1972. Development of *Toxoplasma* oocysts in neotropical Felidae. *Am. J. Trop. Med. Hyg.* **21**, 512-7.
14. Lainson R. 1968. Parasitological studies in British Honduras III. Some coccidial parasites of mammals. *Ann. Trop. Med. Parasitol.* **62**, 252-9.
15. Levine ND. 1973. *Protozoan Parasites of Domestic Animals and of Man*, 2nd Ed., Burgess Publishing Co., Minneapolis.
16. ——— 1977. Taxonomy of *Toxoplasma*. *J. Protozool.* **24**, 36-41.
17. Miller NL, Frenkel JK, Dubey JP. 1972. Oral infections with *Toxoplasma* cysts and oocysts in felines, other mammals and in birds. *J. Parasitol.* **58**, 928-37.
18. Ritchie LS. 1948. An ether sedimentation technic for routine stool examinations. *Bull. U.S. Army Med. Dept.* **8**, 326.
19. Schneider CR. 1967. The distribution of besnoitiosis in Panama, and its transfer to mice. *J. Protozool.* **14**, 647-78.
20. Shah HL. 1970. *Isospora* species of the cat and attempted transmission of *I. felis* Wenyon, 1923 from the cat to the dog. *J. Protozool.* **17**, 603-9.
21. Tadros W, Laarman JJ. 1976. *Sarcocystis* and related coccidian parasites: A brief general review, together with a discussion on some biological aspects of their life cycles and a new proposal for their classification. *Acta Leidensie* **44**, 1-107.
22. Tomimura T. 1957. Experimental studies on coccidiosis in dogs and cats. (1) The morphology of oocyst and sporogony of *Isospora felis*, and its artificial infection in cats. *Jpn. J. Parasitol.* **6**, 12-24.
23. Wallace GD. 1975. Observations on a feline coccidium with some characteristics of *Toxoplasma* and *Sarcocystis*. *Z. Parasitenk.* **46**, 167-78.
24. Webster J. 1960. Investigations into the coccidia of the grey squirrel *Sciurus carolinensis* Gmelin. *J. Protozool.* **7**, 139-46.
25. Wenyon CM. 1923. Coccidiosis of cats and dogs and the status of the *Isospora* of man. *Ann. Trop. Med. Parasitol.* **17**, 231-88.

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Spores of a New Microsporidan Species Parasitizing Molluscan Neurons

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SYNOPSIS. A new species of Microsporida was observed in neurons of the marine mollusc *Aplysia californica* and the ultrastructure of its spores was investigated. The spores were ~ 1.3 μm long and had a thick 3-layered coat. An anchoring disc and polar aperture were present. The polaroplast occupied most of the anterior half of the spore. The vesicular portion included lengths of loosely packed lamellar structures as well as the usual vesicular profiles embedded in fibrillar material; it was entirely surrounded by tightly packed, electron-dense lamellae. The polar tube had 5 or 6 coils. In this stage of the life cycle (mature spores), the parasites did not appear to be disturbing the host cells. The organism was named **Microsporidium aplysiae** sp. n.

Index Key Words: **Microsporidium aplysiae** sp. n.; spores; parasite of marine mollusc, *Aplysia californica*.

DURING a study of certain pigmented neurons in the abdominal ganglion of the marine mollusc *Aplysia californica* (Cooper), we have observed spores of a microsporidan. These organisms have been briefly described before (4), but they were not identified as microsporida or studied in detail. In the present report we describe their ultrastructure.

MATERIALS AND METHODS

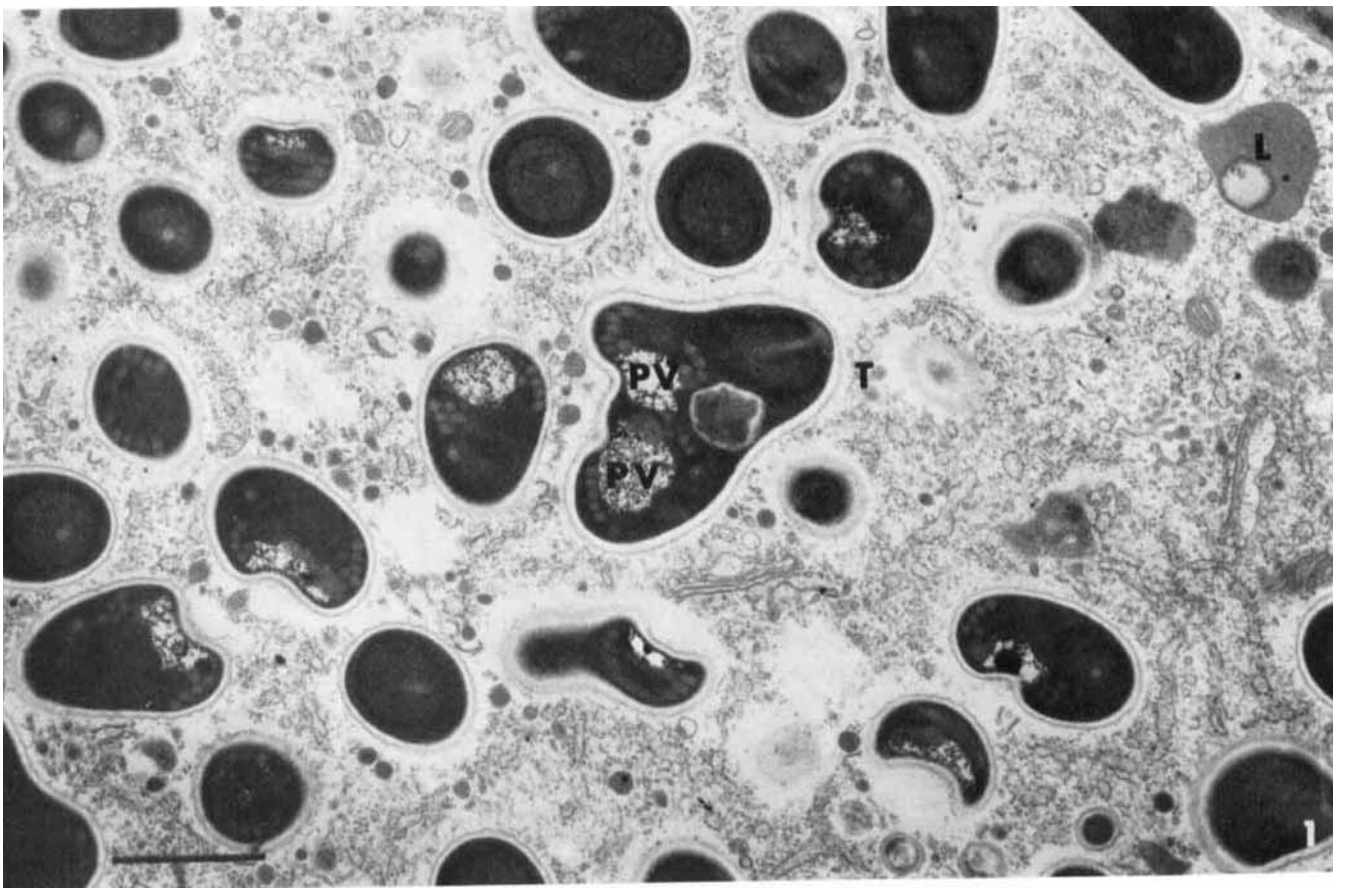
Abdominal ganglia of *A. californica*, obtained from Pacific Bio-Marine, Venice, California, were excised and mounted in a dissection chamber. The connective tissue capsule was cut away to expose the neurons. Before fixation, the preparation was bathed in an artificial sea water solution (3) maintained at 22 C. Each ganglion was either kept in the dark or exposed to light for 20 min; then it was fixed in 3% (v/v) glutaraldehyde in 0.1 M piperazine-*N*,*N*′-bis (2-ethane sulfonic acid) (PIPES) buffer (2), pH 7.6, for 2 h at 22 C. The osmolarity of the fixative was adjusted to 1300 milliosmoles by the addition of sucrose.

The cell bodies of interest were removed from the ganglia and placed in fresh fixative for another hour at room temperature. The cells were then rinsed in PIPES-sucrose, postfixed in 1% (w/v) OsO_4 in 0.1 M PIPES, dehydrated in ethanol series, and embedded in Epon. Thin sections were cut on a DuPont-Sorvall MT-2B ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips EM201 transmission electron microscope.

RESULTS

Spores were observed in the cytoplasm (usually near the plasma membrane) and sometimes in the nuclei of a group of 3 cells called L_{10}, L_{12} and L_{13} (5). They were observed in 6 of 19 cells examined. In our laboratory the spores have never been observed in the giant cell R_2, which has been used most often for electrophysiologic studies. We have not studied the distribution of spores in other neurons of *Aplysia*. Only mature spores were found.

In most cells, the spores seemed to be surrounded by normal cytoplasm (Fig. 1) or nucleoplasm. The neurons contain masses of yellow-orange granules, the lipochondria, which are believed to play a role in the physiologic light response (membrane hyperpolarization) of one of the cells in the group (1). Lipochondria, as well as other host organelles, were found in the infected regions (Fig. 1). Sometimes clear areas were seen around the spores, and occasionally a large clear area was observed near them, but it is possible that these are artifacts of fixation, embedding, and/or sectioning. Cell diameters were measured, and there was no indication that the infected cells had greater volume than uninfected cells. Diameters of the cells were, however, quite variable (110-306 μm), and it was impossible to distinguish among L_{10}, L_{12} and L_{13} on the basis of size or structure; thus individual identified cells could not be compared. Except for the aforementioned clear areas, there was no evidence, at the ultrastructural level, for swelling of the



All figures are of spores of *Microsporidium aplysiae* sp. n. from *Aplysia californica*.

Fig. 1. Spores in host cell cytoplasm, in close proximity to lipochondria (L, upper right) and other organelles. A large teratospore (T) which appears to have 2 polar tubes is present. The anchoring disc (arrow) of one polar tube can be seen. In several spores, 1 or 2 polar tube coils are medial to the rest. Bar, 1.0 μm.

cells, except in one cell into which an electrode had been inserted before fixation.

The spores were typically ovoid or pyriform, measuring 1.3 × 0.7 μm (Fig. 1), on the average. Some profiles (Fig. 2) appeared to be constricted or twisted, and many had an indentation at the posterior end (Fig. 1). A few appeared to have a double complement of some organelles (Fig. 1).

The thick (55 nm) spore coat had a wavy or corrugated surface. It was composed of the 3 layers typical of microsporida: exospore, endospore, and plasma membrane (7). The thin (10-15 nm) exospore was moderately electron dense. The thick (50 nm) endospore was electron lucent, and the plasma membrane was similar in electron density and thickness to the exospore. The endospore was thinner over the anchoring disc than over the rest of the spore (Figs. 1, 3).

At the anterior end of the spore was an anchoring disc (Fig. 3) and a series of light and dark lamellae (septa) that enclosed the polar aperture. The disc extended for some distance along the outside of an annular structure that appeared to consist of tightly packed polaroplast lamellae. Just inside the lamellae was a zone containing several lengths of apparently tubular structures (Figs. 2, 3). The innermost part of the polaroplast contained vesicular profiles surrounded by fibrillar or granular material. This material sometimes appeared quite electron dense, even in unstained sections. The lamellae had an affinity for both uranyl acetate and lead citrate. Less tightly packed lamellae could be seen in other regions of the spores.

The polaroplast was ovoid, with a "pointed" posterior end. The polar tube passed acentrically through the vesicular part of the polaroplast.

The posterior part of the spore was occupied by the coils of the polar tube, a structure thought to be the nucleus, and sometimes a posterior vacuole. The polar tube coiled 5 or 6 times. The coils were usually arranged so that 1 or 2 cross-sectional coil profile(s) was (were) closer to the center of the spore than were the other coil profiles (Fig. 1). The tube was 40-50 nm in diameter. It had several layers. Near the anterior end (Fig. 4), there was an electron-lucent outer layer, a dark layer, another set of light and dark layers, then a layer of moderate electron density separated by a thin, dark line from the inner matrix. A dark spot could sometimes be seen in the middle (Fig. 4). Most of the coils (Fig. 5) lacked the spot and the outer 2 layers, but otherwise were similar to the anterior part. The innermost coil (Fig. 6), however, had a much thicker dark (2nd) layer, and its first layer appeared thinner than that of the other coils. In some sections (Fig. 7), one or more layers appeared to consist of round subunits.

A structure thought to be the nucleus was found in the posterior region, usually anterior to the polar tube coils (Fig. 2). It had an affinity for uranyl acetate. Masses of material similar

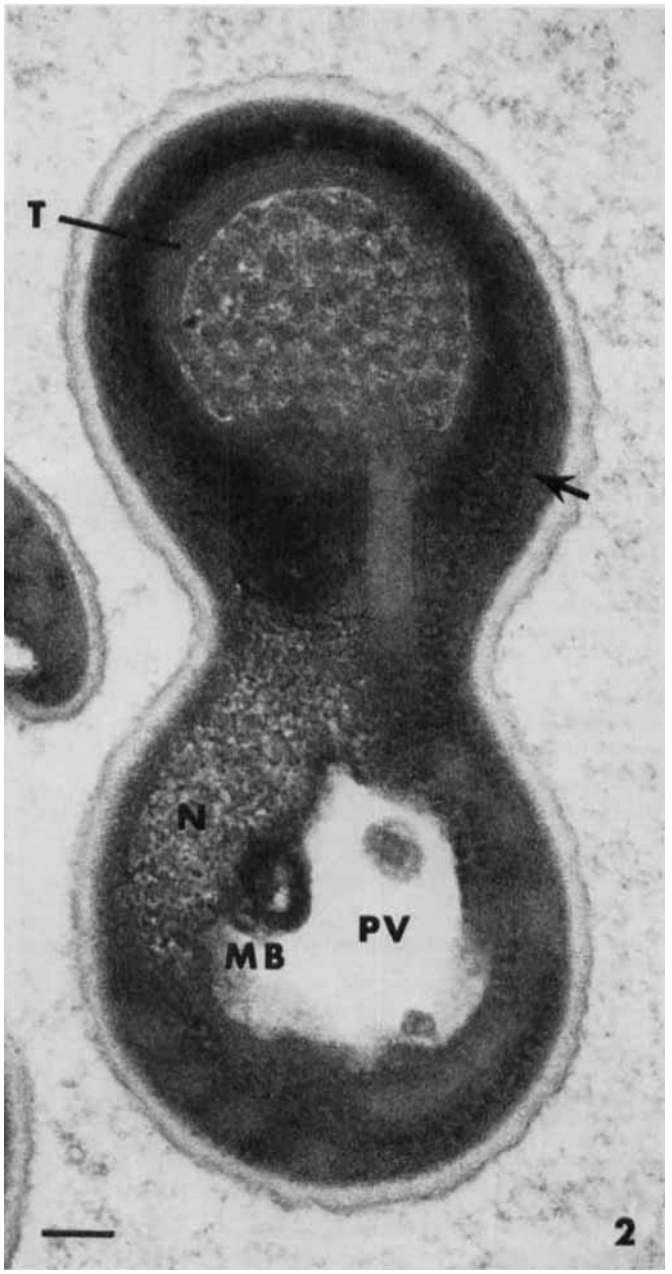


Fig. **2**. Longitudinal section of a teratospore. The presumptive nucleus (N) is in close association with a membranous body (MB) and posterior vacuole (PV). "Transitional" structures (T) can be seen in the polaroplast. Note the rows of ribosomes (arrow). Bar, 0.1 μm.

in electron density to the polar tube, but larger in diameter, were frequently seen in the posterior region.

Posterior vacuoles, some containing granular material, can be seen in Figs. 1 and 2. A membranous body was sometimes seen in the vacuole (Fig. 2). Throughout the spore, including the area around the polar tube coils, small dark granules, probably ribosomes, were observed (Fig. 2).

In many spores small bodies which appeared membrane-bounded were seen in an electron-lucent region. Sometimes a number of these bodies seemed to be connected to form a beaded structure.

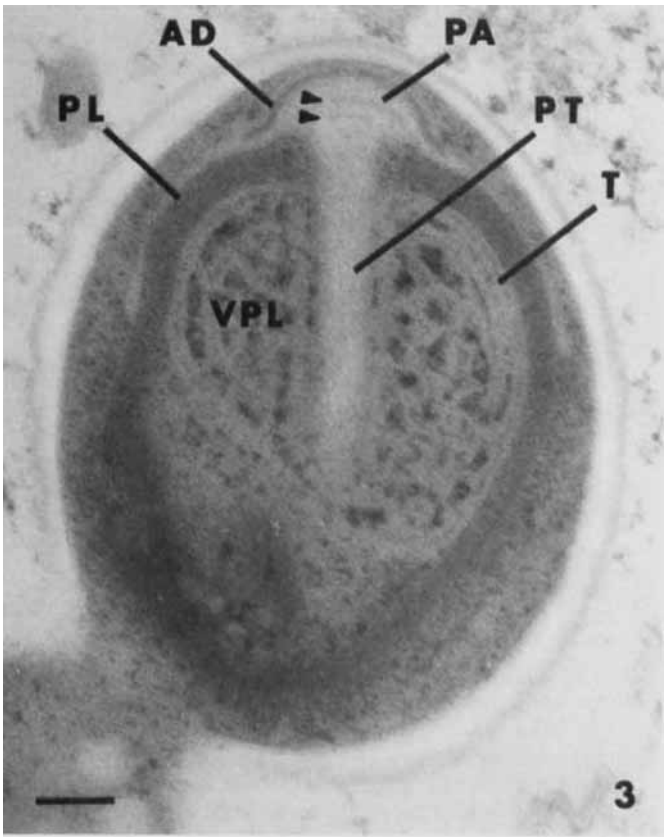
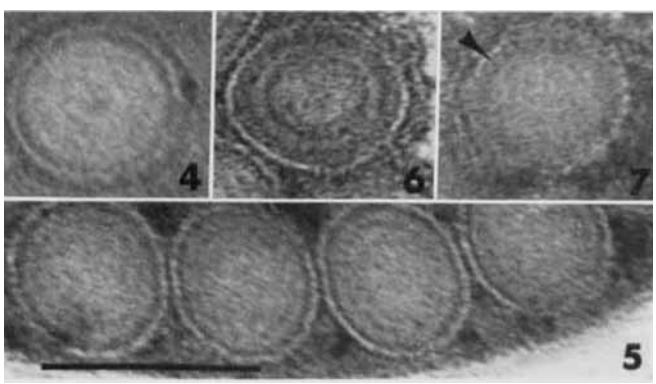


Fig. **3**. The anterior region of a spore. Note the anchoring disc (AD) with polar aperture (PA) and septa (arrowheads), attached to the polar tube (PT). The lamellar (PL), vesicular (VPL), and "transitional" (T) parts of the polaroplast can be clearly distinguished. Bar, 0.1 μm.

TAXONOMIC SUMMARY

Microsporidium aplysiae sp.n.

Diagnosis.—**Spore:** Typically ovoid or pyriform, with mean measurements 1.3 × 0.7 μm; corrugated spore coat 55 nm thick, with the endospore thinner over the anchoring disc than over the rest of the spore; anchoring disc with septa present;



Figs. **4-7**. [Cross sections through various levels of the polar tubes. Bar, 0.1 μm.] **4**. Near the anterior end. **5**. Some of the outer coils. **6**. Innermost coil. **7**. In a polar tube coil, note the apparent subunits (arrow) of a layer of the filament.

typical lamellar and vesicular parts of ovoid polaroplast separated by a "transitional" zone of apparently tubular structures; posterior vacuole, sometimes containing a membranous body, often present; polar tube of uniform diameter (40-50 nm) with 5-6 coils arranged in 2 layers; **vegetative stage** and process of **sporogony** unknown.

Type Host.—*Aplysia californica* (Cooper). Intertidal zone of Pacific Ocean, from Bodega Bay (California) to the Baja Peninsula.

Site in Host.—Nucleus and cytoplasm of abdominal ganglion neurons.

Remarks

This is the only member of Microsporida found in marine molluscs. Its spore size is among the smallest known from the members of the order. Although the known characters of *M. aplysiae* are consistent with those of the species of the genus *Nosema*, some essential characteristics of the latter genus were not observed. The clearly identifiable species seems to be of uncertain generic position. In view of this it has been assigned by us to the "collective group" *Microsporidium*.

DISCUSSION

The present study is the first in which PIPES buffer has been used in the fixation of Microsporida, and some details of the reported ultrastructure may have been influenced by this fact. The posterior indentations of spores (Fig. 1) may be artifacts.

Although Coggeshall (4) reported finding spores only in nuclei of *Aplysia* neurons, we have observed them in both nuclei and cytoplasm. In the stages in which we have observed them, they usually did not seem to be disturbing the cell. Clear areas around some of the spores (the only morphologic evidence for their having an effect on the cells) may be artifacts. The large number of spores in each cell indicated that the spores were formed in these cells, but the lack of damage suggests that the host cells have adapted to the parasite's presence, perhaps to the extent of rendering it almost harmless. The spores with some double organelles (e.g. Fig. 1) are probably teratospores, which could indicate that *Aplysia* is not the preferred host of the parasite under consideration (8).

The vesicular part of the polaroplast did not appear identical to that of any other Microsporida described in the literature. The part of it that appears to be tubular in thin sections is probably lamellar, but in staining properties it resembles the vesicular part of the polaroplast more closely than the lamellar part (Figs. 2, 3). It may be a transitional zone between the 2 parts, similar to that shown in *Nosema nelsoni* Sprague spores (Fig. 6 in Ref. 6) but not previously discussed in the literature.

It is possible that the small oval bodies described by us represent a stage in elaboration of the polar tube, but their accurate interpretation is impossible on the basis of the presently available information. The membranous bodies (Fig. 2), sometimes observed in the posterior vacuole, may be extensions of the polaroplast lamellae.

The species we have described is clearly a member of order Microsporida Balbiani, 1882. It is distinguished by being the only member of this order in marine molluscs. We have been unable to obtain enough information about the organism to assign it to a genus. Therefore, we assigned it to the genus *Microsporidium* and gave it the specific name *aplysiae*.

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REFERENCES

1. Andersen MC, Brown AM. 1978. Photoresponses of a sensitive extraretinal photoreceptor in *Aplysia*. *J. Physiol.* (Lond.), in press.
2. Baur PS, Stacey TR. 1977. The use of PIPES buffer in the fixation of mammalian and marine tissues for electron microscopy. *J. Microsc.* **109**, 315-27.
3. Brown AM, Brodwick MS, Eaton DC. 1977. Intracellular calcium and extra-retinal photoreception in *Aplysia* giant neurons. *J. Neurobiol.* **8**, 1-18.
4. Coggeshall RE. 1967. A light and electron microscope study of the abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.* **30**, 1263-87.
5. Frazier WT, Kandel ER, Kupfermann I, Waziri R, Coggeshall RE. 1967. Morphological and functional properties of identified neurons in the abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.* **30**, 1288-351.
6. Sprague V, Vernick SH. 1969. Light and electron microscope observations on *Nosema nelsoni* Sprague, 1950 (Microsporida, Nosematidae) with particular reference to its Golgi complex. *J. Protozool.* **16**, 264-71.
7. ———, ——— 1971. The ultrastructure of *Encephalitozoon cuniculi* (Microsporida, Nosematidae) and its taxonomic significance. *J. Protozool.* **18**, 560-9.
8. Weiser J. 1953. Parasiten der Raupen der Sonnenblumenmotte, *Homesoma nebulellum* Hbn. mit besonderer Rücksicht zur Art *Mattesia povolnyi* sp. n. *Folia Zool. Entomol.* **15**, 252-64.