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Differentiation of Microsporidian Spore-Tails in *Inodosporus spraguei* Gen. et Sp. N.

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Summary

The new genus *Inodosporus* was erected to accept *I. spraguei*, a new species having eight sporoblasts per pansporoblast with each subsequent spore possessing three or four basal spore-tails and one branched apical one. It is primarily by the apical tail that the species is separated from the only other recognized species, *I. octospora* (Henneguy, 1892) comb. n., formerly *Thelohania octospora*.

Spore-tails of *I. spraguei* are membranous channels which originate within differentiating pansporoblasts during genesis of sporonts into sporoblasts. During the switch from vegetative to spore-forming development, cytoplasmic constituents of *I. spraguei* segregate into two distinctive domains for which we originate the terms "pansporoblast-determinate area" (PDA) and "sporont-determinate area" (SDA). Membrane channels, which form spore-tails, develop within the PDA.

The following observations indicate that the tails of *I. spraguei* are continuous with the outer pansporoblast envelope: lanthanum marker readily penetrates pansporoblasts and localizes in channels, in spore-tail attachment points, and between extra-sporoblast membrane and sporoblasts; a positive reaction for adenosine triphosphatase product accumulates within spore-tails at their sites of attachment to sporoblasts; and spore-tails occasionally remain attached to pansporoblast envelopes after mechanical disruption.

An extensive PAS-positive glycocalyx-like material is found within newly developing pansporoblasts. This observation, plus the presence of an apparent adenosine triphosphatase system on pansporoblast membranes, indicates that the pansporoblast may serve as a molecular or ion transport system during initial phases of sporont differentiation.

Inodosporus spraguei infects each muscle fiber completely until filaments are destroyed, and infections are spread throughout the animal until most fibers are infected. Curiously, uninfected muscle cells seldom show serious pathological changes caused by massive infections of neighboring cells.

Introduction

Thin external membranous appendages, or tails, occur in some unrelated microsporidian species (Vávra, 1963). The tails, at least for the species reported herein, constitute manifestations of a developmental pattern associated with certain pansporoblast-producing microsporidians. Pansporoblasts are growth chambers in which spores develop; currently, the pattern, number, and manner of development of the spores in these chambers represent the means for differentiating taxonomic groupings.

Spore-tails of the present species consist of membranous canals that extend throughout the pansporoblast. They have integrity even after collapse of the pansporoblast, whereas in some other species (Vávra, 1968), the tails readily drop off their associated spores and disintegrate along with the pansporoblastic envelope at the termination of spore-development. Stability of the tail depends upon the nature of the coat at its surface, the surface of the spore, and the surface of the pansporoblastic envelope.

Results on the study of the developmental biology of pansporoblasts, spores, and spore-tails remain recent conquests; nevertheless, the information now available indicates that these approaches are among the best for distinguishing microsporidian categories. In this study of a new microsporidian species that infects muscle tissue of grass shrimps, we found a developmental pattern of the spore-tail and pansporoblast which differs from all previous reports, and we hereby erect a new genus for that species.

Materials and Methods

We examined fresh microsporidian material from three specimens of *Palaemonetes pugio* from estuarine areas in Ocean Springs, Mississippi, using Nomarski interference-contrast microscopy, staining some with Turtox CMC-S mountant or Leifson-modified flagellar stain. Additional cells were fixed for electron microscopy with buffered 3% glutaraldehyde or 10% buffered formalin prior to the glutaraldehyde. After OsO_4 post-fixation, alcohol dehydrations, and propylene oxide transfers, the cells were embedded in Maraglas or Epon. Additional material in *P. kadiakensis* from the Little Brazos River, Texas, was studied exclusively with light-microscopy.

Adenosine triphosphatase activity was visualized by a modification of a method developed by Marchesi and Palade (1967). Cells were fixed for 2 to 4 min in glutaraldehyde, thoroughly washed in Tris-maleate buffer for 12 hrs, and suspended in a working medium as described by Wachstein and Meisel (1957). We used the method for the Periodic acid-Schiff reaction (PAS) presented by Pearse (1968).

To determine whether continuity exists between the exterior envelope of the pansporoblast and the spore or sporoblast, procedures using lanthanum tracers were tested. Following the method of Neaves (1973), lanthanum hydroxide was prepared by slowly adding 0.01N NaOH to 2% $La(NO_3)_3$ until the solution reached pH 7.8. This solution was mixed with an equal volume of cacodylate-buffered glutaraldehyde. We obtained additional preparations using S-collidine (0.2M) pH 8.0 plus 2% $La(NO_3)_3$. Lanthanum was mixed with the fixatives through the washes and through the dehydrations with alcohol.

We measured material with the light-microscope in fractions of a micron and used those values for obtaining averages but often rounded individual values to the nearest μm in the description.

Taxonomy

Codreanu (1966) divided, as we concur, nosematids whose sporonts form eight spores, each of which possesses basal acicular appendages, into a separate genus. He, however, assumed that *Thelohania octospora* Henneguy, 1892, presumably a tailed species, was the type-species of *Thelohania* Henneguy, 1892, and erected *Parathelohania* Codreanu, 1966, with *P. legeri* (Hesse, 1904) as type-species for species with spores having naked walls. Unknown to Codreanu, Gurley (1893) designated *T. giardi* Henneguy, 1892, as the type-species. Sprague (1970a, 1970b) pointed out the mistake and considered *Parathelohania* a synonym of *Thelohania*. He now (personal communication), however, accepts the genus, but only because *P. legeri*, in addition to lacking tails, has two different types of spores (Hazard and Weiser, 1968), a fact which justifies keeping it separate from species of *Thelohania*. We now, assuming *T. giardi* is tailless, erect a genus to accommodate related species with tails.

***Inodosporus* gen. n.**
Diagnosis: Nosematidae. Mother cell developing into eight sporoblasts and subsequently into eight spores. Pansporoblast with persistent membrane. Spore possessing elongated external appendages. *Type species:*

Inodosporus spraguei sp. n.
Description (based on over 100 measurements of most characters using fresh material from three individual hosts from Mississippi with average measurements in parentheses): Pansporoblasts associated with numerous dark chromatophores, those with mature spores nearly spherical, 6 to 9 μm (7.9 μm) long by 6 to 8 μm (7.1 μm) wide; membrane considerably resistant to decay, still intact after pansporoblasts refrigerated several weeks. Spores pyriform, 2.0 to 3.7 μm (2.9 μm) long by 1.7 to 2.5 μm (2.0 μm) wide; refractive ability retained after polar filament extruded. Polar filament uniformly thin, 33 to 50 μm (37.0 μm) long. Sporoplasm transmitted through filament. Basal projections 3 or rarely 4 in number, only 2 situated in same plane, tubular, thin, nearly uniform in thickness unless dried out, then gradually widening to proximal end and much wider throughout, 17 to 33 μm (23.5 μm) long (Figs. 1, 2), resistant to decay. Anterior external projection with proximal portion uniformly wide (wider than basal projections), 0.3 to 1.5 μm (0.9 μm) long, branching to form 2, or possibly more in some individuals, thin inconspicuous filaments 2 to 7 μm (4.5 μm) long; branches usually differing in length.

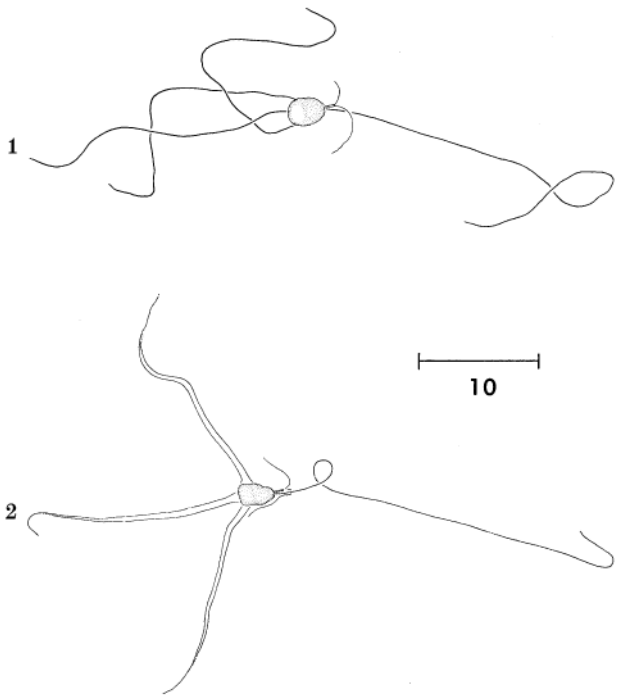


Figure 1. *Inodosporus spraguei*, the same microsporidian as in all figures to follow. Fresh spore with extruded polar filament.

Figure 2. Air-dried specimen illustrating expanded spore-tails. Scale is μm.

Based on electron micrographs: Spore 2.7 to 3.0 μm long by 1.6 to 1.9 μm wide; composed of sporoplasm, extrusion apparatus, and spore-wall. Intraspore sporoplasm dispersed around extrusion apparatus; cytoplasm containing numerous ribosomes; nucleus single, about 1.0 μm in diameter, containing dispersed euchromatin. Extrusion apparatus composed of polar sac, polar aperture (2000 Å in diameter), polaroplast, polar tube, and posterior vacuole. Polar tube (filament) 900 to 1000 Å in diameter, with basic microsporidian pattern, but with 4½ to 5 coils. Vacuole difficult to distinguish except with light-microscopy. Spore-wall consisting of smooth outer coat 250 to 300 Å thick, lucid space 1000 Å thick, and interior plasma membrane.

Hosts and localities: Palaemonetes pugio Holthuis, grass shrimp (Palaemonidae), from Davis Bayou, Ocean Springs, Mississippi (type-host and type-locality); *P. kadiakensis* Rathbun from Little Brazos River, Brazos County, Texas.

Site: Abdominal musculature.

Incidence: 3 of several 1,000 shrimp examined from Mississippi, also rare in Texas.

Syntypes: US National Museum No. 311521. Formalin-fixed material, Leifson-modified flagellar stained material, and epon embedded material.

Remarks: The name *Inodosporus* is derived from the Greek names *inodes* meaning "filamentous" and *sporos* meaning "spore." It is treated as masculine in gender and refers to the tailed spore, a diagnostic character of the genus. The species honors Victor Sprague of

the University of Maryland, Natural Resources Institute, for his pioneering work on decapod microsporidians.

Discussion

*Inodosporus sprague*i, rare in estuaries near Ocean Springs, is probably more common in freshwater habitats. S. K. Johnson graciously sent us on two occasions fixed and fresh material from *Palaemonetes kadiakensis* collected in freshwater from Texas. His material agreed with ours (see Table 1) but included, in addition, a few megaspores 4.4 to 4.9 by 2.9 μm. The bluish-black pigmentation in chromatophores of *P. kadiakensis* was more pronounced than in those of *P. pugio*. Shrinkage of spores due to formalin was minimal, except for the tails, which were considerably shorter. In fixed material, tails rarely remained on spores released from the pansporoblasts but occasionally were observed extending from pansporoblasts. Measurements include those attached directly to spores only.

Table 1. Meristics in μm of 10% formalin-fixed and fresh *Inodosporus spraguei* in *Palaemonetes pugio* from Ocean Springs, Mississippi, and in *P. kadiakensis* from Little Brazos River, Texas

	Fresh				Formalin-fixed			
	Mississippi		Texas		Mississippi		Texas	
	range	average	range	average	range	average	range	average
Pansporoblast, length	6–9	7.9	6–9	6.9	6–7	6.8	6–9	7.4
Pansporoblast, width	6–8	7.1	5–7	6.0	4–7	5.5	5–7	6.0
Spore, length	2–4	2.9	3–4	2.9	2–3	2.6	2–3	2.8
Spore, width	2–3	2.0	2–3	2.1	1–2	1.8	1–2	1.9
Tails	17–33	23.5	14–26	17.8	12–18	16.1	12–16	14.8
Polar filament	33–50	37.0	28–58	37.3				

The only other known related tailed species is *Thelohania octospora*, which we now transfer to the genus *Inodosporus* as *Inodosporus octospora* (Henneguy, 1892) comb. n. Ours differs from it primarily by possessing an anterior appendage. Neither Pixell-Goodrich (1920), the first to observe the tails in *I. octospora*, nor Codreanu (1966) observed such an appendage. It cannot be stated with certainty that either author actually was studying *I. octospora*, a species originally described without tails from France, and it is for that reason and the fact that *I. spraguei* is better described that the latter was chosen as the type-species. Pixell-Goodrich (1920) used dilute iodine and blue-black ink to discern the tails on specimens from England, and Codreanu (1966) measured tails 10 to 18 μm long, shorter than for *I. spraguei*, on spores from the Black Sea. Being as critical as they were, one of them should have noticed an anterior filament, if it was present. Both authors presented measurements similar to ours for spores and polar filaments. Pixell-Goodrich, however, found megaspores 5–6 μm long which were larger than anything in our collections. Typical spores from the United States, England, and the Black Sea measure about 1 μm less than from *I. octospora* as originally described (Thelohan, 1894) (spores 3 to 4 μm long in pansporoblasts

10 μm in diameter). The reported hosts for *I. octospora* are related to, but not in the genus *Palaemonetes*.

While on sabbatical leave in France, Victor Sprague attempted unsuccessfully to find specimens and ascertain for us whether *I. octospora* had an anterior projection and whether *T. giardi* had tails of any type. Both these problems still need solving. Codreanu (1966) suggested unnoticed basal filaments might exist on some of the other species of *Thelohania* from decapods. We know for sure that such appendages do not occur on spores of *T. penaei* Sprague, 1950 in *Penaeus setiferus* or another species of *Thelohania* from *P. pugio* in Mississippi to be described later.

Development

Observations and Results

The vegetative stage of *I. spraguei* is characterized by a single plasma membrane surrounding the cytoplasm, which contains numerous ribosomes and profiles of endoplasmic reticulum. In addition, nuclei are paired (diplokaryons) and mitochondria are absent.

During the switch from vegetative (Fig. 3) to spore-forming development, the cytoplasmic constituents of *I. spraguei* segregate into two distinctive domains (Fig. 4): a pansporoblast-determinate area (PDA) and a sporont-determinate area (SDA). The PDA develops a spatially arranged meshwork of fine dense elements (Figs. 5–7), which measure approximately 180 to 200 Å in diameter and number 6 to 8 per μm^2. These elements are suspended in a homogeneous PAS-positive matrix.

After formation of pansporoblastic elements and matrix, a second membrane organizes around the sporont (Fig. 8). Figs. 9 and 10 are interpreted to reveal the parasite before and after addition of an exosporont membrane (ESM). During the development of the ESM, a network of small membranous channels organize near the dividing sporont (Fig. 8), within the PDA matrix (Fig. 10), and along the perimeter of the pansporoblast (Figs. 8, 10). The channels grow and organize into an elaborate canal-system which extends into the pansporoblastic chamber from the ESM (Figs. 11, 12) and differentiates into the spore-tails (Figs. 10–12).

Early in development, an extramembranous coat forms on the ESM; on the outer surface of the canal-system within the PDA (Fig. 14); and on the outer envelope of the pansporoblast (Figs. 8, 13, 14). Ultrastructural examination indicates that the coat material for these are the same, although the pansporoblast-envelope has an additional canopy of tubular elements extending outwardly and perpendicularly to the exterior surface of the parasite (Fig. 13). The tubules, consistently 400 to 500 Å in length, become assembled during the formation of the extramembranous coat. Other tubular elements considered as microtubules have a diameter of 180 to 200 Å and appear as a network within the funnel-shaped proximal end of the basal spore-tails (Figs. 15a, b); these microtubules, however, are lacking within the truncated apical spore-tail (Figs. 16a, b).

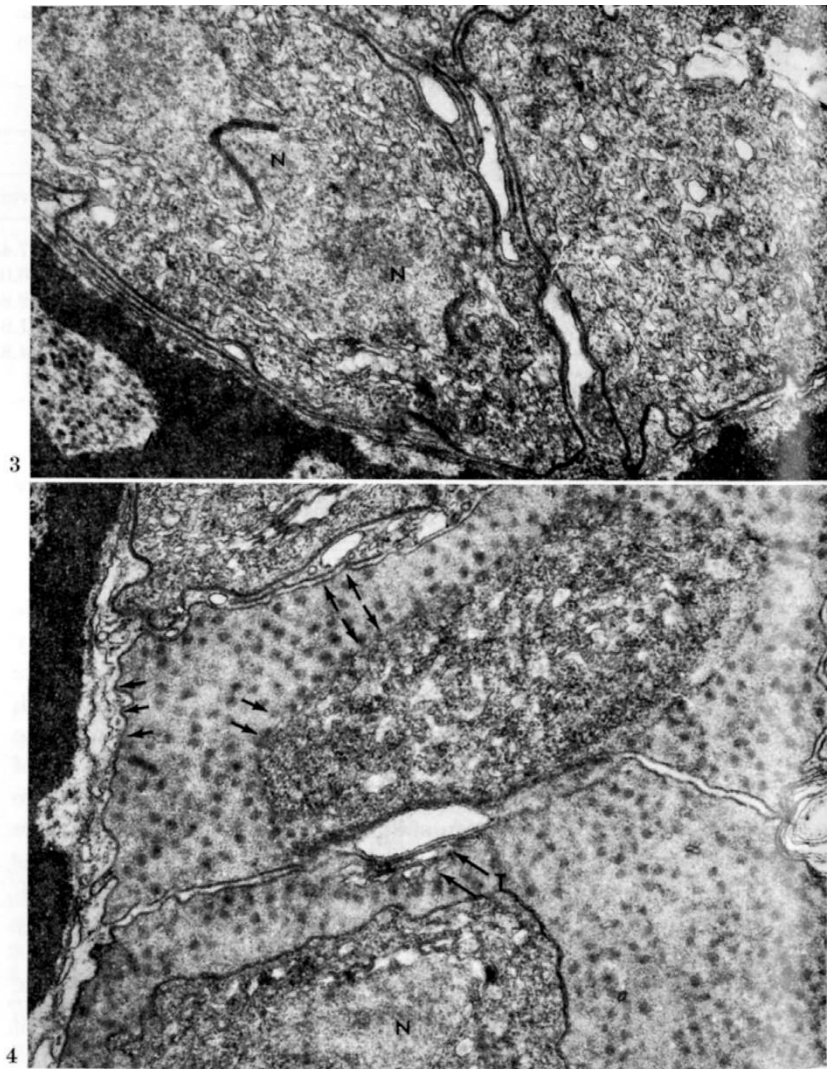


Figure 3. Vegetative stage near nucleus of muscle cell (*M*). Nuclei (*N*) of parasites are paired. ×17600

Figure 4. Vegetative cell segregated into pansporoblast-determinate area, *PDA* (between sets of arrows), and sporont-determinate area, *SDA*. Sporont in lower cell shows nucleus (*N*). Membranous channels (*T*) beginning to form at perimeter of PDA. ×26400

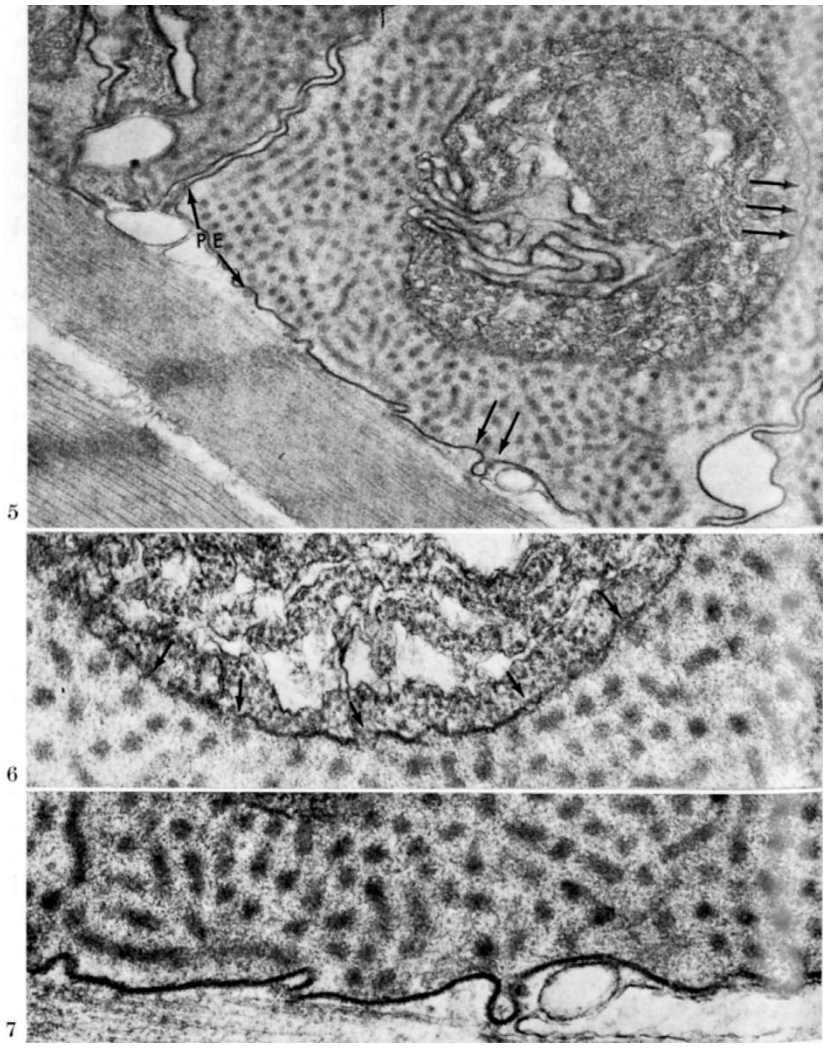


Figure 5. Parasite with *PDA* and *SDA*. Note the network display of small dense elements extending throughout *PDA*. Set of three arrows indicates delimiting membrane of *SDA* and set of two arrows indicates that for *PDA*. Pansporoblast envelope (*PE*) extends to host muscle. ×22880

Figure 6. Enlargement of area with set of three arrows in Figure 5 showing single membrane of *SDA*. There are indications (arrows) that dense elements may extend from *SDA* envelope. ×48400

Figure 7. Enlargement of area with set of two arrows in Figure 5 showing single envelope of *PDA*. Membrane thickness is probably attributable to presence of extraneous coat. The homogeneous material within the *PDA* is *PAS*-positive. ×48400

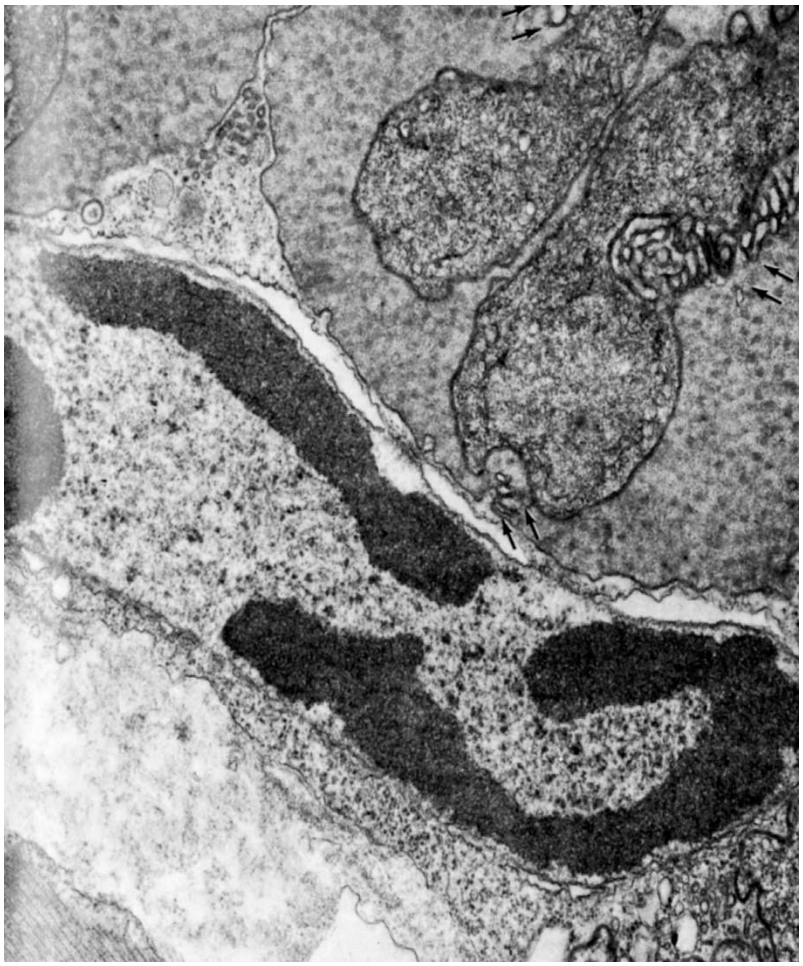
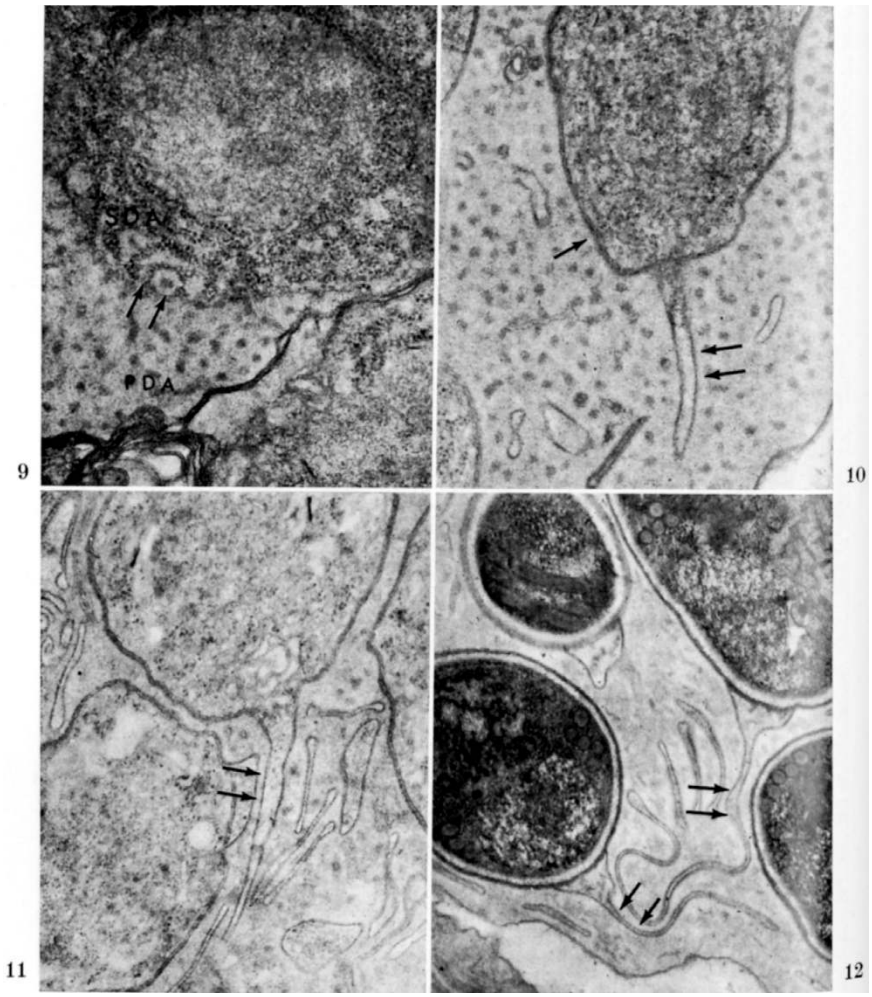


Figure 8. Overall view of *PDA* and *SDA* within host cell. Note a second envelope surrounds the sporont, and a network of small channels appears to be organizing at perimeter of *PDA,* adjacent to *SDA,* and around dense elements (see arrows). Note close proximity of envelope of slightly-hypertrophied host nucleus. ×24600



Figures 9–12. These figures interpret the differentiation of *SDA* and *PDA* into spores and pansporoblasts. Figures 9 and 10 reveal parasite before and after addition of exosporont membrane. Arrows indicate development of originating membrane as a network extending into *PDA*. ×22880. Figures 10–12. Progression of channel development into spore-tails. Latter two ×18480

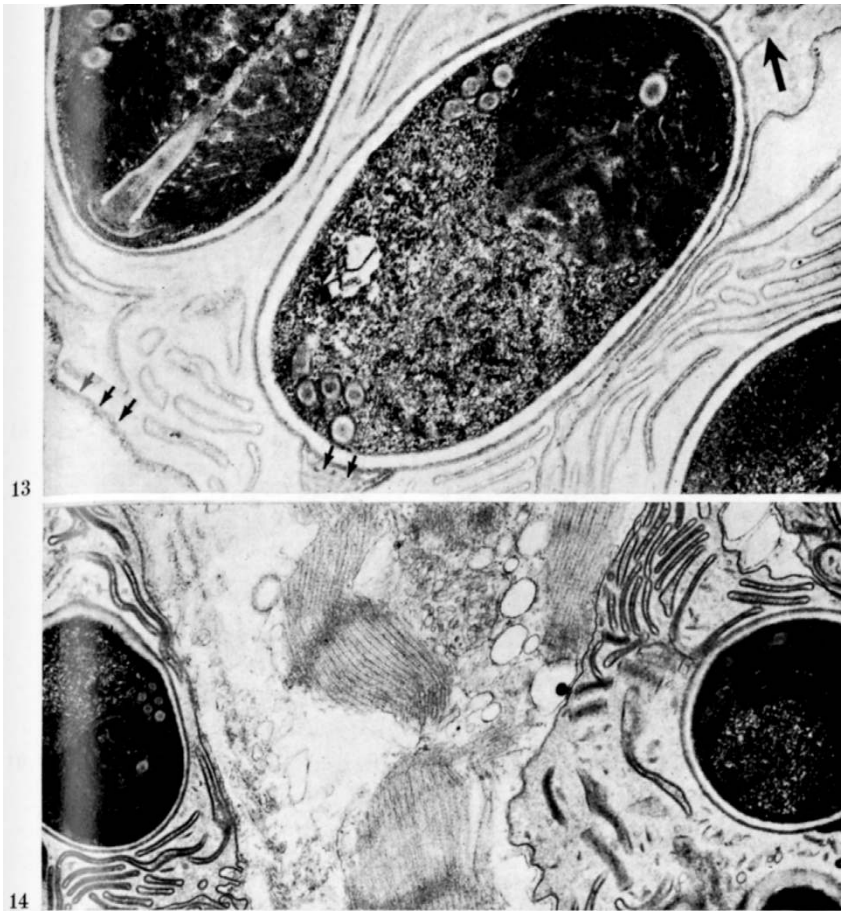
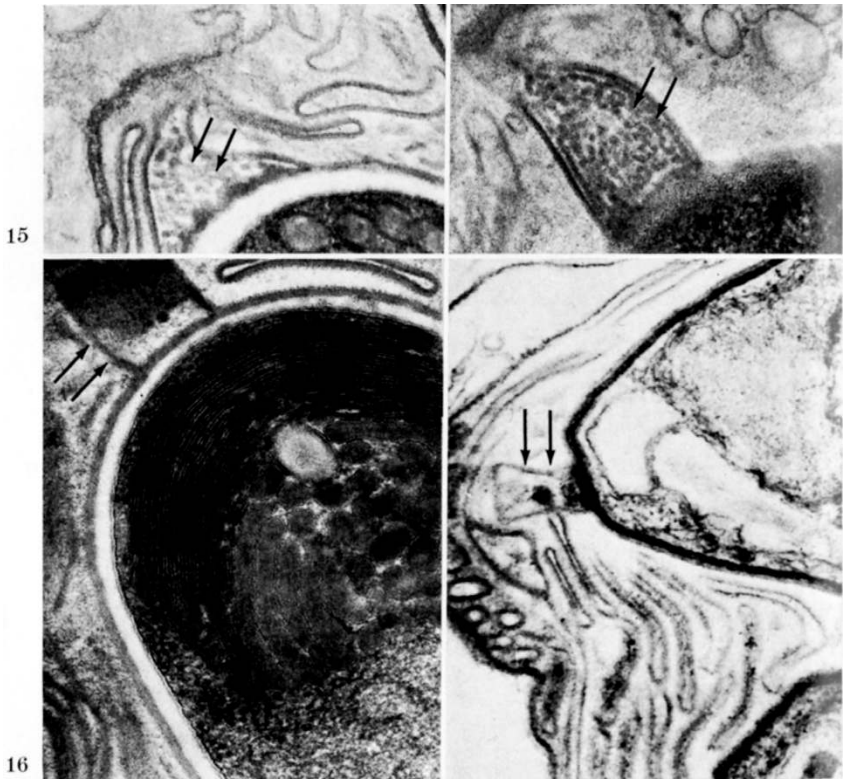


Figure 13. Mature pansporoblast with view of spores. Note microtubules within basal tail where tail attaches (paired arrows), lack of microtubules within apical tail (single large arrow), and microtubule-like structures at perimeter of pansporoblast envelope (set of three arrows). ×33440.

Figure 14. Two mature pansporoblasts with internal profiles of spore-tails separated by degenerating muscle. Note glycocalyces at surface of spore-tail channels. ×17600

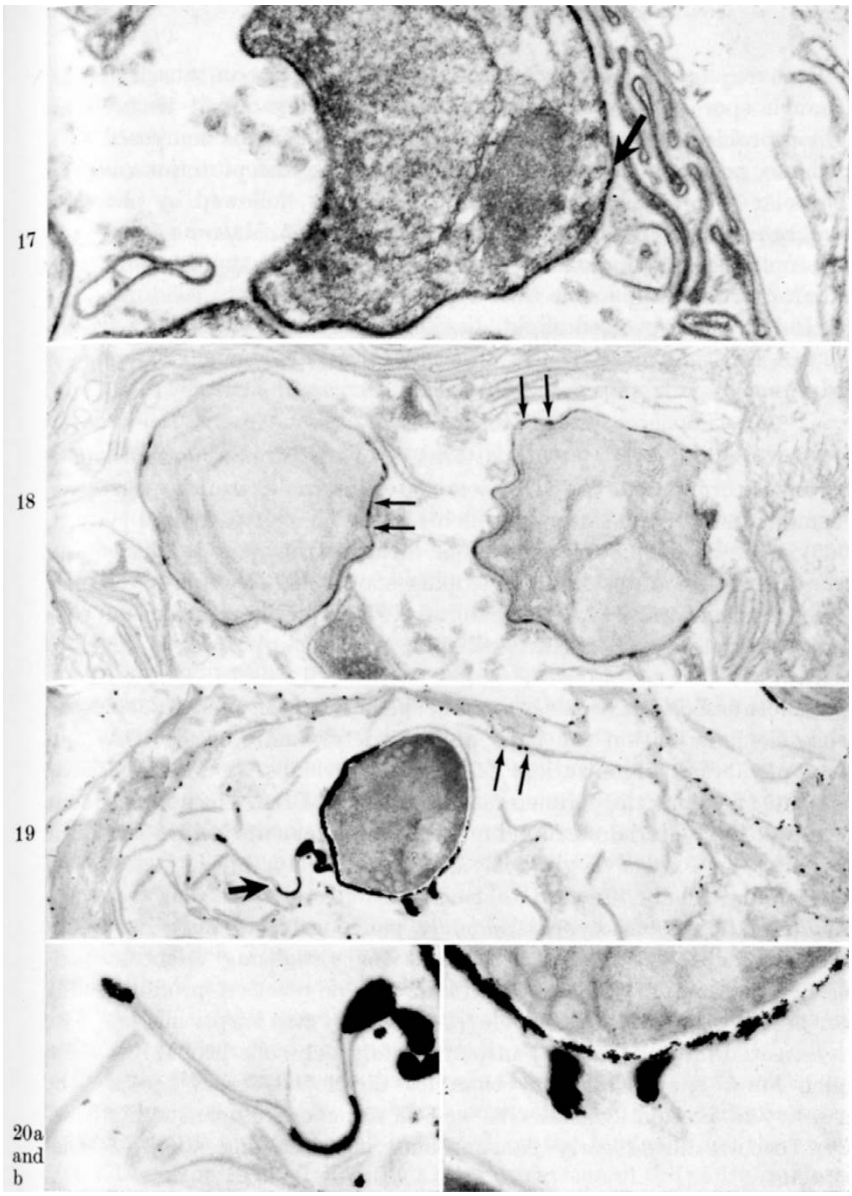


Figures 15a and b. Different views of basal spore-tail proximal regions of mature spores illustrating enclosed microtubules. ×29040

Figures 16a and b. Apical tail at region of attachment (indicated by arrows) devoid of microtubules and more dense than proximal portions of basal tails. Figure 16a shows mature spore with developed polaroplast (×61600), whereas Figure 16b shows sporoblast (×34320)

Solutions of lanthanum hydroxide were used to discern lanthanum's permeability into the pansporoblasts containing sporonts, sporoblasts, or spores. The marker freely penetrated the outer envelope of young pansporoblasts with differentiating sporonts, whereas only traces of lanthanum occurred in mature pansporoblasts containing spores. Most of the marker localized between the sporont, sporoblast, or spore and the ESM (Figs. 17, 18), but some was deposited within the canals in the pansporoblast.

Adenosine triphosphatase activity was measured on nearly mature pansporoblasts (Figs. 19, 20a,b). Reaction product consistently appeared on the inside leaflet of the pansporoblast membrane; moreover, some occurred within the pansporoblastic canals. In or around the membrane of muscle tissue surrounding the maturing pansporoblast, however, the enzyme activity was minimal or lacking. The muscle tissue was often in a state of degeneration.



Figures 17 and 18. Two views of pansporoblast with sporoblasts treated with lanthanum marker. Note lanthanum (arrows) between exosporont membrane and sporoblast. ×22000 and ×20240, respectively.

Figure 19. Positive reaction product for adenosine triphosphatase at perimeter of pansporoblast envelope and within spore-tails. ×14960

Figs. 20a and b. Enlargements of above. ×44880

Both vegetative and spore-forming elements grow in muscle tissue, although spore development dominated in the examined tissue. The eight sporoblasts develop a typical extrusion apparatus composed of a polar sac, polar aperture, polaroplast, polar tube, and

posterior vacuole. The polar tube and polar sac differentiate early, followed by pleats of membrane assembling into the polaroplast (Fig. 13). Mature spores have a sporoplasm consisting of numerous free ribosomes, a typical spore wall, and an extrusion apparatus with five coils of polar tube.

Host muscle revealed slight dissolution of muscle filaments within infected cells and occasionally adjacent ones; also, some of its nuclei underwent hypertrophy.

Discussion

Microsporidia follow two patterns of pansporoblast origin: (1) a sporont-determinate area (SDA) segregates internally from a vegetative plasmodial stage, the latter of which becomes a pansporoblast (Vávra, 1965); or (2) a pansporoblast envelope forms at the sporont surface and subsequently separates from developing sporoblasts, leaving a vacuolar space (Maurand and Vey, 1973; Szollosi, 1971). Our observations indicate that *I. spraguei* pansporoblasts resemble the former pattern of development.

A close association seems to exist between the meshwork of *I. spraguei* dense elements within the pansporoblast-determinate area (PDA) and early membrane organization. Membrane develops around the dense elements and along the perimeter of the SDA and PDA. The homogeneous PAS-positive material surrounding the dense elements within the PDA resembles an extensive glycocalyx, and glycocalyces which surround active cells usually function to facilitate ion and molecular transport (Bennett, 1968). *Inodosporus spraguei* pansporoblasts may have this transport capacity, since our methods for visualizing adenosine triphosphatase activity consistently showed the reaction product along pansporoblast membranes. The level of activity was surprising, since we investigated only maturing pansporoblasts. Such cells probably have a much lower enzyme activity than less-developed forms because the majority of protein synthesis, as well as other energy-consuming processes, occurs during early pansporoblast, sporont, and sporoblast development.

After formation of an extensive membrane system within the PDA, a dramatic shift occurs from a web-network order into membrane channels. The channels, programmed to become spore-tails, are initially continuous with the extra-sporoblast membrane, but this continuity is diminished with sporoblast maturation.

The three or occasionally four microtubule-containing spore-tails, which have funnel-shaped attachment regions at the base of the spore, are a structural contrast from the single, shorter, more electron-dense attachment at the spore's apical end.

Only a few ultrastructural studios on microsporidians have described a structure resembling spore-tails: there are filamentous appendages attached to spores of *Pleistophora debaisieuxi* (see Vávra, 1965); membranous filaments interconnected with spores of *Telomyxa glugeiformis* (see Codreanu and Vávra, 1970); and, spore appendages attached to pansporoblast envelopes of *Stempellia simulii* (see Maurand and Manier, 1968). Other recent accounts of tail-like structures include those by Vávra (1968), Cossins (1973), Vey and Yago (1973), and Maurand and Vey (1973). For the most part, the "tails" of described species seem less channel-like, more densely structured, and less variable in size, and have attachment regions with different structure and shape than on spores of *I. spraguei*.

The following observations indicate that *I. sprague*i spore-tails serve as channels extending from the pansporoblast envelope to the spore. First, after soaking pansporoblasts in lanthanum, the marker readily penetrates into a space between the ESM and the sporoblast. The only connections through which lanthanum could get into this area are the spore-tail connecting points. Second, a positive reaction product for adenosine triphosphatase accumulates within spore-tails, particularly in the attachment points of sporoblasts. Further, upon collapse of the pansporoblast envelope, spore-tails frequently remain attached to disrupted pansporoblast envelopes. Finally, membrane continuity indicated by the morphological similarity of membrane-associated extraneous coats occurs throughout the pansporoblast envelope, spore-tail, and ESM.

The ESM of *I. sprague*i is similar to the envelope surrounding spores of other species. The resistant ESM of *Caudospora simulii* (see Vávra, 1968), like *I. sprague*i, is closely applied to the sporoblast and spore. In contrast to this condition, *Tuzetia infirma* has an ESM which resembles a sac surrounding the sporoblasts or spores (Maurand et al., 1971).

Vávra (1963) assumed that spore-tails served to connect spores within pansporoblasts. No apparent connections exist in maturing pansporoblasts of *I. sprague*i, as indicated by the tendency for the spores to readily disperse in all directions after disruption of the pansporoblastic envelope.

There is also some question about the function of spore-tails on mature spores. Vávra (1963) suggested that the tails could serve as supportive platforms for extraneous mucus spore-coats or provide an easier means to float. A more likely possibility for *I. sprague*i would be that the tails serve as an adaptation to anchor spores to a substratum while in water. Our observations indicate that spore-tails serve as tendrils, since spores moving freely through the water readily become attached to particles by their tails.

Disintegration of muscle induced by different species of .Microsporidia presents a problem. We found *Nosema michaelis*, a parasite of blue crabs lacking a pansporoblast, caused complete dissolution of actin and myosin filaments of infected as well as uninfected muscle fibers. Such infection frequently immobilized the crab. On the other hand, in massive infections in grass shrimp with pansporoblastic *I. sprague*i, uninfected muscle fibers usually remain visibly normal, although neighboring cells are completely taken over by parasites. Such heavily-infected grass shrimp retain the capacity for active swimming. It would be expected that heavy infections of *I. sprague*i would produce a buildup of metabolic waste that might have devastating effects on uninfected cells. Apparently this is the case for infections with *N. michaelis*. One possible explanation for the lack of effect by heavy infections of *I. sprague*i on normal muscle cells may be that the produced metabolic waste materials are not allowed to diffuse as readily into the vicinity of the uninfected cells; perhaps, mature pansporoblast sacs serve as sinks for the biproducts of parasitic metabolism and provide a means of protection for the host. Vernick and Sprague (1970), in a preliminary study, reported a cell-free extract of crab muscle infected with *N. michaelis* produced lysis of normal muscle *in vitro*. The destructive agent could be of either host or parasitic origin.

A similar comparison exists for two other microsporidians, both infecting *Penaeus aztecus* and *P. setiferus*. *Nosema nelsoni*, originally an internal parasite, surrounds muscle bundles and is associated with extensive degenerated tissue, whereas *Pleistophora* sp. usually replaces the muscle tissue, destroying only the infected cells. Overstreet (1973) noted that

shrimps infected with microsporidians seemed to have impaired migratory behavior. The conspicuous presence in particular inshore habitats of a relatively large percentage of infected individuals following migration of most of the populations was especially notable for shrimp containing *N. nelsoni*. The reader should keep in mind, however, that penaeid shrimp infected with *Thelohania penaei*, *T. duorara*, and *Pleistophora* sp., as well as *N. nelsoni*, are all known to succumb readily when placed under stress by bait dealers and biologists.

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