JOURNAL OF INVERTEBRATE PATHOLOGY 62, 220–225 (1993)

In Vitro Germination of *Nosema apis* (Microspora: Nosematidae) Spores and Its Effect on Their αα-Trehalose/D-Glucose Ratio

DIRK C. DE GRAAF,*,[1,2] GEERT MASSCHELEIN,† FRANS VANDERGEYNST,† HUBERT F. DE BRABANDER,‡ AND FRANS J. JACOBS†

Laboratory of Parasitology, and ‡Laboratory of Chemical Analysis of Food from Animal Origin, Faculty of Veterinary Medicine, University of Ghent, Casinoplein 24, 9000 Ghent, Belgium; and †Laboratory of Zoophysiology, Faculty of Sciences, University of Ghent, K.L.Ledeganckstraat 35, 9000 Ghent, Belgium

Received July 9, 1992; accepted March 4, 1993

Nosema apis **spore hatching was examined in different salt solutions. Best results were obtained in 0.5 M NaCl + 0.5 M $NaHCO_3$ at pH 6. No differences could be detected between sodium and potassium salts. The differences in efficacy between monovalent anions were more obvious, and a rank order could be formed: $Cl^- > Br^- > F^- > I^-$. The carbohydrate content of ungerminated and germinated spores was measured by GC–MS analysis. Only a very small shift in the ratio αα-trehalose/D-glucose was found. Scanning electron microscopy of extruded spores was done. No convincing images of released sporoplasms were obtained.** © 1993 Academic Press, Inc.

KEY WORDS: ***Nosema apis; Apis mellifera;*** **spore; germination;** ***in vitro;*** **trehalose.**

INTRODUCTION

Nosema apis (Microspora: Nosematidae) is a cosmopolitan economically important parasite of the honey bee (*Apis mellifera*). Its life cycle is strictly intracellular and the microsporidian is transferred via spores. After ingestion the spores hatch in the midgut lumen of the honeybee. A coiled polar filament inside the spore everts violently and serves as a hollow tube to conduct the sporoplasm directly into or near to the midgut epithelial cells.

The stimulus for the germination of microsporidian spores varies among species and seems to be a reflection of the microenvironment where the spores hatch *in vivo* (Jaronski, 1983). Best results have been achieved with shifts in pH or osmotic pressure, the presence of ions in the medium, and rehydration after desiccation (Malone, 1984; Whitlock and Johnson, 1990; Undeen and Epsky, 1990). Concerning the *in vitro* stimulation of *N. apis* spores, Bailey (1955) reported the extrusion of the polar tube in the gut content fluids. Hydrogen peroxide (Kramer, 1960; Van Laere, 1976), sodium chloride, and rehydration in phosphate-buffered saline (Olsen *et al.*, 1986) also have been used successfully.

In this paper the influence of anions/cations and the pH was examined in order to optimize the *in vitro* germination of *N. apis* spores. Recently Undeen (1990) proposed a new mechanism for microsporidian spore germination based on the endogenous breakdown of the disaccharide αα-trehalose. Therefore, the carbohydrate content of ungerminated and germinated spores was analyzed.

MATERIALS AND METHODS

Purification of Spores

The alimentary tracts were removed from severely infected honeybees by crushing the thorax with tweezers and gently pulling the terminal sclerites away from the remaining abdomen. The collected guts were homogenized in distilled water with an Ultraturrax homogenizer. *N. apis* spores were purified from this crude tissue homogenate by filtration through nylon filters with a decreasing pore size (180, 100, 40, and 10 μm) and centrifugation in a 30–65% sucrose gradient (18,000*g*, 120 min). Before use, the precipitated spores were washed several times in distilled water.

Assessment of Percentage Germination

Four microliters of a spore suspension (1×10^8 spores/ml) were mixed with 100 μl of a germination solution. A small drop (±5 μl) of trypan blue (0.2% in distilled water) was added to differentiate the germinated (blue colored) and the ungerminated (uncolored) spores. Using interference contrast microscopy germination was enumerated every 5 min for 40 min. The experiments were done at room temperature and all repeated 10 times.

[1] To whom correspondence should be addressed.

[2] Supported by an IWONL graduate fellowship.

0022-2011/93 $4.00

Germination Solutions

Different salt solutions at concentrations of 0.5 or 0.25 M were tested for their ability to induce germination. Germinated spores were counted in the presence of NaCl, $NaHCO_3$, NaOH, and KOH. The combination of NaCl + $NaHCO_3$ (pH 8.0), KCl + $KHCO_3$ (pH 8.4), and NaCl + Na_2SO_4 (pH 6.3) was examined. To determine the influence of pH, the 0.5 M NaCl + 0.5 M $NaHCO_3$ solution was adjusted with either 0.1 M H_3PO_4 or 0.1 M NaOH to give a range from pH 4.0 to pH 10.0. The combination of $NaHCO_3$ with other monovalent sodium salts was examined at pH 6.0.

Scanning Electron Microscopy

Nosema spores were attached to coverslips which were pretreated with 1% albumin in glycerol (1:1 v/v). Spore germination was stimulated by submerging the coverslips in 0.5 M NaCl + 0.5 M $NaHCO_3$ for 10 min. Fixation occurred in ice-cooled cacodylate buffer (0.1% cacodylate, 0.12 M sucrose, 0.05% $CaCl_2$, pH 7.4) containing both gluteraldehyde (2%) and paraformaldehyde (2%) (Raes *et al.*, 1989). The samples were dehydrated by an increasing alcohol series and by critical point drying in liquid carbon dioxide. Finally, they were coated with gold and examined with a JEOL JSM 840 scanning microscope.

Preparation for Carbohydrate Analysis

Pellets containing 94×10^7 (or 14×10^7) purified spores were resuspended in either 20 ml germination solution (0.25 M NaCl + 0.25 M $NaHCO_3$, pH 6) or 20 ml water (= ungerminated spores) and incubated in a water bath at 34°C for 30 min. All samples were prepared in triplicate. After the incubation period germinated and ungerminated spore samples were homogenized by violently shaking in a Braun cell homogenizer using an equal volume of glass beads. The specimen chamber of the homogenizer was cooled by spraying with liquid CO_2. The samples were checked by microscopic observations and homogenization was done until most of the spores were broken. The homogenate was centrifuged for 15 min at 15,000*g* in a refrigerated (4°C) Sorvall centrifuge to remove insoluble spore fragments. Ten milliliters of supernatant was filtered (Sartorius minisart, 0.22 μm), lyophilized, and prepared for carbohydrate analysis. Volatile trimethylsilyl ethers were prepared by incubating the lyophilized samples in 5 ml pyridine/hexamethyldisilazane/trimethylchlorosilane (10:2:1 v/v/v) for 45 min at 60°C. Samples were dried with nitrogen and resuspended in 2 ml hexane. Only 1 μl was used for carbohydrate analysis.

Carbohydrate Analysis

Carbohydrate analysis was done with a Finnigan MAT ITS 40 GC–MS in the electron impact mode (70 KeV). Samples were analyzed on a capillary column (25 m × 0.22 mm i.d.) coated with 0.1 μm HT5 (nonpolar packing). The GC oven temperature increased linearly from 100 to 260°C at 12°C/min, and was finally kept for 5 min at 300°C. Measurements were made after a delay of 300 sec and within the mass range 100 to 650 amu at a frequency of 1 scan/sec. The GC–MS response was expressed in "total ion current" (TOT) and related in percentages of the highest peak. Glucose, sucrose, and αα-trehalose standards were obtained from Aldrich Chemie. Peak identification was made by direct GC–MS comparison with silylated carbohydrate standards.

RESULTS

The extrusion of *N. apis* spores is a rapid process and consequently difficult to follow. The expulsion of the polar tube is preceded by a lag phase during which no apparent changes take place. The length of this period was dependent on the stimulation solution that was used. At the end of the lag phase spores started vibrating for some seconds, followed by the violent ejection of the polar filament. In some observations the polar tube left the spore body in a corkscrew motion. Best observations were made in samples where spores germinated at the periphery of trapped air bubbles. The penetration of the air liquid interface by some of the emerging polar filaments caused a magnification of their size and simultaneously a slowing down of the whole extrusion process (Olsen *et al.*, 1986).

When spores were treated with 0.5 M NaCl, only 14% hatched within 40 min (Fig. 1). A 0.5 M $NaHCO_3$ solution caused the extrusion of only 7% of the spores. A solution containing both 0.5 M NaCl and 0.5 M $NaHCO_3$

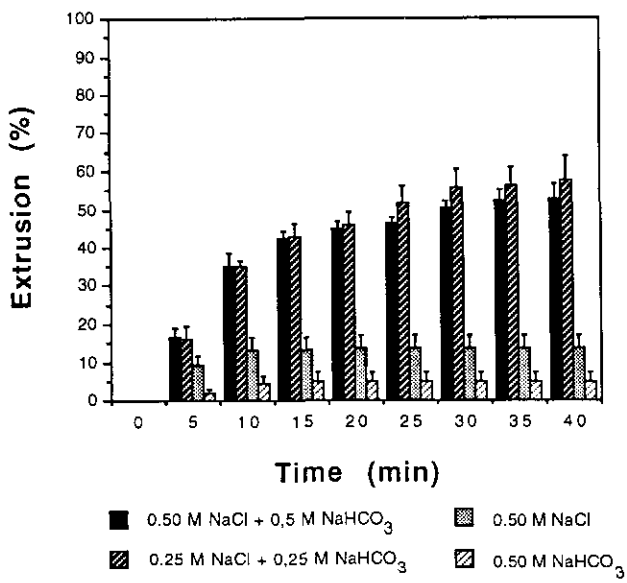

FIG. 1. Mean percentage (±standard error) of germinated *Nosema apis* spores after stimulation with different salt solutions.

gave a much higher frequency of germination (53%), and comparable results were found using the same salts at half this concentration. Sodium salts and potassium salts stimulated spore hatching almost identically (Fig. 2). The combination of NaCl + Na_2SO_4 gave a much lower extrusion percentage than NaCl + $NaHCO_3$. Spores did not hatch at all in a 0.5 M NaOH or 0.5 M KOH solution.

When the pH of the 0.5 M NaCl + 0.5 M $NaHCO_3$ solution was altered between 4 and 10 with 0.1 M NaOH or 0.1 M $NaHCO_3$, germination occurred only between 5 and 9. After 40 min, pH 6, 7, and 8 gave the same final extrusion percentage; however, at pH 6 this level was reached after only 5 min (Fig. 3).

Different monovalent anions in combination with $NaHCO_3$ gave different extrusion stimuli (Fig. 4). Best results were obtained with Cl^-, followed by Br^-, F^-, and I^-.

GC–MS analysis of the carbohydrate content of both germinated and ungerminated spores revealed a major peak of αα-trehalose and minor peaks of α-glucose, β-glucose, and sucrose (Fig. 5). Considerable differences in GC–MS response could be noticed between the spore samples, even when the same spore concentration was used (Table 1). Probably this was due to the varying success of the homogenization technique. Therefore it was irrelevant to estimate the exact carbohydrate concentration by use of internal standards. However, the ratio αα-trehalose/D-glucose was comparable within the samples of germinated and ungerminated spores, respectively. The average ratio αα-trehalose/D-glucose was 27 ± 12 for ungerminated spore samples and 13 ± 4 for germinated spore samples.

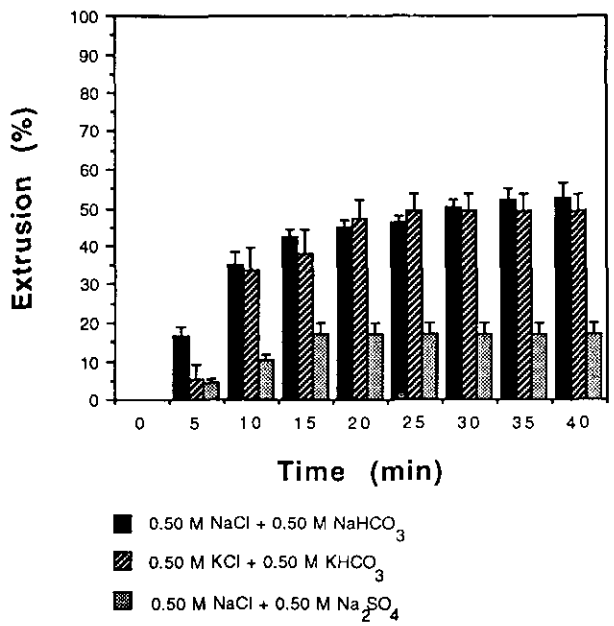

FIG. 2. Mean percentage (±standard error) of germinated *Nosema apis* spores after stimulation with different combinations of salts.

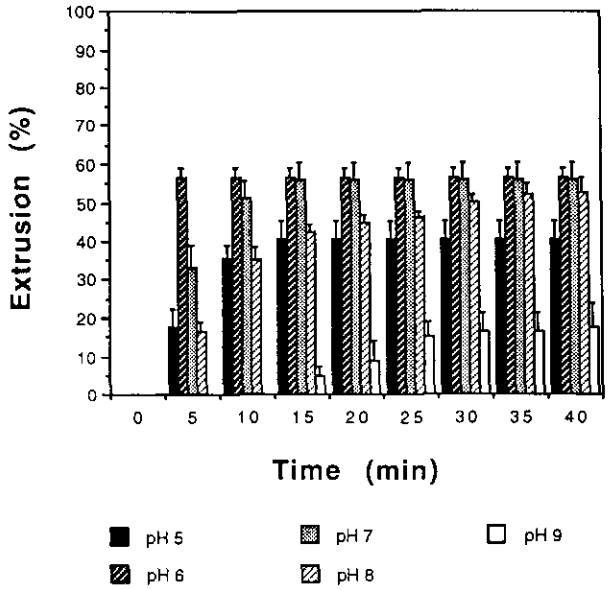

FIG. 3. Mean percentage (±standard error) of extrusion of *Nosema apis* spores in 0.5 M NaCl + 0.5 M $NaHCO_3$ at various pH values. The pH was adjusted with 0.1 M H_3PO_4 or 0.1 M NaOH.

Scanning electron microscopy showed germinated spores and a striking maze of extruded polar tubes (Fig. 6). However, no convincing images of released sporoplasms could be made.

DISCUSSION

Microsporidian spore germination is stated to be an osmotic process (Undeen and Frixione, 1990). This does not necessarily mean that the hatching stimulation is

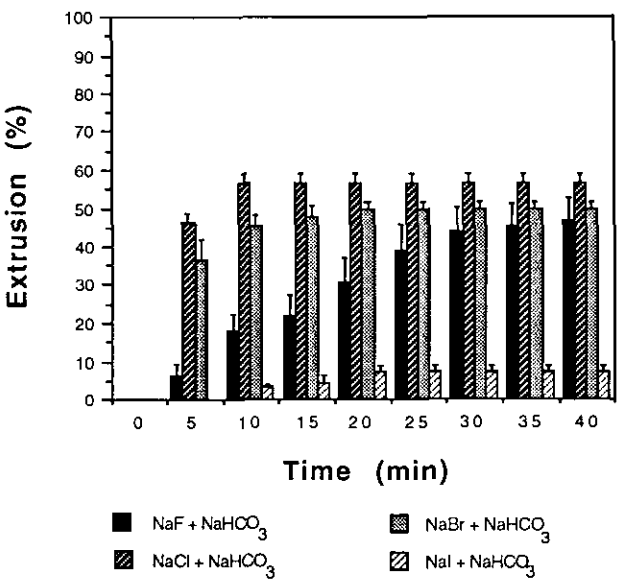

FIG. 4. Mean percentage (±standard error) of extrusion of *Nosema apis* spores in solutions combining $NaHCO_3$ and different monovalent anions at pH 6.

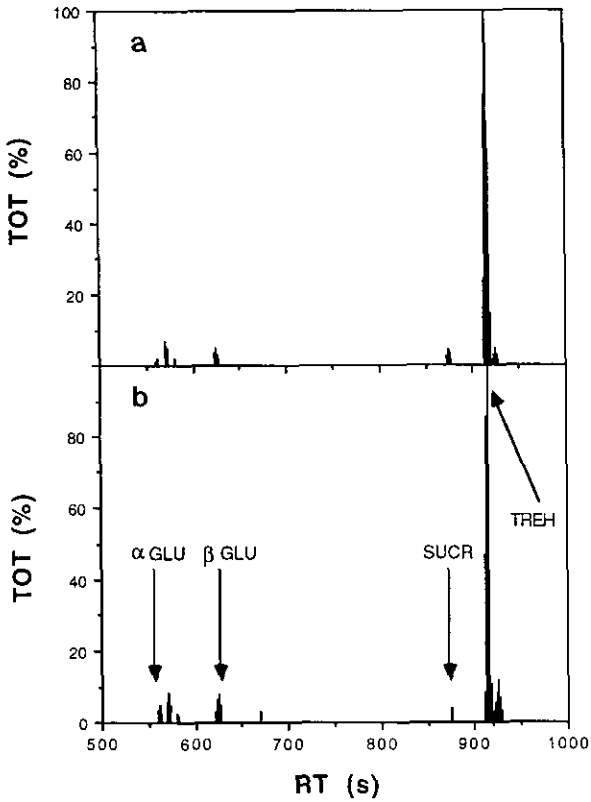

FIG. 5. GC–MS analysis of the *Nosema apis* spore content after incubation in water (a) or in germination solution (b) for 30 min.

osmotically dependant. Kroon *et al.* (1974) proposed that the osmotic shock to which *N. apis* spores are exposed when they are filtered out of the honey stomach and enter the midgut is responsible for spore hatching. However, when *N. apis* spores were shifted from a 30% sucrose solution to distilled water, spore hatching did not occur (results not shown). Olsen *et al.* (1986) earlier described the requirement of salts in the rehydration solution to stimulate *N. apis* spore hatching and in this paper germination was achieved in salt solutions without previous dehydration. Therefore we suggest that for *N. apis* germination is stimulated by the presence of ions and not simply by the alteration of osmolarity.

N. apis spores hatched best in a NaCl + $NaHCO_3$ solution at pH 6. The percentage of spores germinating seems to increase when NaCl and $NaHCO_3$ are simultaneously present in the germination fluid. This increase is not caused by doubling the number of ions because both a 0.50 and a 0.25 molar concentration gave identical results. Possibly there is a synergistic effect between NaCl and $NaHCO_3$. It is not known whether other combinations are possible, but NaCl + Na_2SO_4 did not stimulate hatching to the same extent. The stimulating effect of phosphate-buffered saline, containing a mixture of different salts, is already known (Olsen *et al.*, 1986).

Ishihara (1967) and Undeen (1978) reported an efficacy order of alkali metals in stimulating the germination of *Glugea fumiferanae* ($Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$) and *Nosema algerae* spores ($K^+ > Na^+ > Rb^+ > Cs^+ > Li^+$), respectively. Our results on *N. apis* showed no differences between sodium salts and potassium salts. Whitlock and Johnson (1990) found comparable results for *Nosema locustae*. Differences in efficacy between monovalent anions seemed more obvious for *N. apis* spores, and a rank order could be formed: $Cl^- > Br^- > F^- > I^-$. Undeen and Avery (1988) also reported an efficacy order of anions for *N. algerae* spores: $Br^- > Cl^- > I^- > F^-$. Possibly both anions and cations are involved in the germination-stimulating process.

It was most interesting to notice that the *N. apis* spores hatch the best at pH 6. The buffering power of the contents of the ventriculus of the honeybee at pH 6.2–6.3 is well known (Hoskins and Harrison, 1934) and it seems reasonable to think that the artificial

TABLE 1

GC–MS Analysis of the *Nosema apis* Spore Content after Incubation in Water (a1, a2, and a3) or in Germination Solution (b1, b2, and b3) for 30 min at 34°C

Sample	Suspending solution	Carbohydrate analysis[c] of disrupted spores					Ratio αα-trehalose/ D-glucose
		α-D-glucose	β-D-glucose	D-glucose	Sucrose	αα-Trehalose	
a1[a]	Distilled water	2001174	2410898	4412072	4990273	125196235	28
a2[a]	Distilled water	1949378	2574449	4523827	3116507	67177226	15
a3[b]	Distilled water	459560	253115	712675	968963	26847275	38
							27 ± 12[d]
b1[b]	0.25 M NaCl + 0.25 M $NaHCO_3$, pH 6	1228754	2047229	3275983	1255662	25333477	8
b2[b]	0.25 M NaCl + 0.25 M $NaHCO_3$, pH 6	435881	367093	802974	2236484	11521443	14
b3[b]	0.25 M NaCl + 0.25 M $NaHCO_3$, pH 6	451479	351544	803023	480135	13204884	16
							13 ± 4[d]

[a] Spore sample containing 7×10^6 spores/ml.
[b] Spore sample containing 47×10^6 spores/ml.
[c] GC–MS response of the trimethylsilyl ethers was expressed in total ion current.
[d] Mean ratio αα-trehalose/D-glucose ± standard deviation.

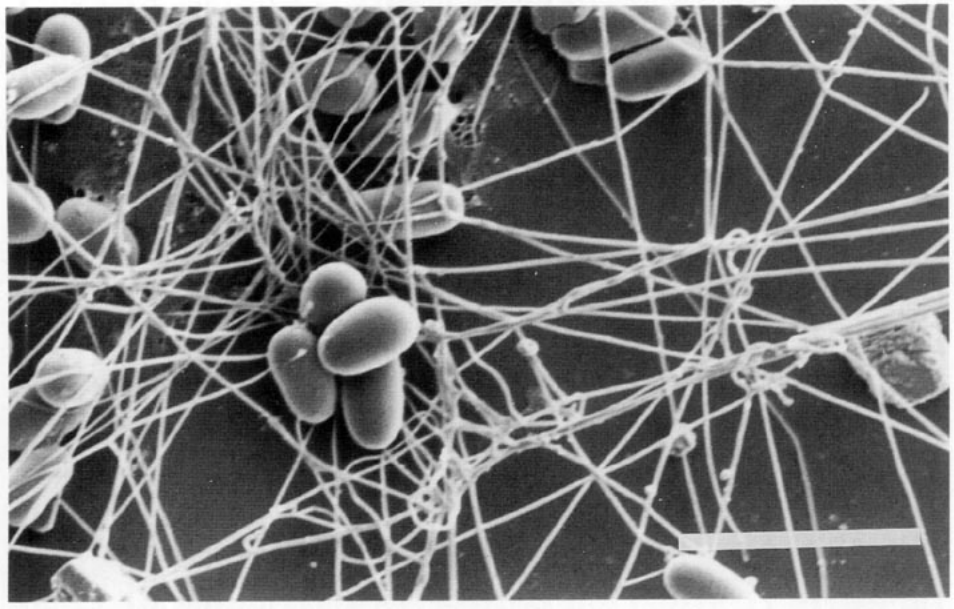

FIG. 6. Scanning electron microscopy of germinated *Nosema apis* spores (bar = 10 μm).

stimulus of *N. apis* spores is to some extent a reflection of the conditions *in vivo* in the honeybee midgut juice.

Different hypotheses have been suggested to explain the expulsion of the polar tube. All of them assume that an internal osmotic potential is built up during the lag phase. Undeen *et al.* (1987) found for *N. algerae* spores that after application of the stimulus, the carbohydrate molar concentration increased by degradation of αα-trehalose into a greater number of smaller molecules. It was suggested that this sudden increase in osmolarity was responsible for a pulse of increased intrasporal pressure which initiated spore germination (Undeen, 1990). *N. apis* spores have abundant stores of αα-trehalose and smaller quantities of glucose and other monosaccharides (Wood *et al.*, 1970). In this paper only a small shift in the ratio trehalose/glucose was measured after germination, suggesting that a huge breakdown of αα-trehalose does not precede the germination process in *N. apis* spores. Therefore it seems unlikely that *N. apis* and *N. algerae* spores have the same mechanism to gain the necessary internal pressure for polar tube expulsion.

It is already known that the biochemical properties of the enzyme trehalase, which is responsible for the cleavage of the disaccharade trehalose into two molecules of glucose, differs among microsporidian species. Trehalase from *N. algerae* spores has a pH optimum of about 5.5 and is almost totally inactive at pH 8.0 (Undeen *et al.*, 1987). Trehalase from *N. apis,* however, has a pH optimum of 7.0, but even so, it was not active above pH 8.0 (Vandermeer and Gochnauer, 1971). Perhaps the differences in trehalase characteristics indicate different germination mechanisms for each of these species. A comparable situation exists in fungal spores. Fungal trehalases have been divided into two classes according to their biochemical characteristics and their functional properties (Thevelein *et al.*, 1984).

Dall (1983) proposed a mechanism in which the internal osmotic pressure of spores was built up by accumulation of cations from the external medium. However, this proton exchange mechanism required an alkaline environment for germination and it was demonstrated that for *N. apis* spores the germination rate was the highest at pH 6. None of the germination mechanisms hitherto proposed for different microsporidian species seems to fit for *N. apis*. It is possible that not only the stimulus but also the mechanism for germination varies among microsporidian species.

REFERENCES

Bailey, L. 1955. The infection of the ventriculus of the adult honeybee by *Nosema apis* (Zander). *Parasitology* 45, 86–94.

Dall, D. J. 1983. A theory for the mechanism of polar filament extrusion in the Microspora. *J. Theor. Biol.* 105, 647–659.

Hoskins, W. M., and Harrison, A. S. 1934. The buffering power of the contents of the ventriculus of the honeybee and its effect upon the toxicity of arsenic. *J. Econ. Entomol.* 28, 924–942.

Ishihara, R. 1967. Stimuli causing extrusion of polar filaments of *Glugea fumiferanae* spores. *Can. J. Microbiol.* 13, 1321–1332.

Jaronski, S. T. 1983. Microsporidia in cell culture. *Adv. Cell Cult.* 3, 183–229.

Kramer, J. P. 1960. Observations on the emergence of the microsporidian sporoplasm. *J. Insect Pathol.* 2, 433–439.

Kroon, G. H., van Praagh, J. P., and Velthuis, H. H. W. 1974. Osmotic shock as a prerequisite to pollen digestion in the alimentary tract of the worker honeybee. *J. Apic. Res.* 13, 177–181.

Malone, L. A. 1984. Factors controlling in vitro hatching of *Vairimorpha plodiae* (Microspora) spores and their infectivity to *Plodia interpunctella, Heliothis virescens,* and *Pieris brassicae. J. Invertebr. Pathol.* 44, 192–197.

Olsen, P. E., Rice, W. A., and Liu, T. P. 1986. *In vitro* germination of *Nosema apis* spores under conditions favorable for the germination and maintenance of sporoplasms. *J. Invertebr. Pathol.* 47, 65–73.

Raes, H., Bohyn, W., De Rycke, P. H., and Jacobs, F. 1989. Membrane-bound iron-rich granules in fat cells and midgut cells of the adult honeybee (*Apis mellifera* L.). *Apidologie* 20, 327–337.

Thevelein, J. M., den Hollander, J. A., and Shulman, R. G. 1984. Trehalase and the control of dormancy and induction of germination in fungal spores. *T.I.B.S.* 9, 495–497.

Undeen, A. H. 1978. Spore-hatching processes in some *Nosema* species with particular reference to *N. algerae* Vavra and Undeen. *Misc. Publ. Entomol. Soc. Am.* 11, 29–48.

Undeen, A. H., ElGazzar, L. M., Vander Meer, R. K., and Narang, S. 1987. Trehalose levels and trehalase activity in germinated and ungerminated spores of *Nosema algerae* (Microspora: Nosematidae). *J. Invertebr. Pathol.* 50, 230–237.

Undeen, A. H., and Avery, S. W. 1988. Effect of anions on the germination of *Nosema algerae* (Microspora: Nosematidae) spores. *J. Invertebr. Pathol.* 52, 84–89.

Undeen, A. H. 1990. A proposed mechanism for the germination of microsporidian (Protozoa: Microspora) spores. *J. Theor. Biol.* 142, 223–235.

Undeen, A. H., and Epsky, N. D. 1990. In vitro and in vivo germination of *Nosema locustae* (Microspora: Nosematidae) spores. *J. Invertebr. Pathol.* 56, 371–379.

Undeen, A. H., and Frixione, E. 1990. The role of osmotic pressure in the germination of *Nosema algerae* spores. *J. Protozool.* 37, 561–567.

Vandermeer, J. W., and Gochnauer, T. A. 1971. Trehalase activity associated with spores of *Nosema apis*. *J. Invertebr. Pathol.* 17, 38–41.

Van Laere, O. 1976. Factors influencing the germination of *Nosema apis* Zander spores. In "Biological Aspects of *Nosema* Disease" (V. Harraj and O. Van Lacre, Ed.), pp. 56–64. Apimondia Publishing House, Bucharest.

Whitlock, V. H., and Johnson, S. 1990. Stimuli for the in vitro germination of *Nosema locusta* (Microspora: Nosematidae) spores. *J. Invertebr. Pathol.* 56, 57–62.

Wood, P. J., Siddiqui, I. R., Vandermeer, J. W., and Gochnauer, T. A. 1970. Carbohydrates of *Nosema apis* spores. *Carbohydr. Res.* 15, 154–158.