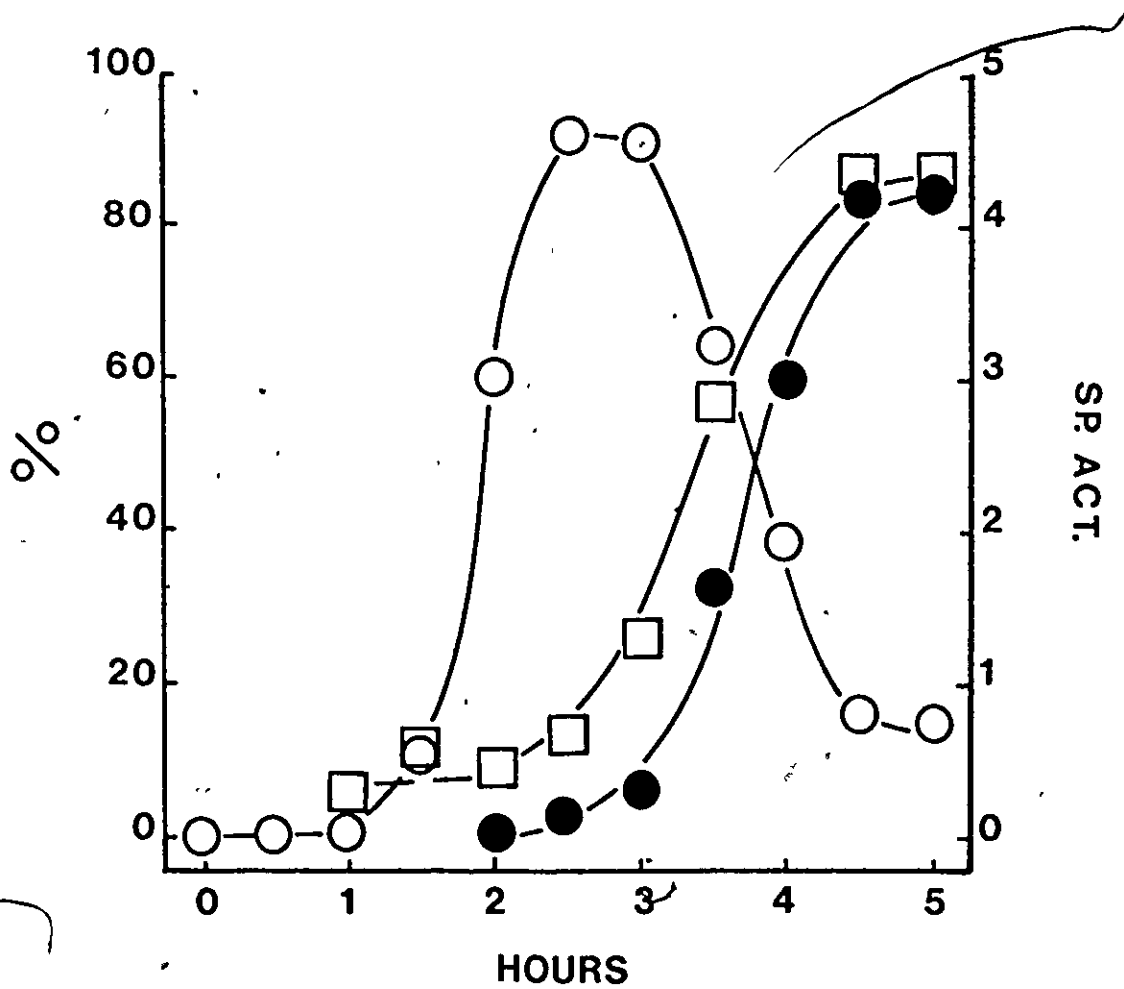


FIGURE 26. Expression of exogenous trehalase in *D. discoideum* strain SG1 spores after heat activation. □, enzymatic activity of endogenous trehalase per 10^7 spores; △, enzymatic activity of exogenous trehalase per 10^7 spores; ○, total enzymatic activity per 10^7 spores.

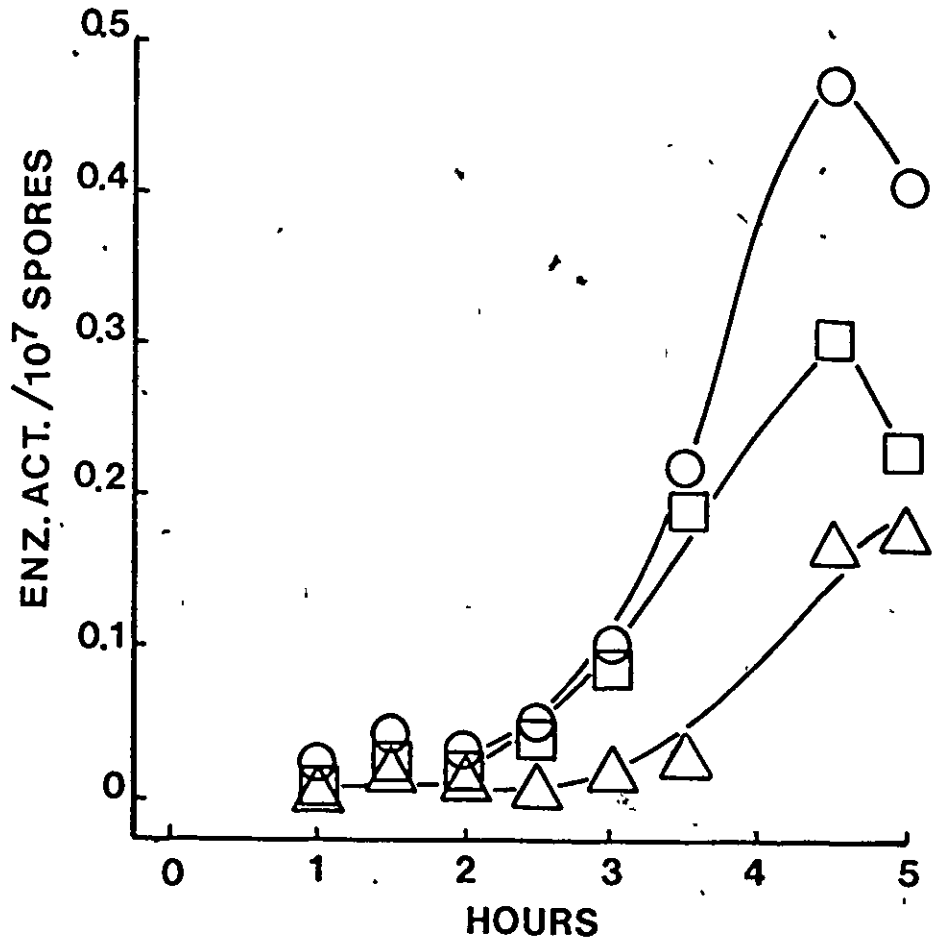


FIGURE 27. Specific activity of endogenous trehalase,
during germination of autoactivated D. discoideum strain
SG1 spores. ○, percent swollen spores; ●, percent
emerged myxamoebae; □, specific activity of trehalase.

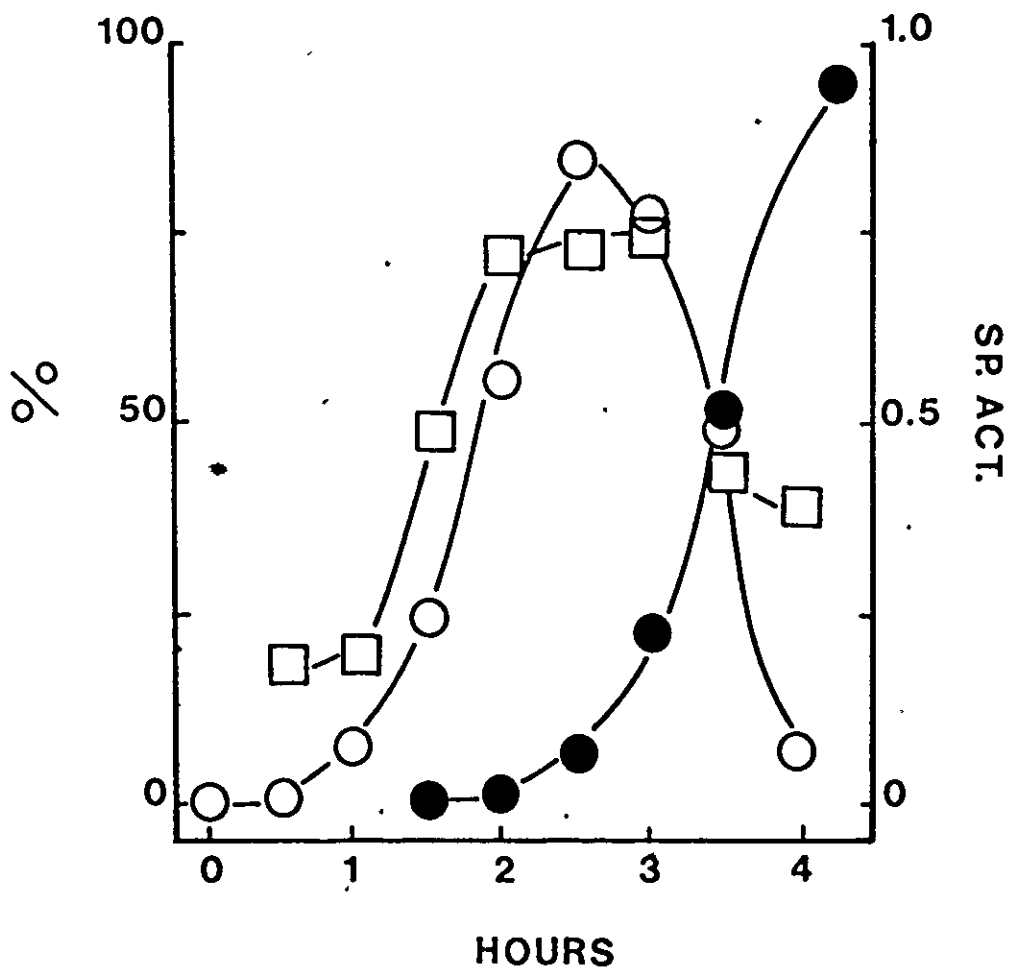


FIGURE 28. Expression of exogenous trehalase in *D. discoideum* strain SG1 spores after autoactivation.

□, enzymatic activity of endogenous trehalase per 10^7 spores; △, enzymatic activity of exogenous trehalase per 10^7 spores; ○, total enzymatic activity per 10^7 spores.

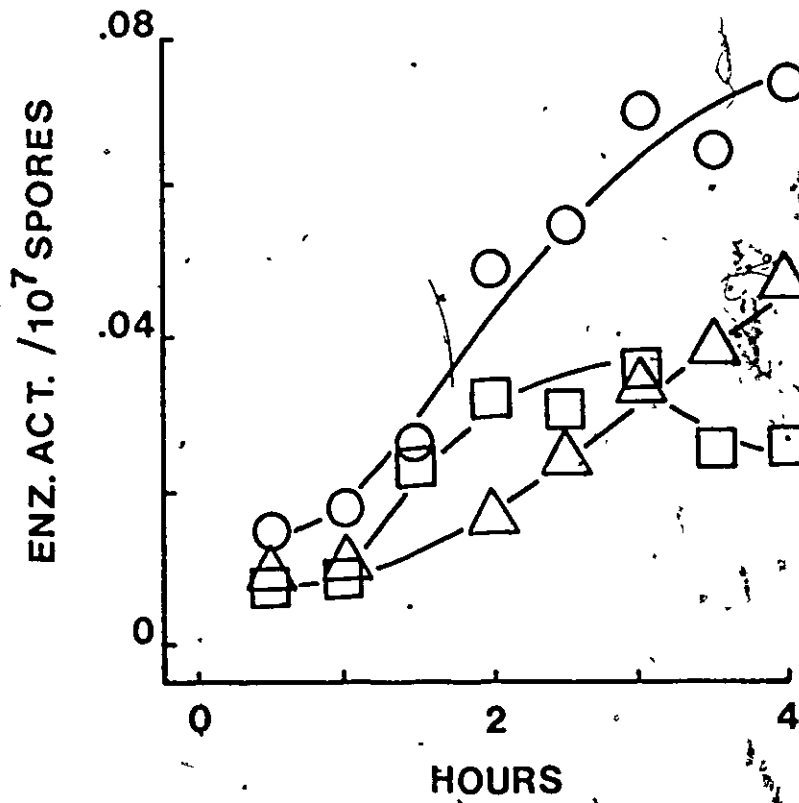


FIGURE 29. Specific activity of endogenous trehalase during germination of DMSO-activated D. discoideum strain SG1 spores. ○, percent swollen spores; ●, percent emerged myxamoebae; □, specific activity of trehalase.

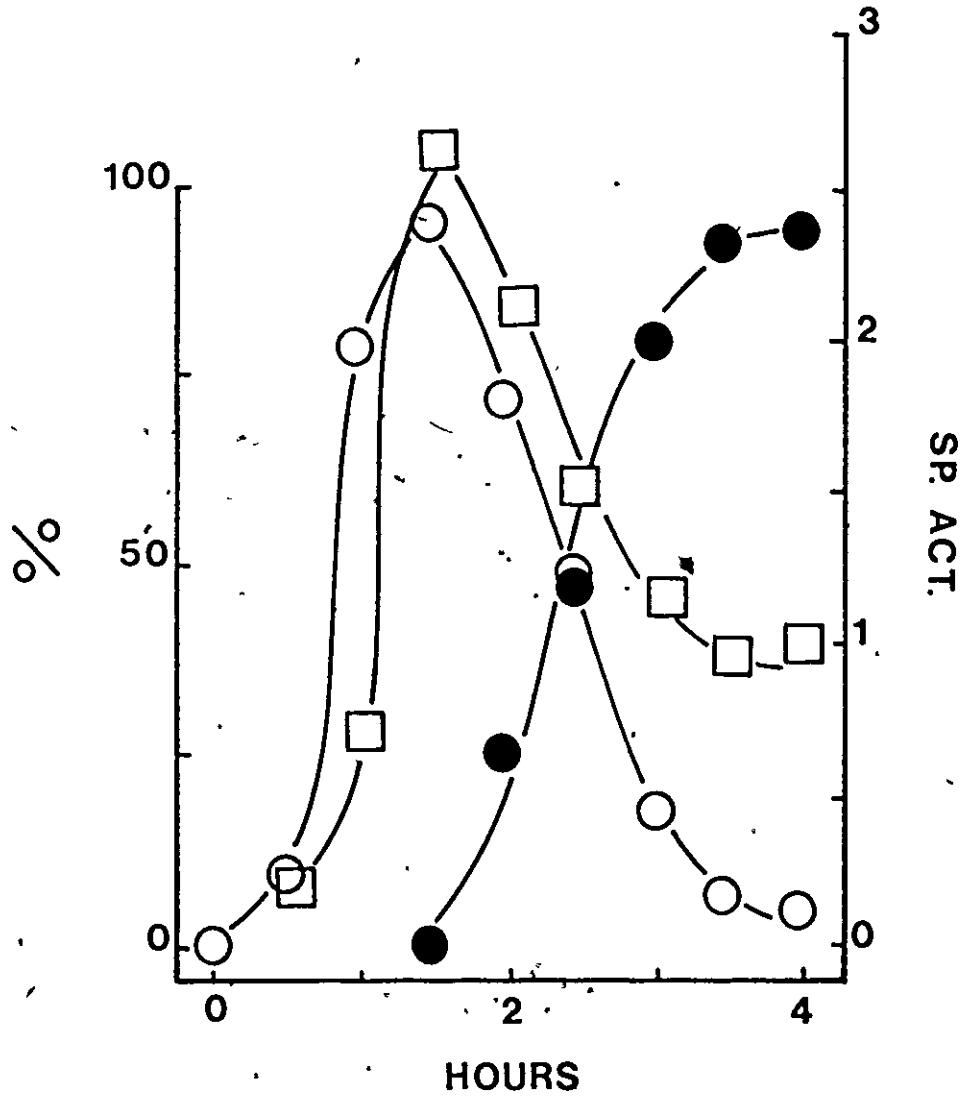


FIGURE 30. Expression of exogenous trehalase in
D. discoideum strain SG1 spores after DMSO activation.

□, enzymatic activity of endogenous trehalase per
 10^7 spores; △, enzymatic activity of exogenous trehalase
per 10^7 spores; ○, total enzymatic activity per
 10^7 spores.

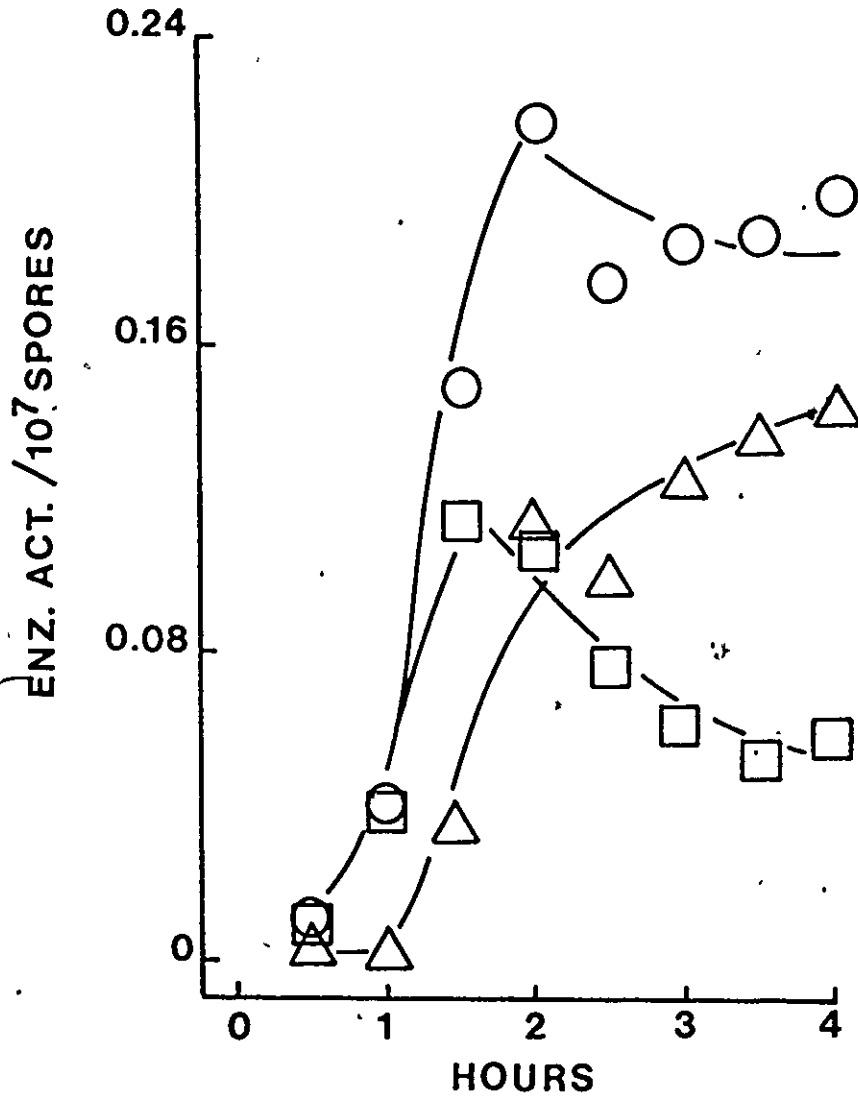


FIGURE 31. Specific activity of endogenous trehalase during germination of urea-activated D. discoideum strain SG1 spores. ○, percent swollen spores; ●, percent emerged myxamoebae; □, specific activity of trehalase.

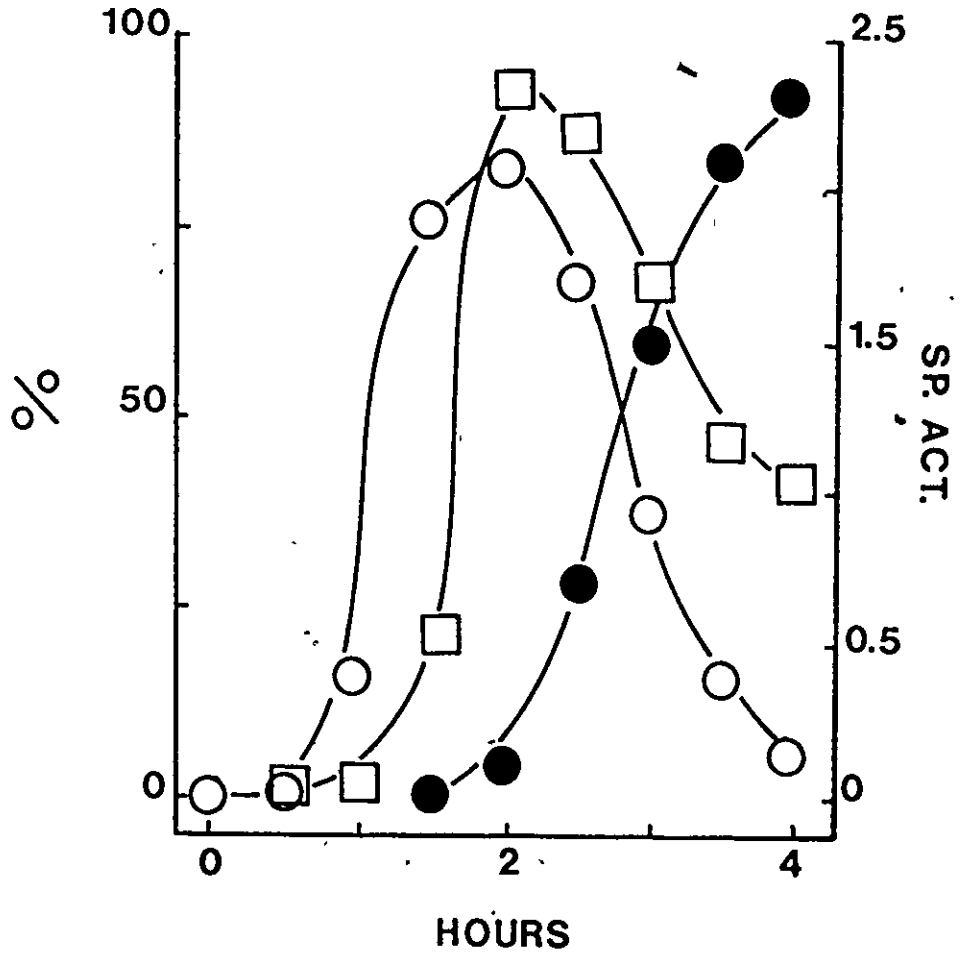
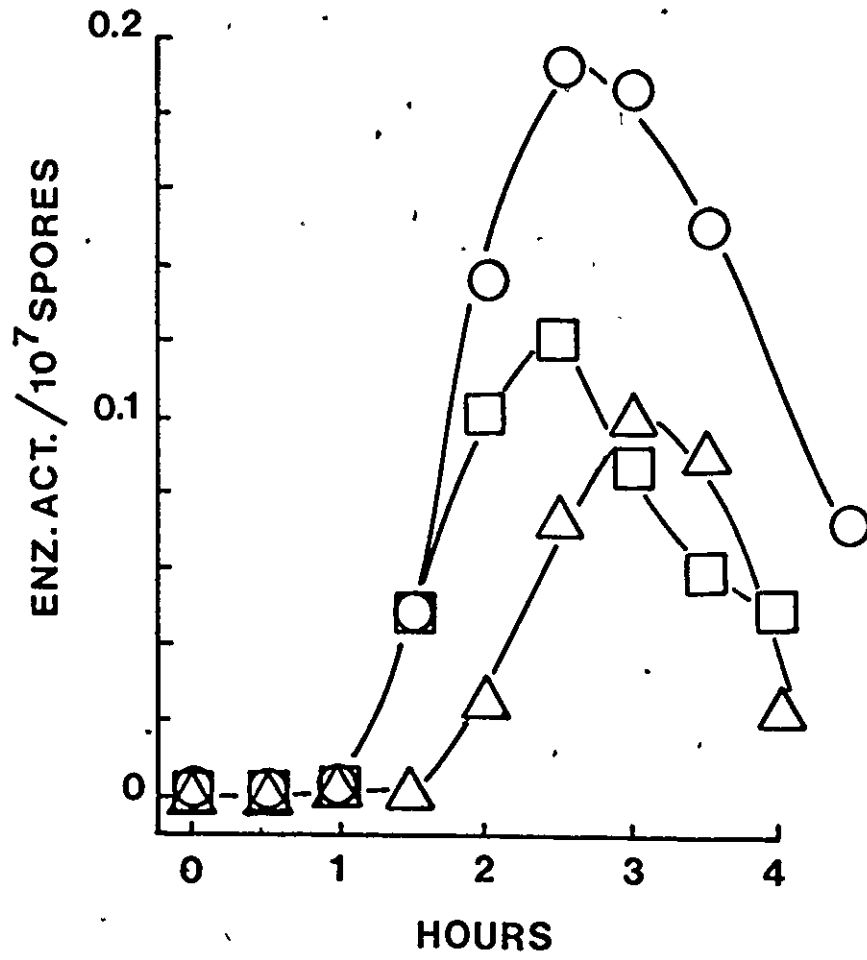


FIGURE 32. Expression of exogenous trehalase in *D. discoideum* strain SG1 spores after urea activation.

□, enzymatic activity of endogenous trehalase per 10^7 spores; △, enzymatic activity of exogenous trehalase per 10^7 spores; ○, total enzymatic activity per 10^7 spores.



The carbohydrate, trehalose, was utilized before maximum swelling (Jackson, personal communication). Since the trehalase specific activity did not increase before late swelling (Figure 22), the enzyme that hydrolyzes the carbohydrate substrate must be that of the pre-formed trehalase. To prove the above hypothesis, spores were either UV irradiated to prevent RNA synthesis or germinated in the presence of cycloheximide to block protein synthesis. Spores under the above conditions would not synthesize the new trehalase (Tisa and Cotter, 1980). If trehalose utilization was monitored under these conditions, the pattern was found to be the same as in the normal germinating spores (Figures 33, 34). Thus, the trehalase which was synthesized during germination was not required for utilization of trehalose present in the spores.

L. Release of glucose into the medium during germination of *D. discoideum* NC4H spores.

When the extracellular medium was monitored for carbohydrates, glucose was found to increase during the germination period (Figure 35). This increase might be due to the release of the carbohydrate from the spores or the breakdown of spore cell walls. The outer wall of *D. discoideum* was mainly composed of cellulose (Hemmes et al., 1972). This carbohydrate could be broken down to cellobiose which eventually might be hydrolyzed to glucose.

When spores were germinated in the presence of cycloheximide to prevent the breakage of the innermost cell

FIGURE 33. Trehalose utilization during germination of UV irradiated D. discoideum strain NC4H spores. Spores were UV irradiated at a fluence of 250 J/m². After irradiation, the spores were heat activated at 45 C for 30 min. Spores were then germinated as described in the Materials and Methods section. Trehalose utilization were expressed as nmoles of glucose released per min at 23.5 C when incubated with 95 U/ml of partially purified trehalase.

○, percent swollen spores; □, nmoles of glucose released (1 unit = 1 nmole).

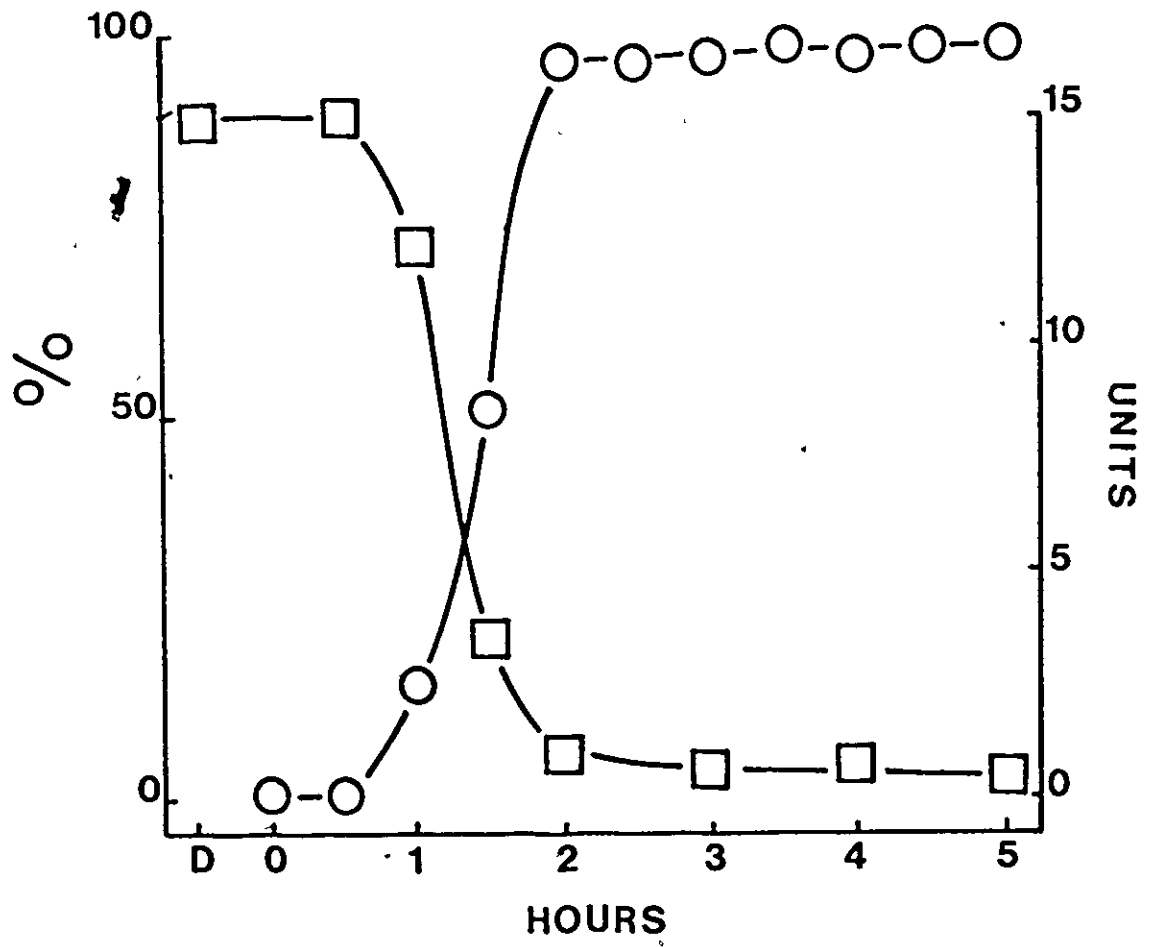


FIGURE 34. Trehalose utilization during germination of cycloheximide treated D. discoideum strain NC4H spores. Spores were heat activated at 45 C for 30 min. and were then germinated in 0.01 M phosphate buffer (pH 6.5) containing 200 ug/ml of cycloheximide. ○, percent swollen spores; □, nmoles of glucose released (1 unit = 1 nmole).

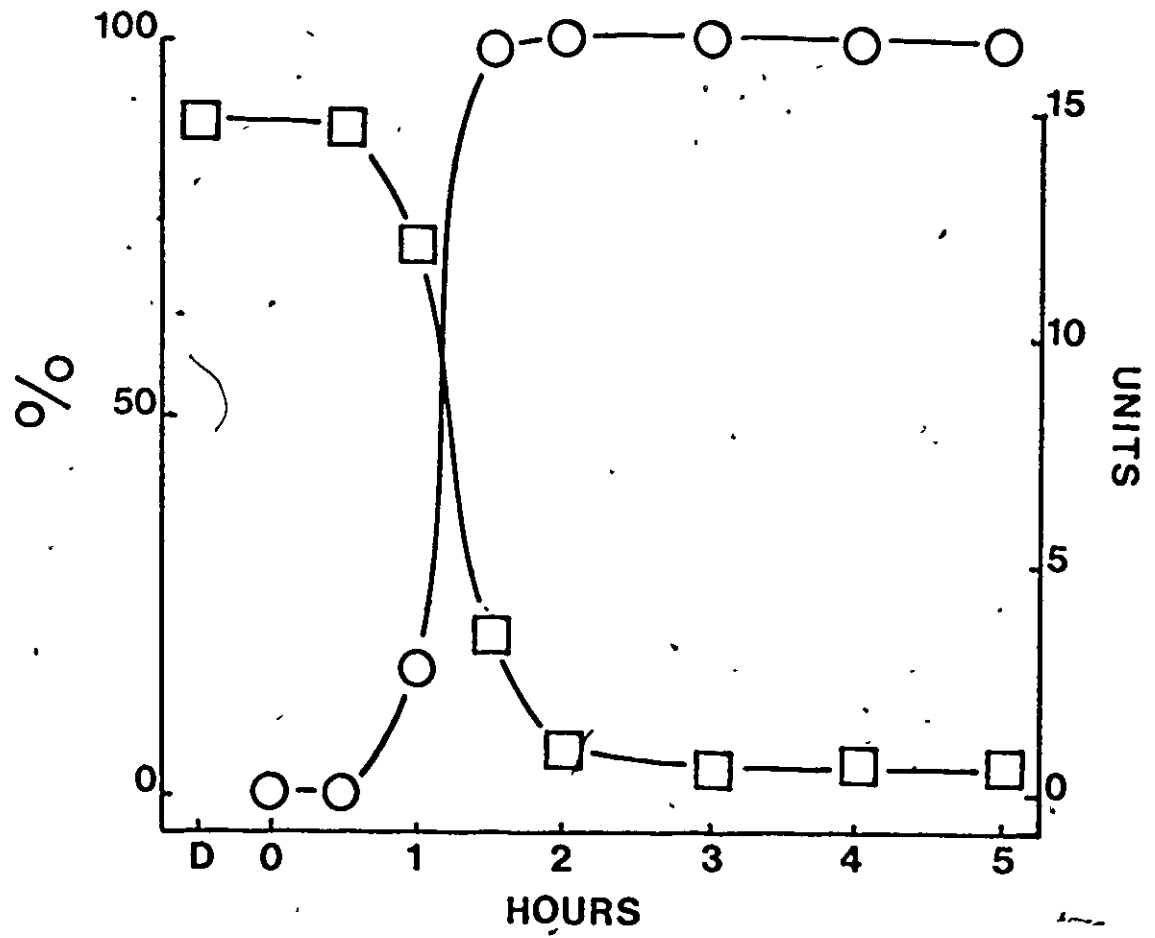
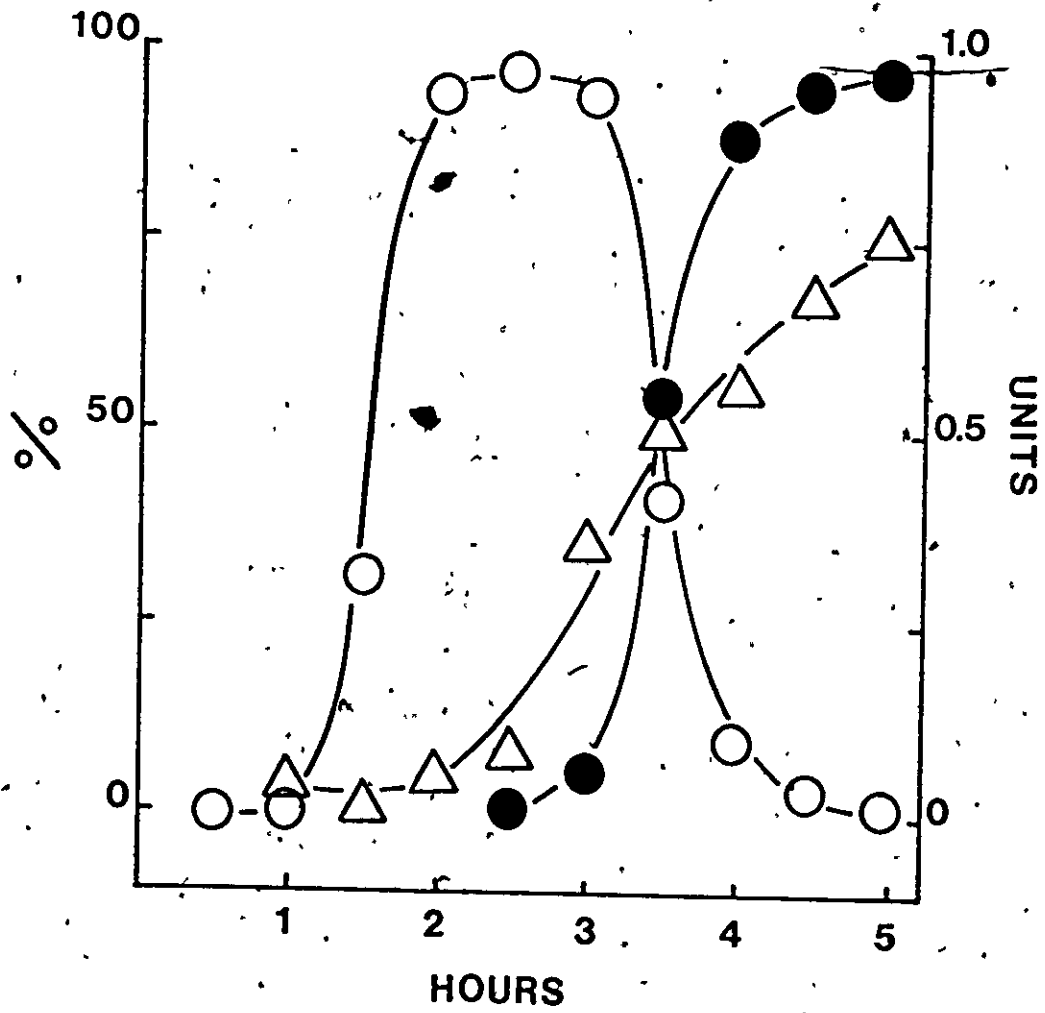


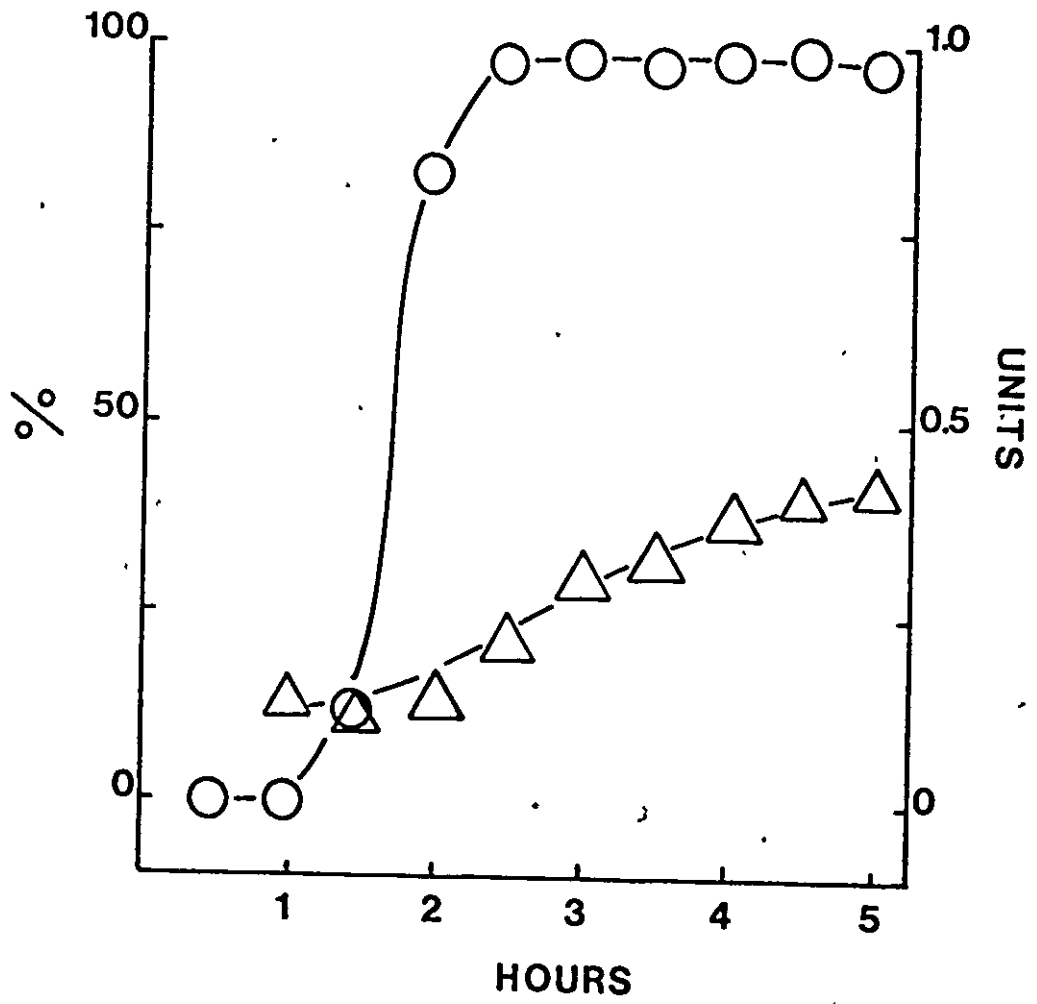
FIGURE 35. Amount of glucose released into the extracellular medium during heat-induced germination of D. discoideum strain NC4H spores. Units are expressed as nmoles of glucose released per min per 10^6 spores under the assay conditions. ○, percent swollen spores; ●, percent emerged myxamoebae; △, amount of glucose per 10^6 spores.



wall layer. The glucose in the extracellular medium was found to decrease (Figure 36). Thus the high glucose level in the medium of the control germinating spores after the third hour, might be due to the breakdown of the innermost layer of the spore wall during germination.

FIGURE 36. Amount of glucose released into the extracellular medium in cycloheximide treated spores. Units are expressed as nmoles of glucose released per min per 10^6 spores under the assay conditions.

○, percent^t swollen spores; △, amount of glucose per 10^6 spores.



DISCUSSION

The enzymes, β -glucosidase and trehalase, increase in specific activity during spore germination of Dictyostelium discoideum (Tisa and Cotter, 1979, 1980). These increases in specific activity occur concurrently or slightly before emergence (Figures 5, 22) which strongly suggests to most workers that these enzymes are required for spore germination (Coston and Loomis, 1969; Cotter and Raper, 1970; Tisa and Cotter, 1980).

Biochemical and genetic studies have shown that β -glucosidase is a developmentally significant enzyme throughout the life cycle of D. discoideum (Coston and Loomis, 1969; Ashworth and Quance, 1972; Jones *et al.*, 1979; Tisa and Cotter, 1979, 1980). Electrophoresis of matrix material from the sori demonstrates the presence of β -glucosidase 2 with a trace amount of β -glucosidase 1 (Figure 4). The enzyme activity of these β -glucosidases is found to be much higher in the matrix than in the dormant spores (Table 1). This may indicate that these enzymes are either residual enzymes derived from pre-stalk cells during fruiting body construction or that they are enzymes derived from spores entering dormancy. Oohata and Takeuchi (1977) have shown that a higher β -glucosidase activity is present in the pre-stalk cells than in the pre-spore cells of D. mucoroides.

β -glucosidase 2 is also found in dormant spores

(Figure 6). Hamilton and Chia (1975) have shown that the mutant P4, which is unable to form spores, synthesizes a reduced β -glucosidase activity during culmination. Coston and Loomis (1969) previously found that β -glucosidase 2 activity is decreased when strain KY-19 culminates; this strain is a mutant which forms fruiting bodies containing normal spores but few stalk cells. Apparently, this form of β -glucosidase is both a pre-spore and pre-stalk enzyme. Therefore, further work is necessary to establish the actual ratios of this enzyme in the two cell types.

The specific activity of β -glucosidase decreases during heat activation; however, upon emergence of myxamoebae, the specific activity of this enzyme increases rapidly (Figure 5). Unlike Polysphondylium pallidum microcysts which release their β -glucosidase activity during germination (O'Day and Paterno, 1979), D. discoideum spores seem to retain the enzyme during germination (Jones et al., 1979; Tisa and Cotter, 1980). This is shown by the lack of β -glucosidase activity in the extracellular medium (Data not shown); and the consistency in the expression of β -glucosidase specific activity inside the myxamoebae after maximum emergence (Figure 9).

Different forms of β -glucosidase are found in the spores and in the myxamoebae, as indicated by polyacrylamide gel electrophoresis (Figure 6). The predominant form of β -glucosidase in the dormant spore is β -glucosidase 2. Upon heat activation, this enzyme

decreases to an undetectable amount in the crude preparations but only decreases by 75% in the enrichment samples. The difference in observations may be due to one or both of the following reasons: (i) the renaturation of the enzyme during gel electrophoresis or (ii) the protection of the enzyme when spores are heat-shocked at very high concentration which may result from lowered water activity. During emergence of myxamoebae, a new form of β -glucosidase is synthesized. The expression of this newly-formed enzyme, β -glucosidase 1, requires both RNA and protein syntheses since UV irradiation (Demsar, personal communication) as well as cycloheximide (Tisa and Cotter, 1980) will block the increase in specific activity of this new enzyme. The fact that denaturation of β -glucosidase 2 occurs during heat-induced activation does not support the assumption that this enzyme is required for splitting of the spore wall during germination (Coston and Loomis, 1969). When dormant spores are trypsin-treated to destroy all β -glucosidase 2 activity (Table II), the spores germinate normally without any change in the rate of swelling or emergence (Data same as in Figure 5). It should also be remembered that heat activated spores can be deactivated and later reactivated with normal germination occurring (Tisa and Cotter, 1980); such secondarily germinating spores would have very little β -glucosidase 2 activity and thus we conclude that this enzyme has no apparent involvement in spore germination. Furthermore,

the activity of β -glucosidase 2 in the matrix is much higher than that in the dormant spore (Table 1). If spores are allowed to age in the intact sorocarps, the enzyme activity of β -glucosidase in both matrix and dormant spores is found to decrease by day 13 (Figure 3), at this time the spores will normally undergo autoactivation (Dahlberg and Cotter, 1978). Thus, it is probable that the enzyme is not required in the dormant spores for germination but is merely the trapped residue which is normally released during terminal development of pre-spore and/or pre-stalk cells. This may also be true for a number of other enzymes, such as alkaline phosphatase, α -glucosidase and α -mannosidase, which also show a decrease in specific activity during heat activation and upon aging (Chan *et al.*, 1981b). These enzymes, along with β -glucosidase, are found mainly in the pre-stalk cells during the developmental cycle of D. discoideum (MacWilliams and Bonner, 1979).

The normal germination of the β -glucosidase 1 minus mutant (Figure 11) and the formation of spheroplasts without the presence of β -glucosidase (Figure 14) suggest that the enzyme is not critical for the emergence of myxamoebae. However, the presence of this form of β -glucosidase does accelerate the splitting of the third spore layer. Thus, unlike cellulase and protease(s), β -glucosidase 1 may only play a minor but dispensable role during spore germination. The retention of the enzyme

within myxamoebae during germination (Figure 9) suggests that β -glucosidase 1 may be used for degradation of ingested bacteria during the vegetative growth of Dictyostelium discoideum.

The other enzyme, trehalase, increases in specific activity before emergence (Figure 22). This enzyme, unlike β -glucosidase 2, is not inactivated upon heat activation (Cotter et al., 1979). Trehalase retains a basal level of enzyme activity in the early stages of germination and by late swelling the enzyme activity begins to increase (Figure 22). The latter increase in trehalase activity requires both RNA and protein syntheses (Cotter et al., 1979; Tisa and Cotter, 1979; Demsar, personal communication).

When the utilization of trehalose is studied, the disaccharide is found to hydrolyze into glucose before maximum swelling (Jackson, personal communication). This indicates that the newly-formed trehalase is not required for the hydrolysis of the storage carbohydrate. To further show the non-requirement of the enzyme, spores were either UV irradiated or germinated in the presence of cycloheximide to block RNA and protein syntheses. Spores germinated under these conditions will not synthesize new trehalase. When the trehalose utilization is monitored, the pattern is found to be similar to that of normal germinating spores (Figure 33, 34). Thus, we conclude

that the newly-formed trehalase enzyme has no apparent involvement in spore germination since the substrate will have been completely hydrolyzed before the synthesis of the enzyme begins (Figures 33, 34). However, further experimentation is needed to establish the relationship between the function of the pre-existing trehalase and spore swelling.

Unlike newly-synthesized β -glucosidase 1 which is retained inside the cells during germination, trehalase is released into the extracellular medium during germination (Figures 23, 25, 27, 29). The release of the enzyme occurs when the newly-formed trehalase is being synthesized. This further supports the idea that the newly-formed enzyme is not critical for spore germination. The reason for the release of the enzyme during spore germination is still unknown. However, the enzyme is also found to undergo preferentially release into the extracellular medium during aggregation (Ceccarini, 1966, 1967).

The dispensable involvement of β -glucosidase 1 and non-involvement of the de novo trehalase suggest that enzymes which are formed late during spore germination are not critical for the process. In other words, not all enzymes synthesized within a particular time period will be important for the morphogenic event(s) occurring at that time. This is especially so for enzymes that are formed late in the terminal development of eukaryotes. Newell (1971) has also previously pointed out that not all enzymes

synthesized during multicellular development of D. discoideum are necessarily produced for the formation of the fruiting bodies. Besides Dictyostelium, there is abundant evidence for increases in specific activities of various enzymes during spore germination in other organisms (Lovett, 1976). Some of these, such as glutamate dehydrogenase (Tuveson et al., 1967), and a post-conidial amino acid transport system (Tisa and DeBusk, 1970) in Neurospora crassa also fail to increase if cells are exposed to cycloheximide. Ohmori and Gottlieb (1965) have shown that a number of metabolic enzymes increase in both specific activity and total amount during germination in species of Trichoderma, Aspergillus, and Penicillium. However, none of these increased enzyme activities have been shown to be necessary for the success of germination.

The cell wall layers of D. discoideum are very complex. They are made up of polysaccharides as well as glycoproteins. Thus, it must be a group of enzymes, and not a particular enzyme, that is responsible for the breakage of the cell wall layers during germination. Enzymes that may be required for the process are the followings: (i) cellulase, which has been shown by Rosness (1968) and Jones et al. (1979) to be a pre-formed enzyme in the dormant spores. This enzyme is released into the extracellular medium during spore germination; (ii) protease(s) which are required to degrade the polypeptide linkages in the cell wall layers (Hemmes et al., 1972); and

(iii) glycosidases which may facilitate the breakage of carbohydrate linkage in the cell wall layers. These enzymes, however, have to be either pre-formed or synthesized early in the spore germination process. Enzymes such as β -galactosidase, which increase during early swelling might be good candidates.

The complexity of the problem is even more apparent when the numbers of newly-synthesized proteins are examined by one and two dimensional gel electrophoresis (Giri and Ennis, 1978; Dowbenko and Ennis, 1980). The large variety of proteins assembled at very early times in spore germination suggested that the probability of finding a single essential protein may be low. So instead of looking for a single enzyme that is required for spore germination, future work should be concentrated on eliminating those enzymes that are non-essential for the process.

Both β -glucosidase and trehalase are found to have a higher enzyme activity in the matrix than in the dormant spores (Figures 3, 21). The above observations are also true for other enzymes such as acid phosphatase, alkaline phosphatase and α -mannosidase (Chan et al., 1981b). These enzymes are considered as being mainly pre-stalk enzymes which accumulate during the developmental cycle (MacWilliams and Bonner, 1979). Other hydrolases such as, N-acetyl- β -D-glucosaminidase, are found to have a higher enzymatic activity in the dormant spores than in the matrix (Chan et al., 1981b). This hydrolase, in turn, is

considered by most workers as a pre-spore enzyme. Thus, these results strongly suggest that the enzymes in the matrix are actually pre-stalk enzymes that are trapped in the extracellular space during the formation of the sorocarps. However, further work using mutants or vital dyes are necessary to confirm the above hypothesis. The low total protein concentration and high β -glucosidase and trehalase enzyme activities in the matrix provide a good starting material for the purification of these enzymes.

Interesting results are observed when spores of SGI are subjected to different types of activation. Temporal as well as a quantitative shifts are observed in the expression of the two enzymes which are dependent on the method of activation. Using the expression of enzyme activity in heat shocked spores as a standard, chemical activation as well as autoactivation results in early synthesis of β -glucosidase and trehalase, with a more pronounced effect on the latter enzyme (Figures 7, 8, 9, 10, 25, 27, 29, 31). The quantitative shift in trehalase is also more obvious than that in β -glucosidase. Again, using different methods of activation, both enzymes exhibit changes in the maximum amount of synthesized enzyme (Figures 7-10, 25, 27, 29, 31).

The actual reason(s) for the change in the temporal and quantitative expression of the enzymes is still unknown. It has been shown that chemicals, such as DMSO, can induce the formation of huge microfilament bundles

(actins) in the interphase nucleus of D. mucoroides (Fukui and Katsumaru, 1978). Such nuclear actins might function in the regulation of gene replication and/or transcription through their contractile nature, thus directly modulating gene expression (Fukui, 1979). This observation is supported by the interesting fact that DMSO can stimulate Friend leukemic mouse cells and rat kangaroo myoblast cells to synthesize hemoglobin (Friend et al., 1971) and collagen (Mirarda et al., 1978) respectively. Since spores of D. discoideum are permeable to DMSO, the temporal and quantitative shifts in enzyme expression may be due to the early induction of genes coding for these enzymes. This may also be true for urea and autoactivator(s). In Tetrahymena, an increased rate of RNA synthesis is observed in cells recovering from an exposure to DMSO (Nilsson, 1976). However, further work at the transcriptional level is necessary to support the above hypothesis.

The above data suggest that one must be cautious in interpreting protein patterns observed by enzyme analysis or two dimensional gel electrophoresis (Giri and Ennis, 1978; Dowbenko and Ennis, 1980). This is because the temporal and quantitative pattern of proteins synthesized during D. discoideum germination may not completely reflect the actual requirements for spore swelling or myxamoebae emergence, but may partially result from the method of activation. Finally, it is expected that the methods developed in this study will allow an indepth analysis of

specific gene regulation during the germination process.

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