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The spore of the Unicellular Organism *Nephridiophaga blattellae*: Ultrastructure and Substances of the Spore Wall

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Summary. Since many aspects of nephridiophagids are still unknown, we investigated their most typical life cycle stage, the spore, using different light and electron microscopic techniques. Cytochemical and fluorescence labelling seemed to indicate the presence of chitin, protein, and calcium as components of the spore wall. Organic solvents, alone or in combination with proteases and chitinases, influenced the staining properties. This led to the assumption that hydrophobic substances at the spore surface and a close association of protein and chitin impede staining and enzymatic actions. Freeze-fracture studies and thin sections showed details of the spore surface and depicted the different wall layers of the spores. The innermost electron-transparent layer bound WGA (indirectly labelled with gold) and thus may contain chitin. A central cap-like structure was revealed to be an opening through which an infectious sporoplasm may emerge. Ultrastructural aspects of spore hatching and hatched sporoplasms were demonstrated for the first time. Spore wall features are reminiscent of microsporidian spores but the similarities are too few to assign the nephridiophagids to any of the known groups of unicellular organisms.

Key words: *Blattella germanica,* chitin, cockroach, Nephridiophagidae, spore, ultrastructure.

INTRODUCTION

The family Nephridiophagidae Sprague (1970) (or Coelosporidiidae, Purrini and Weiser 1990) embraces a group of unicellular spore-forming organisms. It is a fairly unknown group of parasites; therefore the most important features should be briefly mentioned (for more details see Woolever 1966, Toguebaye *et al*. 1986, Purrini and Weiser 1990, Lange 1993, Radek and Herth 1999). Nephridiophagids (a collection of species as defined in Radek and Herth 1999) mainly infect the Malpighian tubules of insects, especially of cockroaches and beetles. Infection is oral *via* uptake of spores. The earliest stages of reproduction are found in the epithelial cells of the Malpighian tubules or in the tubule lumen. Oligo- and multinucleate plasmodia arise, then disintegrate into unicellular merozoite-like stages or undergo sporogony. During the process of sporogony, cisterns of the endoplasmic reticulum (ER) demarcate a portion of cytoplasm around each nucleus, thus generating sporoblasts. Some vegetative nuclei remain in the common cytoplasm and degenerate. A spore wall is formed between the two spore membranes that originate from the fused ER cisterns. Developmental stages with two nuclei [*Nephridiophaga blattellae* (see Radek and Herth 1999)*, N. schalleri* (see Purrini and Rhode 1988)] or even four nuclei [*N. apis* (see Ivanić 1937)*, N. periplanetae* (see Ivanić 1926 and Swarczewsky 1914)] have (controversially) been reported, while the

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mature spores are generally uninucleate (except for *C. binucleata*, see Gibbs 1959). The presence of a spore opening has often been debated, but in some species central, cap-like structures have been suggested to be such an orifice for hatching [*N. blattellae* (see Radek and Herth 1999), *N. blaberae* (see Fabel *et al*. 2001)].

The spore wall (of *N. blattellae*) is resistant to many chemicals and enzymes (Woolever 1966). None of these probes nor mechanical stress (alternate freezing and thawing, pressure on the cover slip) destroyed the spore or caused extrusion of its sporoplasm. The reasons for this enormous resistance are not clear. Results of tests for the presence of chitin were inconclusive (Woolever 1966).

The systematic position of the nephridiophagids is controversial. Due to their appearance under the light microscope, they have been assigned to microsporidia (Perrin 1905, Kowaljowa and Issi 1973), and later, after examining ultrastructural data, to haplosporidia (Woolever 1966, Purrini and Weiser 1990). Other investigators, however, have stated that they do not belong to either taxon (Toguebaye *et al*. 1986, Purrini and Rhode 1988, Purrini *et al*. 1988, Lange 1993, Radek and Herth 1999).

Our main aims were to gain more information on the chemical composition and morphology of the spore wall and hatching spores. Molecular studies were initiated but are not the subject of the present study.

MATERIALS AND METHODS

Specimens of the German cockroach *Blattella germanica* were obtained from cultures of the Federal Health Institute in Berlin. Decapitated specimens were processed for light and routine electron microscopy as recently described (Radek and Herth 1999). Fresh mounts, Giemsa-stained smears, DAPI-stained smears after acid hydrolysis, and paraffin sections stained with Weigert´s iron hematoxylin were used for light microscopy. For transmission electron microscopy, the Malpighian tubules were fixed in 2.5 % glutaraldehyde, rinsed, post-fixed in a 1:1 mixture of 2 % OsO_4 and $K_4[Fe(CN_6)]$, and embedded in Spurr´s medium (Spurr 1969), while the material for freeze-etched preparations was lightly fixed in 0.5 % paraformaldehyde with 0.5 % glutaraldehyde and stored in 20 % glycerol prior to shock freezing in nitrogen slush.

WGA-gold label

For lectin-gold labelling, the Malpighian tubules were fixed in 4 % paraformaldehyde with 2.5 % glutaraldehyde in a 0.1 M phosphate buffer of pH 7.2, dehydrated and embedded in LR-White. Indirect gold labelling of thin sections was performed as follows: rinse in TBS (0.1 M Tris-HCl pH 7.2, 0.15 M NaCl, 2 mM $MnCl_2$, 2 mM $MgSO_4$, 2 mM $CaCl_2$); incubate in 100 µg/ml WGA (lectin from *Triticum vulgaris*, Sigma) in TBS for 1 h; rinse in TBS 2 x 2 min; incubate in fetuin-gold (from fetal calf serum, 10 nm gold particles, Sigma) 1:50 diluted with TBS; rinse in TBS 2 x 2 min; shortly rinse in aqua dest.; routinely stain with uranyl acetate and Reynold´s lead citrate.

Light microscopic detection of chitin, cellulose, protein and calcium in untreated spores

When not otherwise stated, the Malpighian tubules from five cockroaches were collected for each experiment and torn with tweezers to release the spores.

Protein

Detection of α-amino acids by the Ninhydrin-Schiff-reaction (Böck 1989). Paraffin sections (fixation with 4 % formaldehyde) were used instead of native material. Aldehyde groups, which emerged from amino acids by oxidative desamination with Ninhydrin, are stained red-violet with Schiff´s reagent. Negative control: no Ninhydrin.

Calcium

Formaldehyde-fixed paraffin sections and native spore-containing material were used for the detection of calcium.

Silver method according to von Kossa (Böck 1989). Native material was centrifuged at 1000 g for 10 min at each step. Principal of detection: calcium ions in carbonates and phosphates are substituted by silver ions; those are reduced to metallic silver by light resulting in brown-black deposits.

Fura-2. Incubate deparaffinized sections or native spores with 100 µM Fura-2 (Sigma) in 0.1 M phosphate buffer and rinse. The fluorescent marker Fura-2 interacts with Ca^{2+}; the complex thus formed emits visible blue-white light (400-440 nm) after irradiation with ultraviolet light (340-460 nm).

Chitin

Cytochemical assay (Roelofsen and Hoette 1951 in Aronson and Bertke 1987): deacetylation of chitin by cooking in 23 M KOH leads to the formation of chitosan; chitosan transforms to a red-violet substance. Negative control: no KOH treatment.

Fluorescence: add 0.01 % Calcofluor White M2R (fluorescent brightener, Sigma) or 0.01 % FITC-wheat germ agglutinin (FITC-WGA, a fluorescent lectin, Sigma) in a 50 mM phosphate buffer of pH 7.2 to native material for 15 min. Both chemicals interact with β-linked polysaccharides; WGA has a high binding specificity for N-acetylglucosamine, the monomer of chitin (Goldstein and Poretz 1986).

Mannans

0.01 % FITC-Concanavalin A (FITC-Con A) in a 50 mM phosphate buffer of pH 7.2 was given to native material and paraffin sections. The lectin Concanavalin A has a nominal binding specificity for mannose and glucose (Goldstein and Poretz 1986).

Cellulose

Cytochemical method (Jensen 1962 in Aronson *et al*. 1987): Cellulose should be coloured blue-violet. Positive control: Some cellulose fibres of a paper towel processed in the same way.

Cellulose or chitin

Polarisation microscopy: Birefringence of objects may be caused by structure birefringence (e.g. fibres oriented in parallel) and/or inherent birefringence (e.g. uniform orientation of links within the molecules) (Gerlach 1976). While cellulose has a positive structure birefringence and a positive inherent birefringence, while chitin has a positive structure birefringence but negative inherent birefringence. Structure birefringence should disappear if the surrounding medium has the same refraction index. Native spores were suspended in 0.1 M phosphate buffer or glycerol (higher refraction index) and observed with a Reichert Austria microscope equipped with polarization filters and a Brace Köhler compensator.

Application of enzymes, HCl or organic solvents

Selective solubilization of spore wall substances was presumed to result in changed staining properties with fluorescent markers. The following treatments were performed: (1) Control: 0.1 M phosphate buffer pH 7.2, 30 min at room temperature. (2) 0.1 % chitinase (from *Streptomyces griseus*, Sigma) in 0.1 M citrate buffer pH 5.2 overnight at 37 °C. (3) 0.2 % proteinase K (from *Titirachium album*, Sigma) in 0.1 M phosphate buffer pH 7.6 for 60 min at 37 °C. (4) 100 % acetone. (5) 100 % chloroform. (6) 100 % isopropanol. (7) 100 % methanol. (8) 100 % xylene. (9) 1 N HCl. Substances 4 to 9 were left for 30 min at room temperature.

After the incubation all samples were centrifuged at 1000 g for 10 min and washed twice with a 0.1 M phosphate buffer of pH 7.2. Some portions of the pellet were stained with either 0.01 % Calcofluor White or with FITC-WGA. Other portions were first incubated with 0.1 % chitinase in a 0.1 M citrate buffer of pH 5.2 overnight at 37 °C and then stained. The latter test was performed to see whether treatment with proteinase or organic solvents could enhance the attack of chitinase. Both the staining intensity with Calcofluor or FITC-WGA and the percentage of fluorescent spores were estimated using fresh samples.

RESULTS

General appearance of the spores

Since the light and electron microscopic morphology of the life cycle stages of *N. blattellae* have been recently described in detail (Radek and Herth 1999), this study only briefly reports on the general features of the spores. Ten to 40 spores (generally about 25-30) are endogenously formed within a sporogenic plasmodium (= pansporoblast; Fig. 1). Mature spores have an oval, biconcave form and measure about 5.5 x 3.2 µm. Giemsa stain or DAPI cannot diffuse into the intact spores and stain their nuclei, but they do stain the residual, vegetative nuclei in the plasmodial cytoplasm (Fig. 2). The spore nuclei may only be stained after hydrolysis with hot 0.1 N HCl (not shown) or if the spores are broken open as was the case, for example, by the paraffin sections (Fig. 3).

Detection of protein, calcium, chitin, and cellulose in chemically untreated spores

Protein. Application of Ninhydrin-Schiff-reagent stained the spore walls red-violet, meaning that α-amino acids are probably present (Fig. 4).

Calcium. Using the silver method according to von Kossa, the spores had slightly brown-black walls (not shown). Fura-2, however, resulted in a strong fluorescence of the spore walls (Fig. 5). Results were comparable using native spores or paraffin sections.

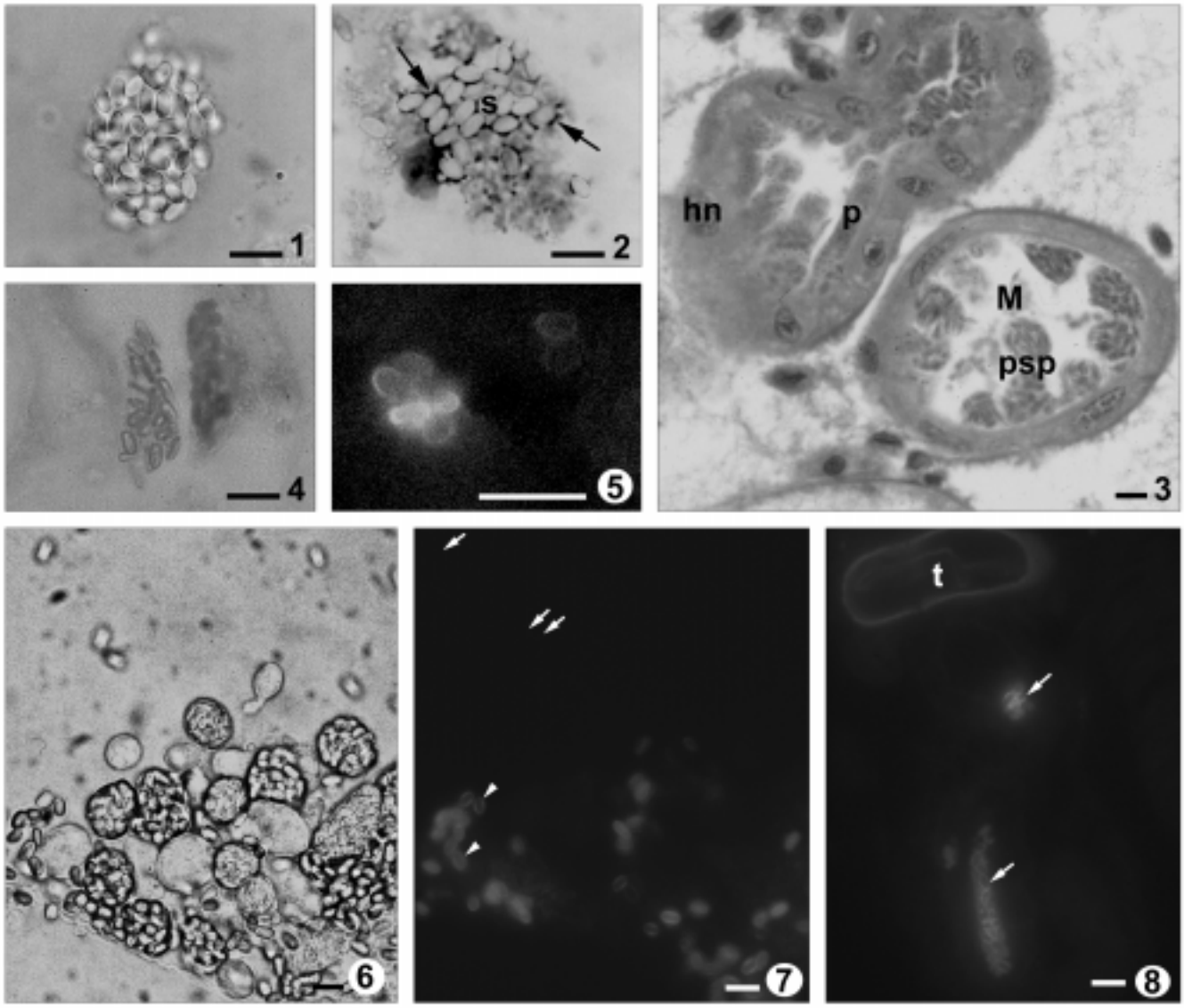
Chitin. The cytochemical method according to Roelofsen and Hoette (1951, in Aronson and Bertke 1987) did not give a positive result with spores. The controls looked the same as the test specimens did.

Fluorescent labelling with Calcofluor White or the fluorescent lectin FITC-WGA stained spores of native preparations to a variable degree. Spores stained heavily, weakly or not at all occurred at the same time, often depending on their position/life cycle stage, such as either single spores free in the medium, or spores within pansporoblasts or in the lumen of Malpighian tubules (Figs 6, 7; Tables 1, 2, controls). Only about 20 % of the spores were stained. Incubation of paraffin sections instead of native smears led to a more intense and complete staining of the spore walls (Fig. 8). Nevertheless, native preparations were used for the further detection methods and experiments (chemical treatments prior to staining) since it cannot be ruled out that spore wall materials are lost or chemically changed during the embedding procedures.

Mannans. FITC-Con A led to a very faint staining of spores in paraffin sections (not shown). The presence of α-mannans remains speculative.

Cellulose. To rule out the possibility that the positive result of the Calcofluor-staining was due to the presence of cellulose instead of chitin, a histochemical test for cellulose was performed. Cellulose could not be detected in the spores; control tests on cellulose fibres, however, were positive.

Chitin/cellulose in polarisation microscopy. A further proof that chitin but not cellulose is present in nephridiophagid spores was given by the use of polarisation microscopy. The relative optical character of the spore walls is positive, meaning that they are birefringent. Birefringence disappeared after the addition of glycerol. The positive structural birefringence



Figs 1-8. Light micrographs of *Nephridiophaga blattellae*. **1.** Mature spores of a pansporoblast in a squash preparation. **2.** Smear stained with Giemsa. The small, residual nuclei of the pansporoblast cytoplasm are stained (arrows), the spore nuclei are not stained. s- spore. **3.** Paraffin section stained with hematoxylin. The lumina of the Malpighian tubules (M) contain pansporoblasts (psp) with spores of different maturity, and multinucleate plasmodia (p) which adhere to the epithelium. hn- host cell nucleus. **4.** The red-violet staining (dark in the black and white photo) of the spore walls after application of Ninhydrin-Schiff-reagent reveals the presence of α-amino acids. **5.** Fura-2-fluorescence depicts Ca^{2+} in the spore walls. **6.** In the fresh squash preparation several pansporoblasts and single spores are visible. **7.** Same preparation as in Fig. 6 showing Calcofluor-fluorescence. Most spore walls of single spores were stained (arrowheads) but not all (arrows). Spores within pansporoblasts did not fluoresce. **8.** Spores in paraffin sections exhibited a more intense and complete Calcofluor-fluorescence (arrows). The chitin-containing tracheas (t) also fluoresced. Scale bars-10 μm

seemed to have been lowered to such a degree that the birefringent nature was abolished by an equivalent negative inherent birefringence. Thus, it seems plausible that chitin is present. If cellulose had been present, a residual positive structural birefringence together with a positive inherent birefringence should have resulted in a fainter but still detectable birefringence.

Staining properties of the spore wall after various chemical treatments

Calcofluor- and FITC-WGA-staining were used to check the influence of different agents on the spore wall material. It was difficult to interpret the results since only a portion of the spores was labelled. Estimation of the

Table 1. Quantitative and qualitative results of staining spores with Calcofluor White M2R or FITC-WGA after treatment with different agents. Quantitative values in %; qualitative data: +++ (intensive), ++ (medium), + (weak), + - (variable), - (none)

	Calcofluor White		FITC-WGA	
	qualitative	quantitative	qualitative	quantitative
0.1 M phosphate buffer pH 7.2 (control)	20	+ -	10	+ -
0.2 % proteinase K	50	+ -	20	+ -
1 N HCl	70	+	50	+ -
acetone 100 %	50	+ -	80	+
chloroform 100 %	70	+ -	50	++
isopropanol 100 %	50	+ -	90	+ -
methanol 100 %	50	+ -	100	+++
xylene	60	+ -	50	+

Table 2. Quantitative and qualitative results of staining spores with Calcofluor White M2R or FITC-WGA after treatment with different agents and subsequent incubation with chitinase. Quantitative values in %; qualitative data: +++ (intensive), ++ (medium), + (weak), + - (variable)

	Calcofluor White		FITC-WGA	
	quantitative	qualitative	quantitative	qualitative
0.1 M phosphate buffer pH 7.2 (control)	20	+ -	20	+ -
0.2 % proteinase K	20	+ -	20	+
acetone 100 %	50	+ -	10	+
chloroform 100%	50	+ -	10	+
isopropanol 100 %	20	+ -	20	+ -
methanol 100 %	50	+ -	10	+
xylene	50	+ -	10	+ -

percentages and fluorescence intensities of marked spores are given (see Tables 1, 2).

Enzymes (proteinase K, chitinase) or 1 N HCl (Tables 1, 2). Incubation of spores with proteinase K prior to Calcofluor-labelling resulted in a higher percentage of stained spores (50 % instead of 20 %, Table 1). A preceding hydrolysis with HCl increased the positive spore yield to 70 %, which demonstrates that Calcofluor can bind to the wall material much better if proteins are removed (at least partially) before staining.

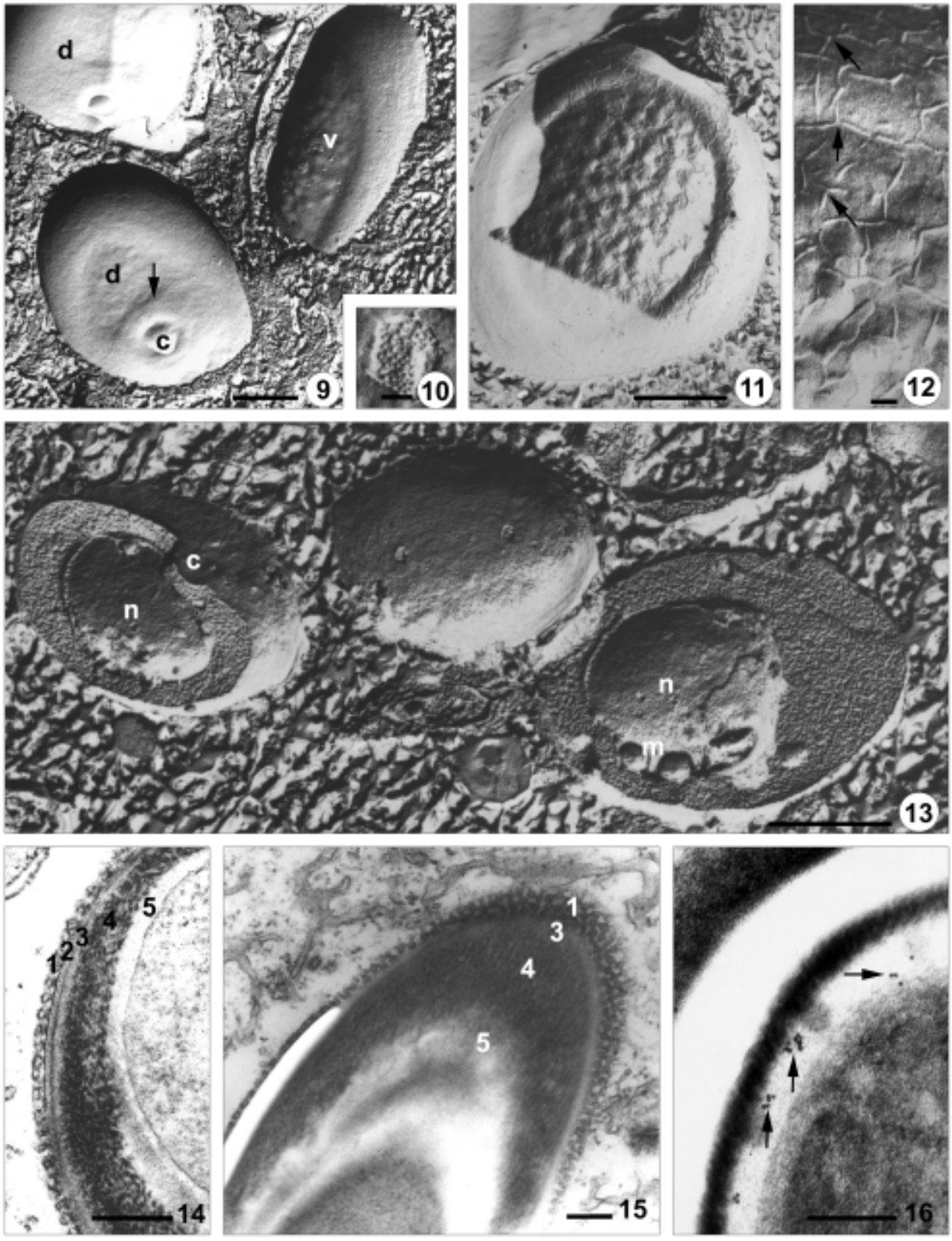
Incubating with chitinase (Table 2, control), however, did not lower the percentage of Calcofluor-stained spores (20 %) compared to untreated specimens.

Proteinase treatment followed by chitinase (Table 2) led to a reduction of stained spores (to 20 %) compared to their number after proteinase incubation alone (50 %).

Organic solvents (Table 1). In order to show whether diffusion barriers such as hydrophobic surface layers (e.g. waxes, lipids) or close physical aggregation and chemical interactions of wall material hampered the diffusion of stains, *Nephridiophaga*-spores were treated with different organic solvents and then stained with Calcofluor or FITC-WGA.

The application of alcohols (methanol, isopropanol) or acetone elevated the percentage of spores stained with Calcofluor to 50 % (control 20 %). Xylene treatment delivered 60 % and chloroform treatment 70 % fluorescent specimens. Labelling with FITC-WGA resulted in an even higher percentage of positive spores (control 10 %) in most cases. Especially after incubation in acetone (80 %), isopropanol (90 %) and methanol (100 %), most spores were stained. The staining intensity was also remarkably strong, particularly in the methanol and chloroform assays.

Organic solvents followed by chitinase treatment (Table 2). The goal of these experiments was to find out whether removal of hydrophobic surface layers could allow chitinase to attack chitin in the spore walls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more effectively. Using Calcofluor, the percentage of positive spores was either the same (acetone and methanol, 50 %) or lower (for chloroform 50 % instead of 70 %; isopropanol 20 % instead of 50 %; xylene 50 % instead of 60%) compared with staining after treatment with solely organic solvents. Labelling with FITC-WGA, however, resulted in a very distinct decline of positive spores in all treatments, and staining intensities also declined (see Table 2). This means that chitinase would function much better if hydrophobic substances were removed from the spore surfaces; the increased degradation of chitin resulted in a fainter staining.

Ultrastructure of spore walls

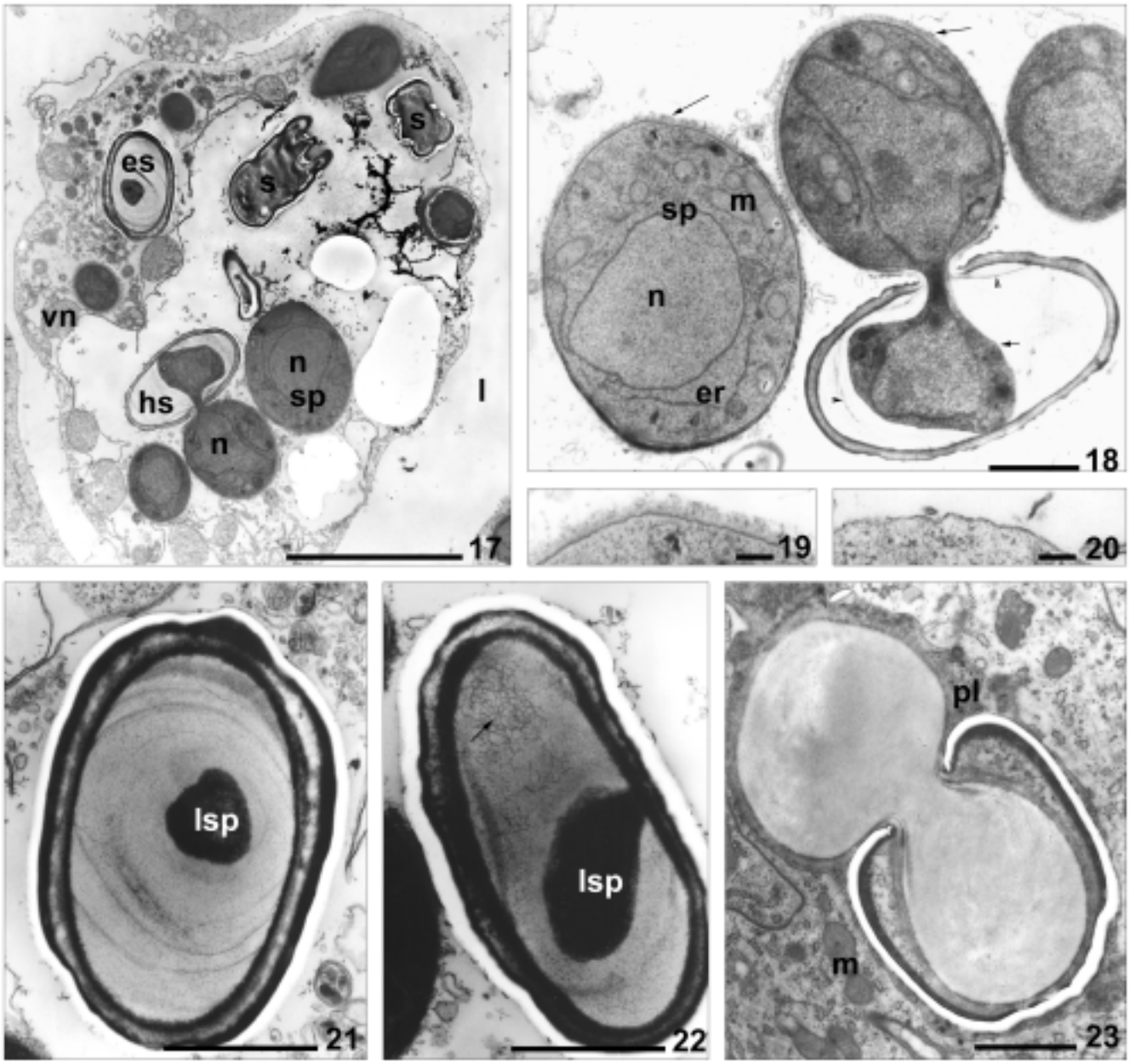
Spore wall morphology in freeze-etched specimens. The oval spores had a thickened rim (resembling an erythrocyte) and two different flattened sides (Fig. 9). One side possessed small swellings as the sole surface structures, while the other side (defined as dorsal) was characterized by a central cap-like structure. The cap lay in the middle of a bridge-like elevation connecting the lateral rims and measured about 0.5 µm in diameter. We obtained only very few freeze-fracture views of internal wall layers. In nearly all cases the fracture plane ran within the bi-layer of the plasma membrane, exhibiting a smooth spore surface with integral membrane proteins (Figs 9, 13). Occasionally residual plaques of a vesicular layer remained at the spore surface (Fig. 10). One spore revealed a fracture plane with conspicuous rods that were arranged in a loose network (Figs 11, 12). Their length varied from about 80 nm to 150 nm (mean value 115 nm) and their diameter was about 20 nm. Most of the strands participated in an association of three strands forming a kind of triskelion with star-like protruding arms (Fig. 12). These triskelions were loosely arranged in a network. Each strand belonged to either two triskelions or serially contacted a strand of a neighbouring triskelion. The small swellings at the ventral side of the spore protruded through the meshes of the network. They had the same dimensions as the swellings, which could be seen in superficially fractured spores. In a few cases spores were broken open, revealing the nucleus and membranous organelles (possibly mitochondria) within the cytoplasm (Fig. 13). Different spore wall layers as were depicted in ultra-thin sections could not be recognized nor could the wall be clearly differentiated from the cytoplasm. The region of the central cap obviously had divergent fracturing properties, since the fracture plane took its course around that structure and not straight through it (Fig. 13).

Spore wall fine structure and immunogold labelling of chitin. Mature spores possess different wall layers (Fig. 14), which may be described as follows (viewed from outside to inside): (1) an outermost complete cover of small vesicles, 20 to 30 nm thick; (2) a unit-membrane or remnants of it; (3) several thin, indistinct homogenous layers of different electron density; (4) a thick layer (about 50 nm) of electron-dense fibres that is extremely thick at the lateral rims of the spore (about 100 nm) and surrounding the dorsal cap (about 150 nm); (5) an inner electron-transparent layer of medium thickness that makes contact to the plasma membrane. The fibrous nature of layer 4 becomes especially clear in grazing sections (Fig. 15). The diameter of the fibres is difficult to measure; they seem to be 8-13 nm thick.

Indirect post embedding labelling for chitin, using wheat germ agglutinin (WGA) and fetuin-gold on LR-White thin sections, resulted in a rather low quantity of bound gold particles. The gold particles were mainly found at the inner electron-transparent layer, i.e. layer 5 (Fig. 16).

Spore hatching (TEM). Different stages of spore hatching could be observed in a section of a sporogenic plasmodium lying in the lumen of a Malpighian tubule (Fig. 17). In addition to one spore in the process of

Figs 9-16. Ultrastructure of the spore and spore wall of *Nephridiophaga blattellae* 9-13. Freeze-fractured and freeze-etched spores. **9.** Surface views of three spores. The flattened, oval spores possess a thickened rim and two different sides. The so-called dorsal side (d) possesses a central cap-like structure (c) that is surrounded by a further elevation (arrow) while the ventral side (v) shows numerous small swellings. **10.** Residual plaques of a vesicular layer occasionally remained on the spores. **11.** This fracture reveals a network of short rods, probably close to the inner margin of the spore wall. **12.** A higher magnification of Fig. 11 better reveals the arrangement of the rods in a rough triskelion pattern (arrows). **13.** Two of the three spores are broken open, revealing nuclei (n) and mitochondria (m). Spore wall layers cannot be recognized but the cap-like structure (c) has divergent fracturing properties; the fracture goes around the cap 14-16. Thin sections of spore walls embedded either in Spurr (Figs 14, 15) or in LR-White (Fig. 16), followed by WGA and fetuin-gold labelling. **14.** The spore wall consists of five layers: 1- outermost cover of small vesicles; 2- unit-membrane; 3- several thin, homogenous zones of different electron density (are not always visible); 4- thick layer of electron-dense fibres; 5- electron-transparent layer of medium thickness. **15.** Numbers as in Fig. 14. The grazing section depicts the vesicular nature of layer 1 and the fibres of layer 4 quite well. **16.** Indirect gold labelling for chitin. Most gold particles (arrows) are found at the inner electron-transparent layer, i.e. layer 5. Scale bars: 9, 11, 13 - 1 µm; 10, 14-16 - 0.2 µm; 12 - 0.1 µm



Figs 17-23. Stages of *Nephridiophaga blattellae* spore hatching depicted in thin sections of infected *Blattella germanica* Malpighian tubules (Figs 17-22) or male accessory glands (Fig. 23). **17.** A sporogenic plasmodium laying in the lumen (l) of a Malpighian tubule contains several stages of spore hatching. The plasmodial cytoplasm is degenerated; some vegetative nuclei (vn) and other cell organelles are left. es - empty spore, hs - hatching spore, n - nucleus, s - spore, sp - sporoplasm. **18.** Serial section to accompany Fig. 17 showing a spore during the process of hatching and a completely hatched sporoplasm (sp). The hatching sporoplasm leaves the spore shell through a small, central aperture, i.e. penetrating the cap-like structure. The cytoplasm including the nucleus is squeezed through the opening. Residues of material remain in the spore shell (arrowheads). The spore wall is of relatively even thickness and consists of only one thick layer with darker borders. While the membrane of the extruding sporoplasm is smooth at the cell portion inside the spore shell (short arrow), it carries a fuzzy coat at the outer cell portion (long arrow). The completely hatched sporoplasm is totally covered by the fuzzy coat (long arrow). Its cytoplasm is not as electron-dense as it is when a sporoplasm lies within a spore, therefore its cell organelles such as nucleus (n), mitochondria (m) and endoplasmic reticulum (er) can be seen clearly. **19.** Plasma membrane of an emerged sporoplasm covered with the fuzzy coat at higher magnification. **20.** Compared to the sporoplasm membrane, the plasma membrane of a merozoite is smooth. **21.** Horizontal section through a nearly emptied spore. Concentric material remnants remain in the spore shell. lsp - leaving sporoplasm. **22.** Material remnants have a network-like appearance (arrow) in a (nearly) median section of a hatching spore. lsp - leaving sporoplasm. **23.** Empty spore inside a sporogenic plasmodium (pl) within the male accessory gland; the sporoplasm left the spore; medial section. The spore wall is thick close to the region of the spore opening. A hollow space remains in the plasmodial cytoplasm where the sporoplasm crept through. m - lamellar mitochondria of a gland cell. Scale bars: 17 - 5 μm; 18, 21-23 - 1 μm; 19, 20 - 0.2 μm

hatching, completely hatched sporoplasms and empty spores were also present. The sporoplasm left its spore shell through a narrow aperture (diameter 0.5 μm) at the centre of one spore side (Figs 17, 18). The spore opening corresponds to the round, cap-like structure of the dorsal surface, as it was seen in the freeze-etched specimens. The shape and dimensions of the spore changed during the hatching process. In median, longitudinal sections, the dormant, mature spores have thickened rims and concave dorsal and ventral sides, measuring about 4.6 to 1.2 μm. Equally sectioned hatching spores showed an oval outline of 4.0 to 2.1 μm. This means that the spore swelled; it became slightly shorter and about twice as thick. At the spore opening, spore wall portions seem to be shifted aside (Figs 17, 18). Apparently, the cap was not folded open but penetrated.

The layered structure of the spore wall was changed in the hatching spore. The outer vesicular layer disappeared, and of the three layers of different electron density (layers 3-5), only one layer with electron-dense borders remained; it had a roughly uniform thickness of about 150 nm and could not unambiguously be assigned to layer 4 or 5 (Figs 18, 21, 22). Fine remnants of material were found between the spore wall and hatching sporoplasm (Fig. 18). A horizontal section through a nearly empty spore revealed concentric material remnants, as if showing consecutive stages of the sporoplasm leaving (Fig. 21). In a nearly median section of a hatching spore, material remnants have a network-like appearance (Fig. 22). Heavily infected cockroaches may harbour sporogony stages intracellularly in male accessory glands. An empty spore was also found here (Fig. 23). Its wall layers appeared to be more intact and were of different thickness in different regions. Obviously the sporoplasm had already left its enclosure and penetrated the host cell where an electron-transparent space remained.

The emerging sporoplasm had to press its total cell mass through the narrow spore opening. Its nucleus seemed to be extremely flexible; it was heavily deformed during hatching (Fig. 18). The cytoplasm of the extruding sporoplasm was lighter than that of the dormant, mature spores, where cell organelles were hidden in the dense cytoplasm. Now the nucleus and other cell organelles such as mitochondria, endoplasmic reticulum, and ribosomes could be easily seen (Fig. 18). Completely hatched sporoplasms were even more electron-transparent, resembling the vegetative stages (Fig. 18). Their sizes seemed to be larger than the volumes of the spores. Sporoplasms closely resembled merozoites but there was at least one difference: sporoplasms possessed a fuzzy, thick membrane coat (Figs 18, 19), whereas the plasma membrane of merozoites appeared to be nearly naked (Fig. 20). The fuzzy coat appeared during the process of hatching. While the portion still inside the spore shell possessed a smooth membrane, its already extruded portion exhibited the fuzzy coat (Fig. 18).

DISCUSSION

Chemical composition of the spore wall

Woolever (1966) performed experiments to cause extrusion of the sporoplasm, but despite incubation in many different chemicals and application of physical stress no hatching took place. Methods known to cause emergence of microsporidian and myxozoan infectious stages also failed. Resistance by the spore wall is probably evoked by the presence of chemically inert and pressure-resistant materials. We tried to identify the main chemical components of the nephridiophagid spore walls.

Spores of microsporidia (Vávra 1976, Perkins 1991) and myxosporidia (Lukeš *et al*. 1993) are known to contain chitin, like the cyst or lorica walls of many ciliates, flagellates and amoebae (Mulisch 1993). Further substances have been reported from cyst and lorica walls of protists such as proteins, glycoproteins, (acid) mucopolysaccharides, silica, iron, pigments, and so on (Mulisch 1993). Woolever´s (1966) tests for chitin in the spores of *N. blattellae* did not give unequivocal results. Our experiments were also difficult to interpret. The cytochemical method (deacetylation of chitin to chitosan followed by J_2KJ - 1 % H_2SO_4) did not reveal chitin. According to Ericson and Blanquet (1969), however, although considered valid when positive, a negative test for chitosan need not be a proof for the absence of chitin. Fluorescent labelling with Calcofluor White and FITC-WGA did stain the spores, meaning that β-linked polysaccharides or more precisely N-acetylglucosamine is present.

A point of criticism when attempting to demonstrate chitin by staining with Calcofluor and WGA is that these agents are not exclusively specific for chitin, although they are often used to label it (Lukeš *et al*. 1993). Calcofluor also interacts with other β-linked D-glucopyranose polysaccharides, especially cellulose (Pringle *et al*. 1989). The interaction with β-1,3-linked polymers is, however, significantly weaker than that with β-1,4-linked

polymers, so that e.g. the glucan of the *S. cerevisiae* cell wall is weakly stained or totally undetectable (Pringle *et al*. 1989). However, the presence of cellulose as the major β-1,4-linked polymer besides chitin, which binds to Calcofluor, was ruled out by the negative histochemical test and by the specific birefringent properties of the spores in the polarizing microscope. The relative optical character of chitin in water is positive, but is negative in highly refractive embedding media (the same applied to the spores), whereas cellulose is birefringent in both media (Schüβler *et al*. 1994). The lectin WGA has a nominal specificity for N-actetylglucosamine, the main component of chitin. However, N-actetylglucosamine may also be part of other saccharides when in its monomeric form, and WGA also has a low binding affinity to other sugars, such as sialic acid residues. In agreement with Lukeš *et al*. (1993), we think that the fact that both stains (Calcofluor and FITC-WGA) label the spore walls strongly supports the hypothesis that chitin is present.

One fact making staining results difficult to interpret is that staining did not follow an "all-or-nothing" pattern; the intensity and the percentage of stained spores varied. We believe that there are several possible factors, which may account for this fact: 1. The maturation status of the developing spores; young spores may not contain enough chitin to be labelled; spores of medium age may be permeable for stains in contrast to impermeable, fully developed spores. 2. Slight injuries of resistant, mature spore layers might be necessary to allow stains to penetrate so that only injured stages can be positively stained. 3. Diffusion of stains may be hampered within sporogenic plasmodia, leading to negative results of intracellular stages. A less intense staining of immature spores was also shown for microsporidian spores (Vávra *et al*. 1993). The same authors showed that staining intensity is dependent on the composition of the Calcofluor-containing medium, on alkaline pre-treatment, and on the age and formulation (fresh, fixed, smear) of the spores. There is also evidence that chitin is not always accessible to WGA (Barkai-Golan and Sharon 1978). In our experiments, judging the percentages of stained spores seemed to deliver clearer results than estimation of fluorescence intensity. FITC-WGA-staining was always fainter than Calcofluor-fluorescence. Maybe the larger size of the lectin [molecular weight of WGA 36000 D (Sigma); WGA dimer measures 4 x 4 x 7 nm (Reeke and Becker 1988)] compared with the smaller Calcofluor-molecule (molecular weight 917 D) makes diffusion of the lectin into the spore wall more difficult.

Chitin is generally associated with other substances, such as extensively cross-linked proteins in animals, or with other polysaccharides in fungal cell walls (Muzzarelli 1977, Blackwell 1982, Giraud-Guille and Bouligand 1986). The protein is thought to protect against attacks by chitinases (Blackwell 1982), and after sclerotization of the proteins, also to give hardness and rigidity to the spores (Muzzarelli 1977). These close associations of chitin with other substances may impede access of the stain to the spore wall and the action of added enzymes on its components, as was the case for example in our chitinase experiments in which the percentage of Calcofluor stained spores was not lowered. Different treatments were performed in order to solubilize obstructing substances and thus ameliorate chitin staining. Protein has histochemically been shown to be a spore wall component (positive Ninhydrin-Schiff-reaction) and, in fact, the removal of at least part of the protein by the application of proteinase K doubled the percentage of stained spores (Table 1). Further treatment with chitinase lowered the amount of positive spores, suggesting that chitinase was then able to degrade part of the chitin (Table 2). In addition to chitin and proteins, β-glucan might also be a component of the matrix as in some fungal cell walls, e.g. oomycetes (Muzzarelli 1977). In this case, treatment with glucanase would have been essential before chitinase could effectively attack the chitin. Alpha-mannans are probably either not present in the spore wall at all or are only a minor component, since the spores stained only very faintly with FITC-Con A (Barkai-Golan and Sharon 1978).

In addition to a close association of proteins with chitin conferring a certain staining resistance to the spores, diffusion of stains may be hampered by hydrophobic substances on the surface of the spores. Deposition of substances such as waxes or lipoproteins on the chitin-protein complexes can make the structures impermeable (Muzzarelli 1977). In order to remove these hydrophobic substances, different organic solvents were applied to spores prior staining and incubation in chitinase. Although these solvents all improved staining, the effect of a subsequent chitinase treatment was ambivalent. Labelling with FITC-WGA was less intense, suggesting that chitinase had acted, but labelling with Calcofluor was rather strong. The explanation could be that the effect of partial chitin degradation was compensated by a better accessibility of remaining molecules to Calcofluor.

Going a step further, we tried to assign the presumed chitin-label to a certain layer of the spore wall. Thin sections indirectly labelled with WGA and fetuin-gold showed a rather faint staining, but most gold particles were found at the inner electron-transparent layer (layer 5), which thus seemed to be the chitin-containing structure. This situation is similar to that found for microsporidia, which possess an outer proteinaceous layer (exospore) and an inner, electron-transparent chitin-containing layer (endospore) (Larsson 1986a, Perkins 1991). In general, chitin fibrils show no contrast in thin sections; the nephridiophagid layer 5 was equally electron-transparent.

Besides chitin and protein, calcium was also demonstrated to be a component of the spores. It probably contributes to the mechanical stability of the spore. The chitinous exoskeleton of invertebrates also possesses a calcified matrix, e.g. in crustacean shells (Blackwell 1982).

Electron microscopy of spore walls

Thin sections revealed different wall layers of the spores including a thick, fibrous layer and a more homogenous, electron-transparent layer reminiscent of the exo- and endospore of microsporidia (Vávra 1976, Larsson 1986a). Spore walls of nephridiophagids are only briefly described in the literature as being an electron-dense granulo-fibrous layer (Toguebaye *et al*. 1986) or a double-layered wall (Purrini *et al*. 1988, Purrini and Weiser 1990). The chemical nature and especially the presence of chitin could not be assigned to any specific layers by routine electron microscopy. Chitin fibres are generally thought to be electron-transparent. For example, microfibrils in the cuticle of arthropods (Giraud-Guille and Bouligand 1986) or the spores of fungi (Schüßler *et al*. 1994) appear as transparent rods surrounded by opaque material, a pattern that according to some authors seems to be evoked by a central chitin crystallite surrounded by protein. However, according to Giraud-Guille and Bouligand (1986), chitin covalently linked with protein *via* aspartic acid (polar amino acid) is incorporated in the dark phase. The irregularly arranged fibres of the *Nephridiophaga*-spores (layer 4) are completely electron-dense. This means that if chitin is contained in these fibres, hydrophilic (contrastable) substances must be associated. The fibre diameter of 8-13 nm fits to the size spectrum of chitin microfibrils: insect cuticles 2.5-3 nm, crustacean and fungi 20-25 nm, lorica of the ciliate *Eufolliculina uhligi* 15-20 nm (Muzzarelli 1977, Mulisch 1993), microsporidian spores 8-9 nm (Vávra *et al*. 1986). Another idea is that the nephridiophagid fibres are proteinaceous. WGA-gold staining results seem to indicate the presence of chitin in the homogenous, electron-transparent wall layer (layer 5) of the spores. The electron-transparent appearance of that wall layer may be due to a high amount of chitin.

The outermost layer of the spores consists of a single layer of small vesicles (20-30 nm in diameter), which appears when the spore coat begins to develop, and disappears when the spores are fully mature (Radek and Herth 1999). The vesicles may transport the spore wall material to the growing spore wall as was also assumed for *N. ormieresi* (Toguebaye *et al*. 1886) and *N. periplanetae* (Lange 1993). In fungi, chitin synthetases are bound to the plasma membrane to which they are transported by small vesicles (diameter less than 100 nm), the so-called chitosomes (Kamada *et al*. 1991); perhaps the nephridiophagid vesicles have a similar function.

Freeze-fracture studies did not reveal many details of the different spore wall layers. Interestingly, one specimen showed rods measuring 80-150 x 20 nm that were arranged in a triskelion-like network. They could represent a sub-membrane skeleton stabilizing the shape of the spore. Rod-like structures of a similar size range (120 x 50 nm) were described from the inner limiting layer (unit-membrane) of a microsporidian spore coat. Different functions have been suggested: anti-slipping device to prevent the spore content from spinning (Liu and Davies 1973); synthetic activity or material transport during spore-coat deposition (Liu 1975).

Spore hatching

Generally, the nephridiophagids are believed to have no spore opening (Toguebaye *et al*. 1986) or lid (Undeen and Vávra 1997). However, Gibbs (1959) saw sporoplasms of *Coleospora binucleata* hatching through a central opening with a plug while empty spores with polar openings were reported for *Nephridiophaga periplanetae* (Ivanić 1926) and *Oryctospora alata* (Purrini and Weiser 1990).

We were able to demonstrate spore hatching at the ultrastructural level and prove the existence of a defined spore opening for the first time. The infectious sporoplasm can emerge at the thin-walled central cap. A zone with thinner or missing wall layers through which an infectious stage can break seems to be a general feature of spores of organisms of different systematic groups [e.g., haplosporidians (Desportes and Nashed 1983),

apicomplexans (Perkins 1991)]. In addition, microsporidian sporoplasms hatch through a thin spore wall region at the anchoring disk of the polar filament (Weiser und Purrini 1985, Larsson 1986b). According to Weidner and Halonen (1993), the outer spore envelope of the microsporidian *Spraguea lophii* completely disassembles at the time of spore activation and the authors suggested that keratin of the spore envelope disassembles and phosphorylates. The spore wall of *N. blattellae* also lost part of its layers during hatching.

The infectious stage of *N. blattellae* seems to incorporate water during hatching; the sporoplasm swells and becomes less electron-dense. In microsporidia a rise in internal osmolarity leads to water uptake and pressure increase, resulting in the emergence of the polar filament through which the infectious sporoplasm exits (Weidner 1976, Perkins 1991).

The hatched sporoplasms of *N. blattellae* possessed a thick, fluffy surface layer at their plasma membranes. At the emerging sporoplasm, this layer was only recognized at the portion that had already left the spore shell. It is uncertain whether this layer is a glycocalyx. A quick synthesis of glycocalyx material during the passage through the spore opening seems unlikely.

Outlook

New information concerning the structure and chemical composition of the nephridiophagid spores and spore hatching could be gathered, but some questions remain. Nothing is known about the triggers of the spore hatching, the mode of movement of the sporoplasm and other stages, and the way in which the infectious sporoplasm takes in the insect body to infect the Malpighian tubules is speculative. The components of the spore walls also need be further studied to finally characterize the substances and their distribution within the wall.

Nephridiophagids share some features with the microsporidia, for example spore wall characteristics (exo-, endospore, presence of chitin and protein) and parts of the life cycle (merogony, sporogony leading to several spores stemming from one mother cell, oral infection). The fine structure of the two spore types, however, is completely different. Unless molecular data is gathered, the systematic affiliation of the Nephridiophagids will remain uncertain.

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