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The Effect of Ultraviolet Radiation on the Germination of *Nosema algerae* Vávra and Undeen (Microsporida: Nosematidae) Spores[1]

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ABSTRACT. Spores of *Nosema algerae* Vávra and Undeen were subjected to various dosages of 254 nm ultraviolet radiation (UV). Very high dosages of UV were required to block germination. Germination was normal immediately after UV dosages of 0.2 to 1.0 J/cm², followed by a delayed effect in which both percentage germination and the intrasporal concentration of trehalose decreased with time after UV exposure. Although a few spores were germinated, most of them were inactivated (rendered temporarily unable to germinate) by exposure to UV of 1.1 J/cm². Ultraviolet radiation between 1.1 and 3.4 J/cm² stimulated spores to germinate. However, spores were completely unable to germinate immediately after exposure to dosages above 3.8 J/cm². Ammonia had little effect on stimulation by UV but was inhibitory to germination after stimulation had occurred. These results demonstrate that UV behaves like a germination stimulus and are discussed in terms of the hypothesis that germination is initiated by the breakdown of barriers between trehalose and trehalase.

Key words. Effect of UV on Protozoa, germination of Microspora, Microspora.

MICROSPORIDIA are parasitic protozoa (phylum Microspora) characterized by spores with an internally coiled polar filament [7]. Most of these spores are environmentally resistant, residing in a dry, terrestrial environment or in water of various and often varying concentrations of salt and other solutes. Upon ingestion by a potential host organism, the spore encounters some unique property of the new environment within the gut which is recognized as a germination stimulus. The stimulus causes a response in the spore resulting in the eversion of its polar filament. As it emerges, the polar filament penetrates the gut wall of the host, forming a tube through which the infective sporoplasm is injected into the host tissues [18]. The stimulus might be simply water, as is the case with *Nosema whitei* Weiser, a parasite of flour beetles occupying a desiccated environment [5]. Spores of the genera *Amblyospora* or *Parathelohania,* from the aquatic stages of mosquitoes, have more complex stimuli which we are still unable to duplicate in vitro [12]. Although the stimuli are necessarily quite diverse among species, depending upon the host and the external environment, the mechanism by which the rapid germination process is accomplished is probably common to all species.

Since the germination mechanism is highly resistant to gamma radiation, microsporidian spore germination apparently does not involve nuclear function [17]. While the spores were unable to infect their mosquito hosts after receiving dosages of gamma radiation above 50 kR, the germination rate remained normal up to 1,000 kR. Above 300 kR germination rates declined with time after irradiation in direct proportion to the radiation dosage [17]. Trehalose is abundant in spores of *Nosema algerae* Vávra and Undeen and is rapidly lost during germination [16]. Trehalose levels within the spores also decreased after spore irradiation, which appears to be responsible for the reduced ability of the spores to germinate [17].

The ions which stimulate *N. algerae* spore germination might act by initiating the metabolism of trehalose. I proposed [10] that stimulation breaks down the compartmentation of trehalose and trehalase, leading to the catabolism of trehalose into a greater number of smaller molecules and an increased intrasporal pressure. Gamma radiation probably damages membranes, causing partial decompartmentation and slow degradation of trehalose [17].

The viability of microsporidian spores, as measured by their ability to infect mosquito larvae, was also destroyed by ultraviolet radiation (UV) [4]. However, the effects of UV on spore germination were never examined. If UV has the same effect on germination as gamma radiation, and if the decompartmentation hypothesis is correct, then spores treated with UV should appear as though they were subjected to a germination stimulus. If UV acts as a stimulus, certain hypotheses can be tested. (1) Germination would be induced by UV irradiation. (2) Lower doses might cause inactivation, analogous to a low level of stimulation [8, 9, 13]. (3) There should be a gradual loss of trehalose after irradiation with appropriate dosages of UV.

This study examines the effect of UV on the germination of *N. algerae* spores, compares it with the effect of gamma radiation, and tests the above hypotheses.

MATERIALS AND METHODS

Spore production. *Nosema algerae* spores were produced in *Heliothis zea* (Boddie) [1]. Infected adult *H. zea* were triturated in a blender and coarsely filtered to remove large debris. The filtrate was processed through a continuous flow centrifuge into Ludox density gradients [11]. Spores from the gradients were rinsed 3 times and stored in deionized water at 5 ± 2° C.

Ultraviolet radiation treatment. Spores were diluted to a concentration of 5×10^7 spores/ml, in water and 1 ml of this suspension was placed on the bottom of an inverted 35-mm plastic petri dish, producing a uniform 1-mm-deep layer of spore suspension. The petri dishes were placed 12 cm beneath an ultraviolet source producing radiation predominantly of 254 nm (General Electric, G15 T8, 15 Watt). The intensity, measured with an Eppley thermopile at 12 cm distance and a nanovoltmeter (Keithley Instruments), was 3,175 μW/cm². The temperature of the spore suspension was measured with a thermistor (BAT-12, Bailey Instrument Co.) after 20 min UV exposure.

Measurement of percentage germination. Throughout this study the spores were stimulated in a standard germination solution (GS) of 0.1 M NaCl with 0.02 M, pH 9.5 Glycine-NaOH buffer in a circulation water bath at 30 ± 0.1° C. Percentage germination was obtained from differential counts of 200 spores. At least 1 h later the germination tubes were shaken, and a 20-μl sample from each was placed on a slide under a coverslip. Counts were made under phase contrast microscopy at ×400 magnification, scoring all spores in each field. Ungerminated spores appeared white and refringent, whereas germinated ones were black, generally with the polar filament still attached.

Direct UV induction of germination was quantified by irradiation of spores for selected periods and taking germination counts after 1 h of incubation in water at 21 or 30° C (two

[1] Mention of a commercial or proprietary product in this paper does not constitute an endorsement of this product by the United States Department of Agriculture.

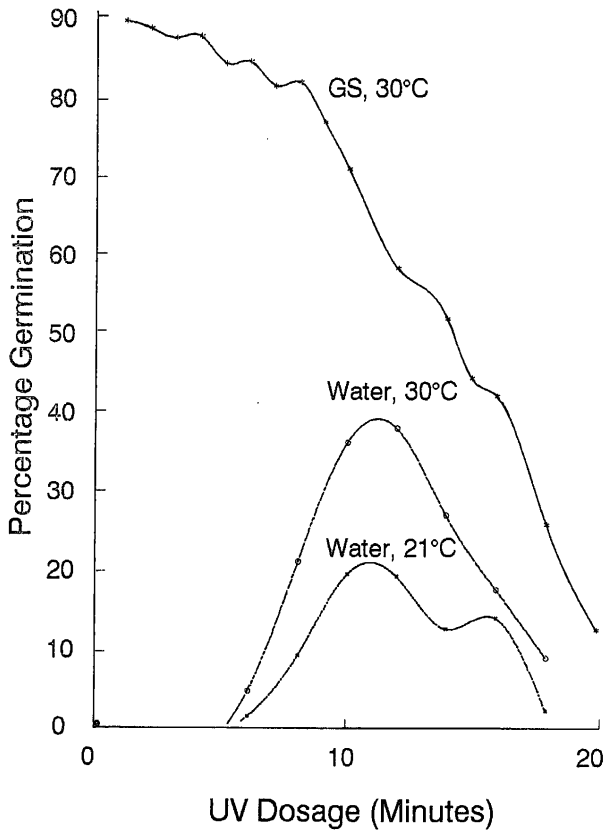

Fig. 1. The effect of UV dosage on the percentage germination of *Nosema algerae* spores. Germination in GS, 2 h after UV irradiation (GS, 30° C), germination in water at 30° C (water, 30° C), germination in water at 21° C (water, 21° C).

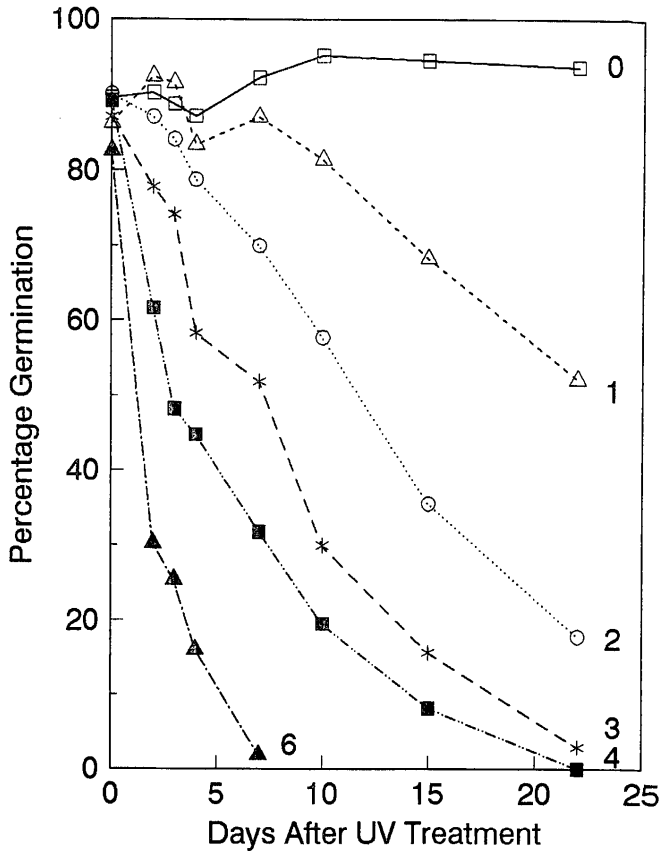

Fig. 2. The decrease in percentage germination of *Nosema algerae* spores with storage time in 5° C after UV treatment. UV dosages, in minutes, as marked on each line.

replicates at each temperature). Spores also were subjected to timed UV exposures and then transferred to GS to determine the effect of UV on GS stimulated germination (three replicates). To determine whether the delayed effect found with gamma radiation [16] is characteristic also for UV, spores were treated with 1- to 6-min UV exposures and then tested for germination in GS over a period of several days.

Stimulation time. To examine the influence of UV on the stimulation time (the duration of exposure to the stimulus necessary for the spores to be irrevocably committed to germination), spores were irradiated 4, 4.7, or 6 min and brought to 30 ± 1° C in a circulation water bath. The spores were then combined with GS and added to 1-ml deionized water in 10μl aliquots at 10-sec intervals. The deionized water had the effect of removing the stimulus by dilution. Percent germination was scored following further incubation of 1 h or more after removal of the stimulus.

Ammonia and alkali inhibition. Ammonia inhibits the germination of spores in GS [15]. To determine whether the germination of spores by UV is inhibited during the process of stimulation by UV or during the incubation phase after UV treatment, the following procedure was used. Spores in water; 0.01 M NH_4Cl; 0.02 M glycine-NaOH buffer, pH 9.5; or solution of pH 9.5, 0.02 M glycine-NaOH buffer plus 0.01 M NH_4Cl (predominantly NH_3 at pH 9.5) were treated with UV for 10 min. Immediately after the UV treatment, the spores from each treatment group were transferred to water; 0.01 M, pH 5.5 phthalate-NaOH buffer (to assure that the ammonia transferred from the treatment solution is converted to ammonium ions, which have no effect upon germination [15]); pH 9.5 buffer or pH 9.5 buffer with NH_4Cl as described above. Percentage germination was scored an hour after completion of the UV treatment. The effect of ammonia on stimulation time in GS [15] was tested by subjecting the spores to both GS and 0.01 M NH_4Cl during the stimulation period and then transferring them, at 10-sec intervals into 1-ml aliquots of low concentration, pH 5.5 phthalate buffer to remove the stimulus and the ammonia and then treated as described above for stimulation time.

Trehalose measurements. Samples were prepared for gas chromatography and analyzed according to the general procedures of Undeen et al. [16, 17]. Spore samples were prepared from 0.1 ml of total spore suspension, containing 5×10^7 spores and 50 μl of a 0.1% sucrose solution as an internal standard. These suspensions were combined in 10 × 75-mm borosilicate culture tubes with equal volumes of glass beads (0.45–0.50 mm, Braun), shaken for 1 min at the highest speed on a vortex mixer (SP, S8220), and then placed on ice. The homogenate was drawn from the beads with a pipette and centrifuged, under refrigeration, at 4,080 *g* for 30 min.

An aliquot of each sample or standard was placed in a 0.3-ml reaction vial and dried at 115° C for 30 min. Carbohydrates were prepared for analysis on the gas chromatograph by silylation with an equal volume of Tri-Sil-Z (trimethylsilylimidazole in dry pyridine, 1.5 mEq, Pierce Chemical Company, Rockford, IL). The silylation reaction was carried out at 70° C for a total of 45 min and the reaction vial was shaken with a vortex mixer at 15 and 30 min to aid dissolution and reaction.

Carbohydrates were analyzed by gas chromatography (GC)

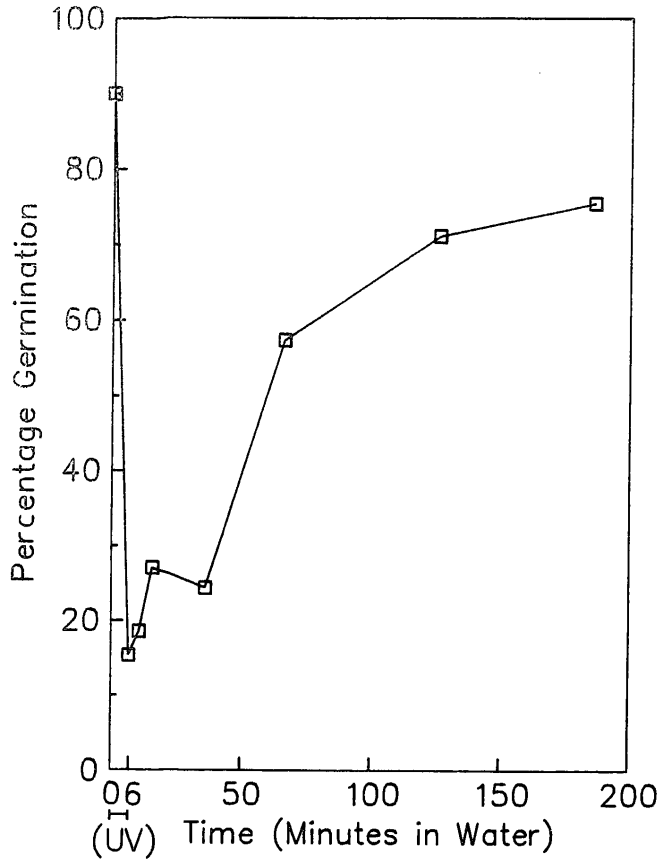

Fig. 3. Inactivation of germination by a 6-min UV treatment followed by reactivation in water (UV treatment was from 0 to 6 min).

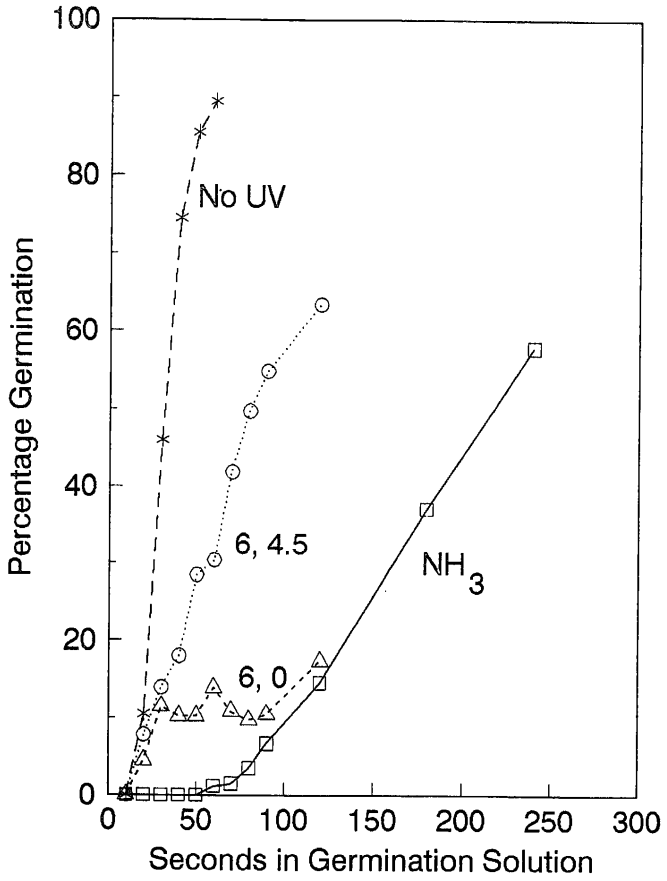

Fig. 4. The effect of ammonia and UV on the time required for stimulation of *Nosema algerae* spores. Stimulation time in GS alone (No UV) is compared with stimulation times in GS + 0.01 M NH_4Cl (NH_3), in GS immediately after a 6-min UV treatment (6, 0), and in GS 4.5 h after a 6-min UV treatment (6, 4.5).

on a Varian 3700 gas chromatograph equipped with a flame ionization detector (Varian Associates, Palo Alto, CA) and a fused silica 30-m DB-1 column; 332 ID, 0.025 μm film thickness (J & W. Scientific, Folsom, CA). The GC oven temperature was programmed from 100 to 250° C at 10° C/min. Qualitative and quantitative data were obtained with a Varian Vista 401 data processor. Sucrose, trehalose, and glucose standards were prepared and peak identification in samples was made by direct comparison with the prepared carbohydrate standards.

RESULTS

Effects of UV on germination. The UV energy delivered to a square centimeter of the 1-mm-deep spore suspension was equivalent to about 0.19 Joules/min. The temperature increased from 22.0 to 25.5° C during 20 min of UV exposure, an increase of insignificant consequence to percentage germination [14]. No attempt was made to evaluate the actual dosage received by single spores or the site at which the UV damage occurred. All dosages are reported in "minutes" at this single radiant energy level. Conversion to Joules/cm^2 (J = W × exposure time in sec) was done for comparative purposes.

Germination of spores continued at a nearly normal level when transferred to GS 2 h after 1–5 min UV treatments. As UV exposures increased to 20 min, the percentage germination decreased to 0 (Fig. 1). Some spores which were transferred to deionized water immediately after 6-20 min irradiation germinated without further stimulation by GS (Fig. 1). Germination in water was enhanced, after the UV treatment (Fig. 1), by higher temperature during incubation.

Germination of UV-irradiated spores declined with storage time at 5° C. This drop in percentage germination was correlated with the UV dosage. Figure 2 displays the results of one test, using five different UV dosage levels, examining the germination percentages over a 22-day period.

Most spores exposed to UV for 6 min and transferred immediately into GS failed to germinate. Incubation of these spores in deionized water at 30° C directly after UV treatment resulted in an increase in the percent germination in GS over the next 3 h. This is indicative of temporary inactivation by the UV radiation (Fig. 3).

Stimulation time. Normal, unirradiated spores were completely stimulated 60 sec after placing them in GS (Fig. 4, 5). Immediately after a 6-min UV treatment, spores were inactive (unable to complete germination) and insensitive to stimulation by GS (Fig. 4). However, after 4.5 h in deionized H_2O, these inactivated spores recovered their ability to be stimulated by GS (Fig. 4). Spores exposed to UV dosages too low to inactivate them (4.0 or 4.7 min) still showed prolonged stimulation times immediately after the UV treatment. Stimulation times decreased to the level of normal spores within a few hours (Fig. 5). Ammonia greatly extended stimulation time in GS (Fig. 4) but did not block it.

Ammonia and alkali inhibition. Immediately after a stimulatory level of radiation, spores germinated at higher percentages in water or pH 5.5 buffer than they did in the normally optimal

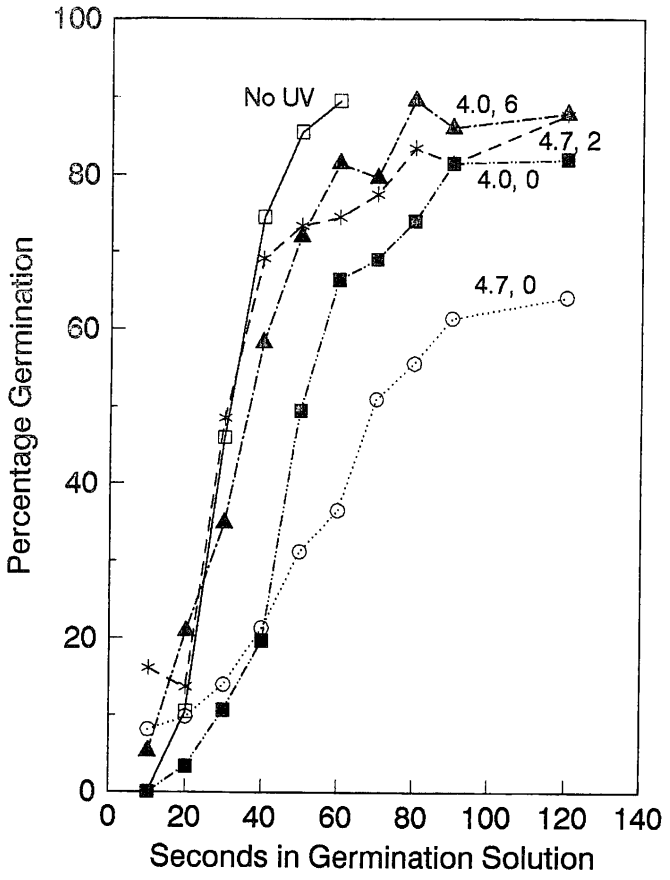

Fig. 5. The effect of ultraviolet radiation on the stimulation time of *Nosema algerae* spores by GS. The stimulation time of unirradiated spores (No UV) is compared with the stimulation times of spores immediately after (4.0, 0) and 6 h after (4.0, 6) a 4.0-min UV treatment and immediately after (4.7, 0) and 2 h after a 4.7-min UV treatment, (4.7, 2).

pH 9.5 buffer (Table 1). There was little inhibition of germination when the pH 9.5-buffered NH_4Cl (ammonia) or the pH 9.5 buffer alone was applied during the UV treatment whether germination occurred in water or pH 5.5 buffer (Table 1). After UV stimulation, ammonia completely inhibited germination, and the pH 9.5 buffer was a partial inhibitor (Table 2). A highly alkaline solution inhibited germination of irradiated spores more rapidly than unirradiated ones (Table 1).

Trehalose measurements. The intrasporal concentration of trehalose was significantly reduced immediately after the 20-min UV treatment. After milder UV treatments, most of the trehalose loss occurred later (Table 3).

DISCUSSION

In order to understand and properly interpret the effect of UV on spore germination, both the UV dosage and the elapsed time between UV treatment and the germination stimulus must be taken into account. The ability of the spores to germinate was completely and permanently lost after exposure to high dosages of UV, whereas the effect of the lowest dosages became obvious only after several days. At intermediate dosages the response was even more complex. Immediately after the 6-min UV treatment, most of the spores failed to germinate in GS. The recovery of their ability to germinate after 2 h in water indicated that the UV had caused inactivation [8, 9, 13]. The effect of UV irradiation on spore germination is, therefore, highly dependant upon the interaction of both UV dosage and time intervening between the UV treatment and the measurement of germination. This was true also for gamma radiation [16].

Table 1. Percentage germination of *Nosema algerae* spores induced by a 10-min UV treatment without GS and its inhibition by alkaline conditions and ammonia.

	Percentage germination in:			
	H_2O	pH 9.5 + NH_4Cl	pH 5.5[a]	pH 9.5[b]
UV treatment in:				
H_2O	32	0	46	8
0.01 M NH_4Cl	37	0	44	1
pH 9.5	23	0	26	11
pH 9.5 + NH_4Cl	20	0	22	1

[a] 0.01 M, pH 5.5 phthalate-NaOH buffer.
[b] 0.01 M, pH 9.5 glycine-NaOH buffer.

Considerably more ultraviolet radiation was needed to prevent germination than to destroy the viability (infectivity) of the spores. According to Kelly & Anthony [4], 8 min at 121 $\mu W/cm^2$ (0.06 J/cm^2) reduced the infection rate of *N. algerae* to almost 0. Our results showed that 20 min at 3175 mW/cm^2 (3.8 J/cm^2) was required to produce an equivalent, immediate and permanent reduction in percentage germination. Spores of other organisms were killed by approximately the same UV dosages in *N. algerae*. About 50% of *Bacillus thuringiensis* var. *thuringiensis* spores were killed by 0.03 J/cm^2 [3] and 0.05 J/cm^2 UV destroyed over 99% of *Bacillus sphaericus* Neide 1593 spores [2]. The *B. sphaericus* spores were still able to germinate, as indicated by their conversion to the phase-dark form, however, Burke et al. [2] did not report the UV dosage required to prevent bacterial spore germination. The highest dosage (1.1 J/cm^2) used on *B. sphaericus* [2] (within the range where *N. algerae* spore germination gradually declined) did not affect the ability of its proteinaceous toxin to kill mosquito larvae.

Tests of the effects of UV on bacterial and fungal spores frequently failed to differentiate between viability and germination. Although Cantwell [3] used the label "numbers of spores germinating" on his tables, his plate counts scored the numbers which formed colonies and were, in fact, a measure of viability. Rotem et al. [6] tested the effect of UV on fungal spores, estimating mortality by the proportion of spores which failed to germinate after termination of UV exposures. Effective dosages (>95% germination reduction) for three species of fungi ranged

Table 2. The effect of alkaline conditions (0.01 M NaOH) on the germination of *Nosema algerae* spores irradiated with ultraviolet light (5 min).

Treatment	GS[a] pH	Minutes in NaOH or water			
		1	30	75	360
		% Germination			
No UV, NaOH	5.5	88	88	81	12
No UV, NaOH	9.5	91	94	88	60
UV, water	5.5	59	80	86	88
UV, water	9.5	77	83	89	92
UV, NaOH	5.5	66	30	21	1
UV, NaOH	9.5	68	49	28	1

[a] Germination solutions were 0.1 M NaCl, 30° C; pH 9.5 by 0.01 glycine-NaOH or pH 5.5 by 0.01 M phthalate-NaOH buffers.

Table 3. Trehalose levels ($\mu g/4.5 \times 10^8$ spores ± SE, n = 3) in untreated *Nosema algerae* spores and spores treated with ultraviolet radiation and stored at 5° C.

	Hours at 5° C after UV treatment		
Min UV	0	2	120
0	22.35 ± 2.74	–	14.70 ± 5.42
6	12.97 ± 2.69	24.80 ± 3.77	9.63 ± 0.68
12	18.47 ± 4.43	4.39 ± 0.41	2.99 ± 0.59
20	7.97 ± 3.65	1.29 ± 0.69	2.55 ± 1.06

from 0.3 to greater than 2.0 J/cm² of bactericidal UV radiation. This amount of UV energy is closer to a germination-lethal dose for *N. algerae* than to the dosages which prevent either bacterial reproduction or infection by microsporidian spores. Growth of the fungus, after germination, would represent a more realistic measure of viability. Perhaps a greater exposure to UV is also needed to stop fungal spore germination than to prevent growth.

Treatment of *N. algerae* spores with between 0.2 and 1.0 J/cm² (1–5 min) had no immediate effect on percent germination but caused a loss of germination ability over a period of several days. Both the relative insensitivity to radiation and the delayed effect on germination are also characteristic of gamma radiation [17]. In any germination tests following either UV or gamma radiation, the time factor must be taken into account—probably for other organisms as well as microsporidia.

Characteristic spore responses to germination stimuli [14, 15] were also detected. These effects were not observable in gamma radiation studies because of the extended times (4–12 h) required to deliver equivalent gamma radiation dosages [17]. One of these responses was the moderate levels of germination directly induced by UV exposures in the range of 1.1 to 3.4 J/cm². Another was the reversible inactivation caused by the 1.1 J/cm² UV treatment. This temporary loss of germination was similar to inactivation caused by low concentration of stimulant ions or GS at cold temperature [8]. The most striking difference was that UV inactivation required only 6 min (1.1 J/cm²), whereas inactivation by low levels of other stimuli took about 3-5 h. Reactivation followed the same time course after both UV and suboptimal stimulus inactivation [8, 13].

At dosages lower than 1.1 J/cm², the stimulation phase of germination was temporarily slowed although percent germination was normal. The stimulation rate increased to near normal levels within a few hours, showing that inactivation by UV operated during or very soon after stimulation. Spores irradiated at levels which affected the stimulation times eventually lost their ability to germinate. However, this loss occurred over a period of days, while the recovery of stimulation occurred within a few hours.

Trehalose concentrations appear to be related to the ability of the spores to germinate after both UV and gamma radiation [17]. When the concentration of trehalose is low, the spores cannot germinate. Unconfirmed observations indicate that trehalose levels decrease during inactivation and then increase during reactivation. This appears to be the case here after UV inactivation as well (6-min exposure on Table 3) and is an attractive explanation for inactivation, however, we found that trehalose concentration also increased after the spores were warmed to 30° C for a few hours and then decreased again after remaining overnight in the refrigerator (unpublished results). As a result of this apparent lability of trehalose, we are unwilling to give too much credence to anything less than a two-fold change in trehalose concentration.

The exact nature of the UV damage that affects germination is unknown. Germination was induced by UV, beginning after 6 min exposure, reaching a maximum after about 15 min and then declining to 0 after 20 min under UV. Initially it could physically damage the compartmentation, causing contact between trehalose and trehalase, thereby initiating the germination process. Between 15 and 20 min is the same UV exposure range through which spores also lose their ability to germinate in GS immediately after radiation. This might be the result of damage other than the one which stimulates germination. However, the concentration of trehalose found after a 20-min UV treatment is low enough to explain the inability of the spores to complete germination.

Germination of *N. algerae* spores is optimal in an external medium with a pH of about 9.5 [8] and germination at lower pH is generally enhanced by pretreatment of the spores in an alkaline solution [13]. Ammonia inhibits germination at an early stage by increasing the intrasporal pH [15]. These results show that, although stimulation by GS is slowed in the presence of ammonia, it is not completely blocked. Ammonia was ineffective in blocking the stimulation of germination by UV but was very effective in stopping germination once UV stimulation had occurred. These results are all consistent with the view that ammonia blocks germination at a stage immediately following stimulation, possibly by making intrasporal conditions too alkaline for the essential enzymes to function. For example, the trehalase in *N. algerae* has a pH optimum of 5.5 [16] and is completely inactive at pH 9.5, the optimal external pH for germination. Germination induced by UV, without GS, was also adversely affected by high pH in the external medium. Spores subjected to a level of UV too low to induce germination (5 min) also germinated more poorly following treatment in an alkaline solution. Ultraviolet radiation possibly damaged membranes to the extent that the spore had a reduced ability to maintain its internal pH against a much higher external pH. The pH within the spore might then increase rapidly without the requirement for an intermediary such as ammonia.

Spores which were unable to germinate after irradiation might have failed simply because there was inadequate trehalose remaining to effect germination. This interpretation is particularly compelling if germination is the result of pressure increase brought about by an increased intrasporal solute concentration from rapid cleavage of trehalose molecules into more numerous smaller molecules [10]. Trehalose is probably degraded by the same pathway after UV irradiation as after the ionic stimulus. The germination induced by the 8- to 18-min UV exposures is evidence of this. Insufficient pressure would be generated after lower UV dosages because the catabolism of trehalose is too slow. This slow loss of trehalose would eventually result in trehalose levels too low to effect germination.

Ultraviolet radiation probably does not break compartmentation in the same way as an ionic stimulus. This is indicated by the different dynamics of inactivation (6 min vs. 4 h) and the fact that lower percentages of spores were induced to germinate by the action of UV. Otherwise, UV behaves in every observed way like a germination stimulus, supporting the decompartmentation hypothesis for the stimulation of microsporidia spore germination.

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