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Trehalose Levels and Trehalase Activity in Germinated and Ungerminated Spores of *Nosema algerae* (Microspora: Nosematidae)[1]

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Gas chromatographic analysis of carbohydrates in ungerminated *Nosema algerae* spores revealed trehalose at concentrations above 0.4 M and traces of glucose. After germination in KCl at *p*H 9.5, only one-third of the trehalose remained, and a large increase in glucose was found. A significant amount of glucose was also detected when germination was inhibited by NH_4Cl, but the trehalose concentration was unchanged. Trehalase activity in the soluble and insoluble fractions of crude *N. algerae* spore homogenate, its response to temperature, *p*H, and trehalose concentration, and its relationship to spore germination were investigated. The temperature optima are 43.7°C for the supernatant and 43.9°C for the residue fraction. The K_m's are 26 mM for the supernatant and 25 mM for the residue. Thirty-minute temperature tolerance tests showed 50% activity reductions at 35°C for the supernatant and 38°C for the residue. The *p*H optima were 5.5 for the supernatant and 5.25 for the residue fractions. A significantly greater proportion of the total enzyme activity was associated with the supernatant fraction in germinated than in ungerminated spores. Neither NH_4Cl nor γ radiation appeared to exert inhibitory effects directly through trehalase.

KEY WORDS: *Nosema algerae;* trehalose; spore germination.

INTRODUCTION

Germination of *Nosema algerae* spores in KCl begins after a latent period of as little as 1 min, and after about 5 to 20 min the maximum percentage germination (80–95%) is reached (Undeen, 1978). During the germination process, a filament coiled within the spore everts to form a tube through which the sporoplasm, composed of two nuclei and a small amount of cytoplasm, is forced. The whole process requires only about 1–2 sec. When it occurs in a host gut, the filament penetrates the gut wall and injects the sporoplasm into a host cell. The sporoplasm initiates the infection in the host, and the first nuclear division occurs about 24 hr later (Undeen, 1975; Avery and Anthony, 1983).

Germination of *N. algerae* spores is stimulated in vitro by chlorides of potassium, sodium, rubidium, and cesium. A variety of monovalent anions can be substituted for chloride. Divalent ions are ineffective or even inhibitory (Undeen, 1978). *Nosema algerae* has an optimal in vitro germination *p*H of 9.5. Ammonium chloride is a potent inhibitor of spore germination at *p*H 9.5 in KCl or NaCl, at concentrations sufficient for germination (20–200 mM) (Undeen, 1978). Gamma radiation inhibits spore germination with a further decline in percentage germination with time after irradiation (Undeen et al., 1983).

So far, the mechanisms proposed to explain spore germination implicate either a pressure increase within the spore or a weakening of the polar cap. No mechanisms have been proposed that explain the in vitro inhibition and inactivation by low

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salt concentrations observed (Undeen, 1978) in *N. algerae*. Wood et al. (1970) found that trehalose was the major carbohydrate in *Nosema apis* spores. Vandermeer and Gochnauer (1971) examined the trehalase activity in extracts from *N. apis* spores and suggested that trehalose might have a role in the infective process.

N. algerae spores are easily germinated in vitro under the influence of a number of controllable variables. Consequently, this species was chosen as a model for studies on the mechanisms of microsporidian spore germination. This paper presents our findings on spore carbohydrate composition and the differences that occur among germinated, ungerminated, and NH_4Cl-inhibited spores. Trehalase catalyzes the first step in trehalose metabolism, the conversion of trehalose to two molecules of glucose. The present work also describes *N. algerae* spore trehalase and examines the hypothesis that germination stimuli or inhibitors might exert their influence on trehalase.

MATERIALS AND METHODS

Spore Production

Spores of *N. algerae* from *Anopheles quadrimaculatus* were fed to starved third or fourth instar *Heliothis zea* larvae for mass production (Anthony et al., 1978). The infected *H. zea* adults were triturated in a blender, filtered through Miracloth or Pellon, and the resulting spore suspension was purified by continuous flow-density gradient centrifugation (Undeen and Avery, 1983). The spores were stored at 5°C for 4–7 weeks and washed again in one 5-ml aliquot of deionized water before use.

Spores were counted in a hemacytometer and measured with a Vickers image-splitting eyepiece. Spore volume was calculated by the equation for a prolate spheroid: $[4.189 \times ((\text{length} + \text{width})/4)^3]$.

Trehalose Measurement

Spore samples were prepared from 1.05 ml of spore suspension and the appropriate additives required to give a total volume of 1.5 ml. The following samples were incubated in a water bath at 30 ± 1°C for 15 min: (1) ungerminated spores suspended in deionized water; (2) germinated spores suspended in 200 mM KCl and 20 mM 9.5 glycine buffer; (3) inhibited spores prepared by suspending normal spores in 200 mM KCl, 20 mM glycine, *p*H 9.5 buffer, and 25 mM NH_4Cl. All samples were prepared in triplicate.

After the incubation period, all samples were brought to a volume of 2 ml by the addition of 0.5 ml 1% sucrose solution. Sucrose was added as an internal standard to minimize analytical error during subsequent sample preparations.

An additional three samples of inhibited spores were washed twice with 15 ml of water after which they were resuspended in 1.5 ml of water and finally brought to a volume of 2 ml with 1% sucrose solution.

The 2-ml spore suspensions were crushed in a Braun homogenizer using an equal volume of glass beads (50 sec shaking time). The specimen chamber of the homogenizer was cooled with liquid CO_2. The homogenate was removed and centrifuged for 30 min at 4080*g* in a refrigerated centrifuge at 4 ± 1°C. All samples were kept on ice or frozen at −15°C until carbohydrate analysis.

An aliquot of each sample or standard was placed in a 0.2-ml vial and dried in an oven at 100–110°C. Carbohydrates were prepared for gas chromatographic (GC) analysis by silylation with an equal volume of Tri-Sil Z (trimethylsilylimidazole in dry pyridine, 1.5 mEq, Pierce Chemical Company, Rockford, IL). Each sample was heated at 70°C for a total of 45 min with a vortex treatment at 30 min incubation.

Carbohydrates were analyzed by GC on a Varian 3700 equipped with a flame ionization detector (Varian Associates, Palo Alto, CA) and a 1.8 m × 2-mm i.d. glass column packed with 3% OV-17 on 120/140 mesh Gas-Chrom (Applied Science Laboratories, State College, PA). The GC oven tempera-

ture was programmed from 100 to 250°C at 10°C/min. Qualitative and quantitative data were obtained using a Varian Vista 401 data processor. Sucrose, glucose, and trehalose standards were obtained from Calbiochem, La Jolla, California, and Sigma Chemical Company, St. Louis, Missouri. Quantitative data were collected using a sucrose internal standard, and peak identification was made by direct GC comparison with silylated carbohydrate standards.

Trehalase Assay

Germinated spore samples were prepared by incubating aliquots of spores in 100 mM KCl at *p*H 9.5 with 10 mM glycine buffer for 30 min at room temperature (23° ± 2°C). Ungerminated spores were kept in deionized water. Two- to four-ml aliquots of spore suspensions were crushed in a Braun homogenizer using an equal volume of glass beads (0.45–0.50 mm) for 1 min as described above. The homogenates were removed and centrifuged for 30 min at 4000*g* in a refrigerated centrifuge (4° ± 1°C). The pellet was suspended in an aliquot of deionized water equal to the volume of the supernatant. The resuspended pellet (bound trehalase) and the supernatant (free trehalase) were either used immediately or stored frozen in 25- to 50-μl aliquots at −15°C for a week or less until used.

In all studies, unless otherwise specified, trehalase activity was determined by incubating samples containing free or bound trehalase with 100 mM solutions of trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) dissolved in 50 mM potassium phosphate buffer, *p*H 5.5, in a total volume of 1 ml at 37°C for 30 min. The reaction was stopped by placing the samples in a boiling water bath for 5 min. After cooling, 1 ml of statzyme glucose solution (Worthington, Freehold, NJ) was added and the samples were incubated for 20 min at 37°C. Absorbency by the resulting red color was measured on a Perkin–Elmer spectrophotometer at 500 nm and was proportional to the amount of glucose in the sample. Along with each run, a reagent blank and boiled enzyme samples were assayed as controls. Boiled samples were found to have produced negligible amounts of glucose.

Under these experimental conditions, trehalase activity was directly proportional to the sample concentration in the reaction mixture and the incubation time.

For *p*H studies, 30 mM sodium citrate/HCl or NaOH buffer systems were used in the *p*H range from 2 to 6.5 and 5 mM potassium phosphate buffer in the *p*H range from 5 to 8. The effect of *p*H on trehalase activity was evaluated statistically by a quadratic regression analysis independently for each of the seven replicates (activity = (b_1) (pH) + (b_2) (pH^2) + c). The *p*H value where the slope (first derivative) of the respective quadratic equation equalled zero was used to estimate the optimum *p*H ($pH = -b_1/2\ b_2$).

To assay the effect of temperature on trehalase activity, enzyme samples from residue and supernatant fractions of ungerminated spores were allowed to catalyze trehalose at temperatures between 8° and 64°C. This test was replicated three times and the temperature optimum calculated as described for the *p*H optimum. Trehalase tolerance to heat was studied by preincubating enzyme samples mixed with buffer solutions at temperatures between 8° and 70°C for 30 min. Unheated control samples were used along with the incubated ones and the activity of the heat-treated samples was presented as a percentage of the control sample activity. The curves in Figure 3 are based on the mean of seven replicates. Probit analysis was used to estimate the temperature at which half the activity was lost.

Effect of substrate (trehalose) concentration on the enzyme activity was examined by incubating trehalase samples with concentrations of trehalose between 10 and 750 mM for 30 min. Determinations of K_m and V_{max} values were made from Lineweaver–

burke plots (Irwin, 1968) for both free and bound trehalase.

Trehalase activity in both pellet and supernatant fractions was assayed in homogenates of ungerminated and germinated spores. In a separate test, germination solutions were also added to homogenates of ungerminated spores prior to centrifugation to control for the effect of salt solutions and *p*H, unrelated to germination. Each test was replicated four times.

Ammonium chloride is known to inhibit the germination of *N. algerae* spores. Its effect upon trehalase activity was assessed by adding 20 mM NH_4Cl to the assay mixture.

Two-milliliter aliquots of spores were irradiated in a Gammacell-200 cobalt-60 irradiator with a dose rate of 2100 R/min for total dosages of 100 Kr. The activity of trehalase in homogenates of these spores was tested immediately after the radiation treatment and again 10 days later. These results are compared with trehalase activity from unirradiated spores and the percentage germination of the spores.

Statistical analyses were carried out by multiple regression analysis and probit analysis, using Statistical Analysis System (Version 79.6) from SAS Institute, Cary, North Carolina, and the Northeast Regional Data Center, University of Florida.

RESULTS

The amounts of glucose and trehalose found in the samples tested are presented in Table 1. The supernatant from crushed, normal *N. algerae* spores contained small amounts of glucose relative to the high concentrations of trehalose that were detected. The supernatants from germinated spores showed both a marked increase in glucose concentrations and a decrease in trehalose concentrations. When spore germination is inhibited by NH_4Cl, the supernatant from crushed spores had elevated levels of glucose but no significant change in trehalose level. Washing the inhibited spores to remove both KCl and NH_4Cl decreased the glucose to minute quantities.

TABLE 1
CARBOHYDRATE COMPOSITION IN NORMAL, GERMINATED, AND INHIBITED *Nosema algerae* SPORES

Treatment	Medium	Sample (μg/ml)[a]	
		Trehalose	Glucose
Normal	Water	538 ± 42	0.7 ± 0.7
Germinated	0.2 M KCl, 0.02 M glycine, *p*H 9.5	160 ± 36	109 ± 49
Inhibited	0.2 M KCl, 0.02 M glycine, *p*H 9.5, 0.025 M NH_4Cl	543 ± 42	74 ± 29
Inhibited, and then washed	Water	537 ± 35	0.2 ± 0.2

[a] Mean and standard error of three replicates.

The hemacytometer counts showed a spore concentration of 3.01×10^8 spores/ml in the test suspensions. The ovocylindrical spores measured 3.6 ± 0.04 μm in length and 2.1 ± 0.09 μm in diameter. Based upon these dimensions and the approximation of a prolate spheroid, the mean spore volume is 12.1 μm^3. The percentage of water in the spore has not yet been determined, but the minimum molar concentration of trehalose is 0.44 M, assuming the spore to be 100% water. However, if a valve of 50% is used as a maximum value for water content, then the minimum trehalose concentration would be 0.87 M in normal *N. algerae* spores.

The effects of *p*H on trehalase activity from the supernatant (free trehalase) and from the residue (bound trehalase) are shown in Fig. 1. The mean *p*H optimum (rounded to the precision of the measurements) was 5.5 ± 0.06 for the free enzyme and 5.2 ± 0.01 for the bound enzyme. The difference between the two optima is significant at the 0.001 level.

There were no significant differences between the activities of the bound and free trehalase through a temperature range of 8–64°C (Fig. 2). The optimum temperatures are 44° ± 1°C for free enzyme and 44° ± 1° C for the enzyme in the residue.

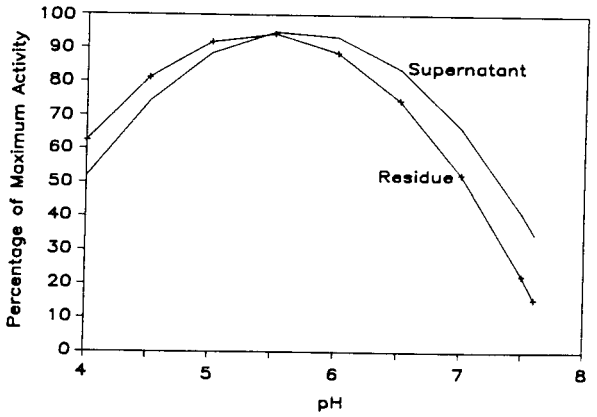

FIG. 1. Activity of trehalase in the residue(+) and supernatant fractions of *Nosema algerae* spore homogenates as a function of *p*H.

At the enzyme and substrate concentrations used in these assays, glucose production was linear with time for at least 2 hr. The 30-min assay, therefore, provided a reasonable estimate of the initial reaction rate. From Lineweaver–Burke plots of free trehalose-catalyzed reactions, a K_m of 26 ± 3 mM and a V_{max} of 23 ± 6 mM/min were obtained. For the bound enzyme K_m was 25 ± 6 mM and V_{max} was 14 ± 3 mM/min. The differences were not significant at the 0.05 level. The reaction rate reached its maximum at 200 mM trehalose, remaining nearly constant to 750 mM, the maximum concentration tested.

The effect of a 30-min incubation at temperatures between 8° and 70°C upon the subsequent activity of the enzyme is shown in Fig. 3. Bound trehalase appeared more stable than free trehalase at temperatures below 47°C, but the difference was not significant. One-half of the initial activity was lost after incubation at 35°C for the free enzyme and at 38°C for the bound enzyme. At 47°C, ca. 72% loss in the activity was noticed in both supernatant and residue samples. At higher temperatures, free and bound enzymes continued to lose activity more or less identically.

Addition of germination solution to homogenized ungerminated samples had no significant effect on either the total activity or the relative distribution of trehalase in supernatant and residue fractions (Table 2). However, the germination process significantly increased the relative activity of the supernatant.

Addition of KCl (a germination stimulus) did not enhance and ammonium chloride (NH_4^+) did not reduce the activity of either the bound or the soluble trehalase in the *p*H 5.5 assay system (Table 3). Trehalase activity was so low above *p*H 8 that inhibition by NH_3 could not be tested.

Trehalase activity in extracts and residues were unchanged by 1000-Kr γ radiation immediately before extraction (Table 4). Ten days later, germination had declined to only 15% in the irradiated spores

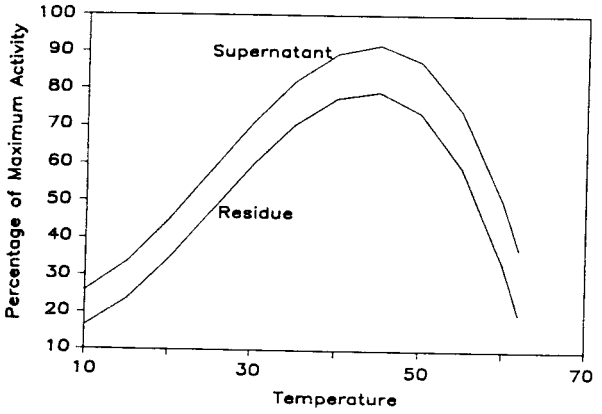

FIG. 2. Activity of trehalase in the residue and supernatant fractions of *Nosema algerae* spore homogenates as a function of temperature.

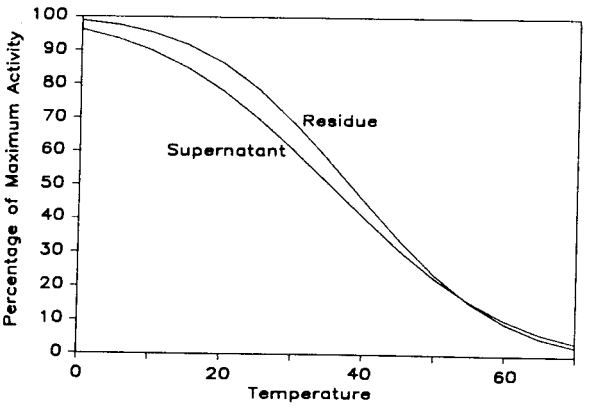

FIG. 3. Activity of trehalase in the residue and supernatant fractions of *Nosema algerae* spore homogenates after 30-min heat treatments.

TABLE 2
EFFECT OF SPORE GERMINATION ON ENZYME ACTIVITY IN SUPERNATANT AND RESIDUE FRACTIONS

Homogenates of	Optical density units (S + P)	Optical density units S:P Ratio
Ungerminated spores	2.5 ± 0.6 A	0.03 ± 0.005 A
Ungerminated spores + 100 mM KCl, *p*H 9.5, 10 mM glycine	1.7 ± 0.4 AB	0.13 ± 0.04 A
Spores germinated in 100 mM KCl, *p*H 9.5, 10 mM glycine	0.9 ± 0.2 B	0.5 ± 0.1 B

Note. S, supernatant; P, pellet. Values by the same letter in each column are not significantly different at the 0.05 level.

without significant change in trehalase activity or ratios between the bound and the free enzyme.

DISCUSSION

Like *N. apis* (see Wood et al., 1970), *N. algerae* spore extracts contain large amounts of trehalose (Undeen et al., 1983). As only the percentage concentration was presented by Wood et al., no comparison of intrasporal trehalose concentrations can be made. Small amounts of glucose were found in both *N. apis* and *N. algerae* but the larger quantities of glucitol and the hepitol reported by Wood et al. were not present in the *N. algerae* spore extracts. Other carbohydrates must surely exist, at least as intermediates in many metabolic pathways but at quantities too small to be detected in this study. The nonadditive nature of the results shown in Table 1 indicates that the major end products of trehalose metabolism are not detected by the techniques used or that some sugars are sequestered onto membranes and remain in the residue rather than in the supernatant.

N. algerae spores have extremely high internal pressures (Undeen, 1978). The spore content was found to be ca. 2.0 OSM by determining the concentration of sucrose required to prevent germination (Lom and Vavra, 1963; Undeen, 1978). Trehalose constitutes a significant portion of the osmoticum.

Germination begins 1–5 min after application of the stimulus, during which time the carbohydrate molar concentration could have increased by degradation of trehalose into a greater number of smaller molecules. This sudden increase in osmolarity could be responsible for a pulse of increased intrasporal pressure to initiate spore germination as suggested by Undeen (1978). A short-term increase in carbohydrate concentration has not, however, been directly observed.

It is interesting that almost as much glucose was found in supernatants from crushed inhibited spores as from the germinated spores. Washing the inhibited spores in water before crushing them eliminates the glucose, indicating that it was outside the spores, most likely from the few spores which might have germinated.

TABLE 3
THE EFFECT OF NH_4Cl ON *Nosema algerae* TREHALASE ACTIVITY AT *p*H 5.5 *IN VITRO*

	Optical density units with indicated assay conditions			
	Normal	+ 100 mM KCl	+ 10 mM NH_4Cl	Tris buffer, *p*H 8.0
Supernatant	0.354 ± 0.250	0.482 ± 0.077	0.467 ± 0.079	0.024 ± 0.011
Residue	0.572 ± 0.064	0.682 ± 0.232	0.623 ± 0.177	0.026 ± 0.009
Whole	0.320 ± 0.076	0.338 ± 0.064	0.323 ± 0.044	0.010 ± 0.000

TABLE 4
THE EFFECT OF γ IRRADIATION OF *Nosema algerae* SPORES ON THE ACTIVITY OF TREHALASE IN THE SPORE HOMOGENATES

	Optical density units at indicated time postexposure			
	0 Kr		1000 Kr	
	Day 0	Day 10	Day 0	Day 10
Supernatant	0.165 ± 0.002	0.252 ± 0.037	0.178 ± 0.003	0.319 ± 0.012
Residue	1.39 ± 0.02	1.33 ± 0.16	1.95 ± 0.05	1.52 ± 0.14
% Germination	94	89	93	15

However, based on the amount of glucose found in germinated spore supernatant, much less glucose would be expected from minor germination in the inhibited spore samples. Interestingly, the material balance of trehalose and glucose gained in germinated spores indicates that further glucose catabolism occurs during normal spore germination. In the case of ammonium chloride inhibition, the breakdown of glucose may be blocked, thus creating abnormally high levels of glucose.

Trehalose metabolism is an important first step in the germination of many fungal spores and is initiated by two classes of trehalase, one regulatory and the other nonregulatory (Thevelein, 1984). Germination processes in fungal spores are slower and not analogous to microsporidia spores, however, and trehalose metabolism is unlikely to play the same role in germination. In *N. algerae* the entire process of germination is completed in 1–5 min while the trehalose loss in germinating fungal spores is not complete until after 1 hr or more (Thevelein et al., 1983). Microsporidia spores appear to have all the structures necessary for germination and the process itself involves the deformation of existing structures rather than the synthesis of new ones.

The similarities between the free and the bound *N. algerae* trehalase suggests that they are the same enzyme. The slightly lower *p*H optimum for the bound enzyme might be due to the presence of spore membranes to which some enzyme molecules are bound. More analytical studies and perhaps electrophoretic studies would be required to prove the identity of the enzymes in the two fractions.

Since trehalase is the first enzyme in the trehalose degradation pathway, it is a likely target for the germination stimulus. These results show the total trehalase activity to be lower in the germinated than in the ungerminated spore preparations. The other significant difference between enzyme levels in germinated and ungerminated spores is the distribution between the bound and the soluble fractions, suggesting that the stimulus might somehow act on membranes, causing release of enzyme molecules previously bound and either inactive or isolated from the substrate. The relative increase in soluble enzyme is greater in the germinated spores than in samples to which the germination stimulus was added after grinding, demonstrating that the process of release occurs inside the intact, germinating spore and is not entirely the action of the buffered salts on the homogenate.

The *p*H optimum of 5.5 reported here is more acidic than the optimum of *p*H 7 for trehalase from *Nosema apis* (see Vandermeer and Gochnauer, 1971) and similar to the *p*H optimum of 5.5–6.5 reported for trehalase from *Culex quinquefasciatus* (see Giebel and Domnas, 1976) and a mosquito-parasitizing fungus, *Lagenidium* sp. (see McInnis and Domnas, 1973). Optimal *p*H

for *N. algerae* spore germination is near the *p*H maximum in the mosquito larval midgut (Undeen, 1976) and considerably more alkaline than the optimum for trehalase activity. This indicates that a steep *p*H gradient is maintained across the spore membranes and that an internal *p*H shift is unlikely to be the germination stimulus.

Trehalase was not inhibited by NH_4Cl in vitro at the enzyme's *p*H optimum of 5.5. At the germination *p*H optimum of 9.5, NH_3 predominates over NH_4^+ and, as an uncharged molecule, would enter the spore more easily than would NH_4^+. If the spore cytoplasmic *p*H were near 7, NH_3 would protonate to NH_4^+ after entering. This could cause an increase in *p*H to a level above which many enzymes, including trehalase, would function, thereby indirectly inhibiting germination and trehalose metabolism. Tests for the in vitro effect of NH_3 on trehalase are precluded because the *p*H required is too high for trehalase activity.

Trehalose levels and percentage germination decline as a result of γ radiation (Undeen et al., 1983). The decrease in trehalose after irradiation does not seem to be in response either to a change in trehalase activity or to a shift in the ratio of soluble to bound trehalase. Radiation might cause a gradual decline in trehalose levels through membrane damage, which results in contact between enzyme and substrate.

In conclusion, trehalose metabolism appears to be involved in the germination process. Other than the relative increase of enzyme in the soluble fraction of the homogenates from germinated spores, there is no evidence of a germination-regulatory role for trehalase. The germination stimulus must act independent of trehalase, perhaps indirectly resulting in its release or activation in a later step.

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