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Conversion of Intrasporal Trehalose into Reducing Sugars During Germination of *Nosema algerae* (Protista: Microspora) Spores: A Quantitative Study

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ABSTRACT. Carbohydrates were extracted from dormant, stimulated and germinated spores of *Nosema algerae*. Concentrations of total sugars were measured by the Anthrone test. Non-reducing sugars were quantified by NaOH hydrolysis followed by the Anthrone reaction, and reducing sugars by the Nelson's test. Glucose was measured by the *o*-toluidine test and a glucose oxidase assay. The concentrations of trehalose in the cytoplasm of the dormant, ungerminated spore was estimated to be in excess of 1.0 M. Trehalose decreased by 70% during the five-minute course of germination. All of the lost trehalose was converted to reducing sugar of which 70–78% was glucose. The osmotic potential increase caused by catabolism of trehalose appears to be sufficient for germination.

Supplementary key words. Carbohydrates, glucose, Microsporida, osmotic potential.

MICROSPORIDIA (phylum Microspora) are intracellular, obligate, parasitic protists characterized by spores containing an internally coiled polar filament. After ingestion by a host organism, a set of stimuli within the gut activates a series of events terminating in the rapid eversion of the polar tube [8]. The polar tube penetrates the host gut barriers and the infective sporoplasm is injected through the tube and into the host tissues. Development of this parasite is entirely intracellular, culminating in the formation of spores.

Monovalent ions stimulate the germination of *Nosema algerae* spores, setting into motion a series of events that lead to increased hydrostatic pressure within the spore [6, 8, 14]. In an alkaline solution of 0.1 M NaCl at 30° C stimulation occurs in 10–60 s, after which germination in the group of spores ensues for ca. four min [10–12]. Discharge of the polar tube and sporoplasm takes only two s [5]. The disaccharide trehalose is abundant in microsporidian spores and is thought to play a role in germination [14, 17]. Microsporidian spores contain the enzyme trehalose, which appears to be activated or released during germination [13, 17]. In the spores of *N. algerae*, trehalose is rapidly depleted during germination [13, 16]. Gas chromatographic measurements of sugar concentrations before and after germination indicated that no more than 20% of the original quantity of trehalose was converted to glucose during germination while 30% of it was still recoverable as trehalose. This left about 50% of the trehalose unaccounted for [13, 16].

This research describes the timing and stoichiometry of trehalose catabolism during germination. Specifically, measurements are made of the quantity of trehalose lost and reducing sugar gained. The resultant change in osmotic potential is calculated and compared with changes predicted by the chemiosmotic hypothesis of spore germination.

MATERIALS AND METHODS

Spore germination and extraction. Spores of *N. algerae* were mass-produced in *Helicoverpa zea* [1], harvested from the adults and purified by continuous flow Ludox density gradient centrifugation [9] and stored at 5 ± 2° C. Immediately before use the spores were washed and resuspended in deionized water at 2×10^9 spores/ml. Spores were germinated in a solution of 0.1 M NaCl buffered to a pH of 9.5 with 0.02 M glycine-NaOH buffer at 30 ± 0.1° C in a circulating water bath for up to 15 min. These procedures were performed in 10 × 75-mm borosilicate culture tubes with a total volume of 200 µl, including 50–200 µl of the spore suspension. Germinated and ungerminated spores were disrupted in the culture tubes by adding 200 µl of 0.45-mm glass beads (B. Braun Biotech, Inc., Allentown, PA) and shaking for one min at the highest speed on a vortex mixer (SP S8220). The homogenate (ca. 100 µl) was withdrawn from the beads. The beads were washed with an additional 100 µl deionized water, which was withdrawn and combined with the homogenate in a 1.5-ml Eppendorf tube (Eppendorf North America Inc., Madison, WI). Further enzymatic activity was prevented by placing the samples in boiling water for 5 min. Each was centrifuged at high speed (Eppendorf, model #5415) for 5 min. The supernatant, hereafter called the spore extract, was removed and stored at −30° C for future sugar analysis.

Complex carbohydrates. Tests were conducted to determine the presence and relative abundance of polysaccharides such as glycogen in *N. algerae* spores. Saturated sodium sulfate was added (2%) in 80% methanol to precipitate complex carbohydrates from a suspension of crushed spores. Total carbohydrate content was determined in both the methanol fractions and residue fractions with the Anthrone test. The same test was also applied to the spore extract for assurance that simple sugars predominated in the extracts used for the rest of the study.

Residue sugars. Residues from crushed spores, after centrifugation to obtain the spore extract, were subjected to an additional extraction with water. Both the extract and the residues were tested for total carbohydrates in order to measure and correct for the sugars that remained in the residues after extraction.

Sugar changes during germination. The quantities of total sugar, non-reducing sugar, reducing sugar and glucose in the extracts from germinated and ungerminated spores were measured. Sugars were also measured at close intervals while spores were germinating. For this procedure, the spores were combined with the glass beads, germination solution added and the process of germination stopped at intervals of zero, one, two, three, four and six min. Germination was terminated by removing a tube from the 30° C water bath and immediately grinding the spores.

Reagents. The anthrone reagent for determination of total carbohydrates was prepared by dissolving 750 mg of anthrone in 530 ml of 70% sulfuric acid [19] and stored at 5 ± 3° C. The strong acid hydrolyses the glycosidic bonds and the resulting monosaccharides react with anthrone, producing a blue-green color. The Nelson's test is specific for carbohydrates with free reducing groups; reagents were prepared according to Clark [3]. Glucose was measured with a Statzyme glucose® kit (Worthington Biochemical Corp., Freehold, NJ) and *o*-toluidine reagent. The kit contained glucose oxidase, highly specific for glucose, and was used with modifications described by Undeen et al. [12]. The *o*-toluidine reagent (6% (v/v) *o*-toluidine in glacial acetic acid) was obtained commercially (Sigma Chemical Co., St. Louis, MO) and used as described by Van Handel [18], who

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obtained identical results with both glucose oxidase and *o*-toluidine in glucose assays in serum. The quantity of carbohydrate present in each test sample was estimated by comparison with a standard curve obtained from a two-fold dilution series of known amounts of glucose standards (6–200 μg) run at the same time. The minimum sensitivity of all the colorimetric tests was considered to be about 6 μg sugar.

Total carbohydrate. Five-ml aliquots of anthrone reagent were added to each aliquot of spore extract and incubated at 90 ± 0.1° C for 17 min in a circulating water bath. The tubes were cooled and the optical density (OD) was measured on a spectrophotometer (Bausch & Lamb Spec 20) set at a wavelength of 625 nm.

Non-reducing sugars. Reducing sugars were removed from spore extract samples by incubating them for 7 min at 90° C with 1.5 × their volume of 1.0 M NaOH [19]. The samples were then tested as described above for total carbohydrate.

Reducing sugars. Nelson's reagent was prepared just before use by combining 0.5 ml of Nelson's B with 12.5 ml of Nelson's A [3]. Aliquots of the spore homogenates and standard glucose solution were brought to a volume of 1 ml with H_2O and combined with 1 ml of Nelson's reagent in 15 × 100-mm culture tubes. This solution was incubated for 20 min in a boiling water bath, cooled and then 1 ml of the arsenomolybdate reagent was added. After five min, 7 ml of water was added with occasional agitation, and the OD was measured at 540 nm.

Glucose. The glucose content was assayed by adding 1 ml of the Statzyme Glucose® kit reagent to the sample and incubating it for 20 min at 37° C. Optical density was measured at 500 nm and compared with a standard glucose curve obtained with standards run concurrently [13]. In a separate experiment, glucose was measured by the *o*-toluidine test. Glucose standards and spore extracts were placed in test tubes, adjusted to 50 μl volume with water, and reacted with2 ml of *o*-toluidine reagent (Sigma) for 10 min at 100° C; the tubes were cooled and the OD was read at 630 nm.

Calculation of osmotic potential. The volume of the spore was calculated assuming its shape to be that of a prolate spheroid [14]. Volume of the cytoplasm was calculated from dimensions obtained by subtracting 2 × the thickness (ca. 0.19 μm [15]) of the spore wall from the dimensions of the entire spore. Concentrative properties for reducing sugar were obtained from the table for glucose and trehalose values were estimated from tabulated values for sucrose [20]. Calculations of osmotic potential, in atmospheres, were by the equation: Pressure = mRT, m = molal concentration, $R = 0.0821$ (ideal gas constant in units of L atm/K mol for pressure in atm) and T = temperature in degrees Kelvin [7].

RESULTS

Complex carbohydrates. Tests for the presence and relative abundance of polysaccharides in crushed spores showed that only 12.7 ± 0.7% (n = 7) of the total carbohydrate could be classified as complex carbohydrates. The remaining carbohydrates (87.3 ± 1.0%) were methanol-soluble, simple sugars. The same test, applied to the spore extracts as used in the rest of this study, showed only 3.0 ± 0.6% of total sugars to be complex carbohydrates (n = 3), thus 97 ± 1.6% of the extract sugars were simple sugars.

Residue sugars. The residues of crushed spores remaining after the spore extract was removed contained 16.6 ± 1.3% (n = 2) of the total carbohydrate from the spores. The residue carbohydrates were composed of complex and simple carbohydrates that represented 9.8% and 6.8% respectively of the total carbohydrate.

Spore extract sugar. Glucose and trehalose controls were unaffected by experimental conditions imposed on the spores prior to the various sugar assays (Table 1). The Anthrone test yielded the same results with equal quantities of glucose and trehalose. The Nelson's test failed to detect any reducing sugar in samples of trehalose. The disruption and extraction procedure used to obtain the spore extract did not significantly affect the quantitative results for glucose standards, thus all standards were run without sham extractions.

The 7-min NaOH treatment had little effect on the control trehalose and did not change the amount of anthrone-reactive sugar in ungerminated spore extracts. The NaOH treatment eliminated reducing sugar in the glucose control (Table 1) and reduced its level in germinated spore extracts (Tables 1, 2).

The amount of reducing sugar present after germination was equal to the quantity of trehalose lost during germination (Table 2). Likewise, the sum of the reducing and non-reducing sugar after germination was equal to the quantity of total sugar found in ungerminated spores (Table 2).

Both the Statzyme glucose test and the *o*-toluidine tests revealed that most, but not all, of the reducing sugar was glucose (69.8% and 77.7% respectively) (Table 2). The conversion of trehalose to glucose began one–two min post-stimulation and was complete after four–six min (Table 3).

DISCUSSION

Increased hydrostatic pressure is thought to be responsible for microsporidian spore germination. This pressure has been ascribed to increased permeability to water [6] or increased solute concentration. Dall [4] suggested that ion concentrations were increased by proton exchange across the spore wall in an alkaline environment, thereby increasing osmotic potential to the extent necessary for germination. Alternatively, the solute concentration, and osmotic potential, might be increased endogenously by the cleavage of larger molecules into smaller ones [8]. Processes other than increased pressure might also be involved in the initiation of germination. Structural weakening of the polar cap region or "liquefaction" of the cytoplasm are two processes that might obviate or reduce the requirement for increased pressure.

The loss of the disaccharide trehalose during the rapid germination of *N. algerae* spores [13, 16] led to the hypothesis that internal pressure increased due to increased solute concentration from the cleavage of trehalose into smaller molecules [8]. In measurements of sugars by gas chromatography, less than 20% of the trehalose lost during germination was recovered as glucose [13, 16]. We thought that the glucose, in turn, was catabolized further. Until the products of trehalose degradation were quantitatively identified, the extent and functional significance of solute concentrations, and osmotic pressure change remained open.

The primary supernatant (spore extract) contained about 93% of the simple sugar extractable from the crushed spores. The concentration of sugars in the spores—a mean of all tests for spore extracted sugars—was 65 μg/3.75 × 10^7 spores. Considering the volume of the *N. algerae* spore to be 10.9 μm^3 [14], the concentration of trehalose is 0.45 M. This value is consistent with concentration reported in previous studies [13, 16]. Taken together, the results of all five chemical tests for sugars show that, before germination, almost all of the sugar is non-reducing, probably trehalose. Cleavage of the glycocytic bond between the two glucose molecules of trehalose is expected to yield two glucose molecules that would be detected as reducing sugars by the Nelson's test. In contrast with previous work [13, 16], glucose accounts for most (70–80%) of the trehalose that was lost. The

Table 1. Measurements of anthrone reactivity of sugars from *N. algerae* spore extracts before and after germination. Reaction with NaOH before the anthrone test was to eliminate any reducing sugar. Trehalose and glucose were controls for the assay conditions.

Test: / Sugar:	N[a]	Anthrone test Total sugar[b]	NaOH-anthrone Trehalose
Ungerminated spores	5	60.1 ± 6.3	65.8 ± 9.6
Germinated spores	5	60.4 ± 6.3	8.8 ± 3.7
Controls			
100 μg trehalose[c]	1	100.4	95.6
100 μg glucose[c]	1	105.2	0

[a] Number of individual assays.
[b] Units are μg/3.75 × 10^7 spores ± SE.
[c] Sugars as controls for assay conditions.

Table 2. Total carbohydrates, non-reducing sugar, reducing sugar and glucose in extracts of *N. algerae* spores before and after germination. Spores were germinated in 0.1 M NaCl with 0.02 M, pH 9.5, glycine-NaOH buffer for 10 min at 30° C.

Sugar	N[a]	Ungerminated spore	Germinated spore
		Experiment #1	
Total	6	73 3 ± 4 2[b]	72.0 ± 1.7
Non-reducing	6	67.3 ± 5.2	19.0 ± 3.7
Reducing	6	5.1 ± 0	56.5 ± 2.2
Glucose[c]	6	3.6 ± 5.3	39.5 ± 2.8
		Experiment #2	
Total	9	64.3 ± 1.9	6.74 ± 1.9
Non-reducing	9	61.2 ± 1.7	21.3 ± 0.7
Reducing	9	3.2 ± 1.8	47.2 ± 5.2
Glucose[d]	10	1.6 ± 1.6	35.8 ± 3.2

[a] Number of individual assays.
[b] μg glucose/3.75 × 10^7 spores ± SE.
[c] Glucose oxidase test.
[d] *o*-toluidine test.

rest of the sugars are also reducing sugars, perhaps isomers of glucose, such as fructose or mannose. Thus, the sum of the non-reducing and reducing sugar after germination was equal to the sum of these two categories before germination, accounting for all of the post-germination sugar. The other reducing sugars have not yet been identified. We have no explanation for the low and variable levels of reducing sugars detected by gas chromotography. The fact that all of the trehalose lost during germination reappeared as reducing sugar indicates that the sugar is not part of a biochemical energy-producing pathway.

The conversion of trehalose to glucose is catabolized by the enzyme trehalase, known to occur in microsporidian spores [13, 17]. Overall trehalase in a suspension of crushed germinated spores appears to be less active than the enzyme from similarly treated ungerminated spores. Trehalase activity was also measured in both the residue and supernatant fractions of germinated and ungerminated spores. After germination a significantly greater fraction of trehalase activity was found in the supernatant [13]. This observation supports the hypothesis that trehalase is bound or compartmented to isolate it from trehalose and that the stimulus leads to the release of trehalase.

Germination has long been assumed to be caused by increased pressure [4, 6, 8]. Such an increase was demonstrated for *N. algerae* [14]. The experiments described here are the first to quantitatively determine the products of trehalose catabolism, thus providing the means for estimating the increase in osmotic potential.

The conversion of trehalose to reducing sugar was concurrent with the 5-min duration of spore germination at the same ionic, pH and temperature conditions used previously [10–12]. Table 2 reveals that no changes in sugar occurred the first minute, before any spores begin to germinate. The first minute after introduction of the stimulant has been shown to represent the period of stimulation, during which the stimulatory ions are thought to enter the spore [11, 12]. After stimulation *N. algerae* spores continue the process of germination in absence of the stimulus [11, 12, 14]. No further changes in sugars occurred beyond the time that all spores had completed germination. The changes in sugars closely match the germination curves obtained spectrophotometrically [10–12]. Thus, the osmotic potential increase from trehalose degradation occurs during the period predicted by the chemi-osmotic hypothesis of spore germination [8, 14].

An increase in intrasporal solutes should increase the internal hydrostatic pressure. Once the anterior spore wall (polar cap) ruptures and the polar tube begins to emerge, the contraction of the elastic spore coat and the osmotic movement of water into the spore should be sufficient to expel the contents of the spore, without any further increase in pressure. This expulsion phase of germination lasts only two s, a small fraction of the one–four-min lag time between stimulation and expulsion, during which all of the sugar activity occurs. Three factors must be considered before arriving at an estimate of the trehalose concentration within the spore and the pressure induced when trehalose is cleaved to glucose. 1) The osmotically active materials should be within the plasmalemma, which lies against the inner face of the spore wall. Most likely, it is in or near the posterior vacuole, so that it would emerge last from the polar tube. 2) The entire volume of the cytoplasm is not "solvent volume." 3) Other solutes contribute to the osmotic potential within the spore.

The last two factors complicate estimating the osmotic potential of dormant spores. However, having expelled the polar tube, polaroplast, cytoplasm and nuclei, the spore case appears almost empty after germination. Therefore, calculation of the solute concentration within the empty spore case is subject to less uncertainty. The spore case is smaller [14] and its internal osmotic potential is at least equal to the osmotic potential of the solution in which it germinated. This is demonstrated when spores germinate in solutions of polyethylene glycol (25–50 Atm); the empty cases are initially turgid and then quickly become flat as the internal solutes and water are lost.

The total volume of an empty spore case was estimated to be 9.26 μm^3 [14]. This value, minus the spore wall volume of 3.51 μm^3 (calculated from a mean spore wall thickness of 0.190 μm

Table 3. The dynamics of conversion of non-reducing sugar, trehalose, to glucose within spores of *N. algerae* during germination in a solution of 0.1 M NaCl buffered to pH 9.5 with 0.02 M Glycine-NaOH.

Minutes after	Sugars in spore extracts		
	Total sugar	Non-reducing	Glucose
0[a]	63.9 ± 3.4[b]	63.2 ± 3.8	0
1	74.4 ± 16.9	63.2 ± 1.0	0
2	68.2 ± 9.5	48.9 ± 10.1	9.9 ± 5.2
3	63.9 ± 3.4	19.2 ± 5.1	33.6 ± 1.0
4	64.2 ± 5.3	14.7 ± 3.1	39.0 ± 1.2
6	63.2 ± 3.8	10.2 ± 4.5	41.0 ± 2.8

[a] Time = 0, germination solution was added after the spores were ground; all others, time includes the one-min grinding time.
[b] Units are μg glucose/3.75 × 10^7 spores ± SE, mean of 3 replicates.

[15]), yields an inner volume of 5.75 μm^3. The concentration of sugar in germinated spores of this volume would be about 1.51 M. The midpoint of the sucrose concentration range through which *N. algerae* spore germination is osmotically inhibited is about 1.5 M [14]. Even assuming volume to be precisely estimated, the concentration of sugar is still not adequately defined. The residue from the spore extract contains an additional 6.8% extractable sugar, increasing the concentration to 1.63 M. Furthermore, the empty spore case is not exactly empty; it contains a diffuse substance in the center with a wide layer of denser material peripherally, next to the plasmalemma [2]. If this were to reduce the water content of the spore by only 5%, the concentration of sugars would rise to 1.7 M. Consequently, the concentration of osmotically active sugars appears to be well within the range required for germination.

Electron micrographs of dormant ungerminated microsporidian spores show a dense cytoplasm. These solids subtract from the volume available to intrasporal water. Apart from that which the membranes and colloids add to the osmotic potential of the spore (matric potential), there are also unknown quantities of solutes, other than sugars, which further add to the osmotic potential. These components, added to the large quantities of trehalose, could confer upon the spores an extremely high turgor pressure. When the concentration of solutes is increased by cleavage of each trehalose molecule into two molecules of glucose, the pressure increase might be sufficient to rupture the polar cap, initiating the expulsion phase of germination even against the high concentrations of sucrose or polyethylene glycol observed in other reports [6, 14].

Assuming values for cytoplasmic solvent space and the concentration of other solutes, estimates of osmotic potentials can be made. For example: postulate the same spore wall volume as for the germinated spore case, 65 $\mu g/3.75 \times 10^7$ spores, that 70% of the cytoplasmic volume is water, and that the concentration of all other solutes is 0.1 M. The cytoplasmic volume of a 10.9 μm^3 *N. algerae* spore would be 7.39 μm^3 and the concentration of osmotically active solutes would be about 1.2 M, increasing to 1.9 M when the spores germinate. These concentrations, without considering matric potential [7], would give the spore a turgor pressure of about 37 atm; it would increase to 64 atm after the cleavage of trehalose as described here. This system is highly sensitive to solvent concentration. A decrease of the available solvent to 60% raises the turgor pressure to 54 and the germination pressure to 79 atm. Spores that have a lower water content because of shrinkage in hypersomotic solutions [14] would have higher initial osmotic potential changes. Osmotic potential should decrease as the spore regains its normal size.

These results demonstrate that trehalose is quantitatively cleaved to glucose and other reducing sugars concurrent with the time that spores are germinating. Also, the rapid cleavage of trehalose with the accompanying hydrostatic pressure increase is great enough to provide a plausible explanation for spore germination. Further information now needs to be obtained about other factors that influence osmotic potential, such as the water content of spores.

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