ADENOSINE TRIPHOSPHATE IN THE EXTRACELLULAR SURVIVAL OF AN INTRACELLULAR PARASITE (*NOSEMA MICHAELIS*, MICROSPORIDIA)

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INTRODUCTION

The resistant spores of *Nosema michaelis*, a parasite of the blue crab *Callinectes sapidus*, can be made to hatch by priming them with an alkaline buffer and then exposing them to a tissue culture medium (Weidner, 1970, 1972). In the hatching process the polar tubule coiled within the spore is extruded suddenly and the sporoplasm is forced through the lumen of the tubule and appears at its distal end (Oshima, 1937; Lom and Vavra, 1963; Weidner, 1972). If the tissue culture medium contains live cells, some of the sporoplasms are injected into these cells. Such sporoplasms retain their morphological integrity (as judged by their fine structure) at least for several hours, whereas those ejected into the medium appear dead and disintegrating within less than half an hour (Fig. 2).

Since the avian malaria parasite *Plasmodium lophurae* can be removed from its host erythrocyte and maintained alive and developing extracellularly in vitro in a special medium (Trager, 1971), it seemed of interest to determine how well the sporoplasms of *N. michaelis* might survive in this medium. It was soon found that sporoplasms extruded into the complete medium as used for *P. lophurae* retained their morphological integrity in vitro at 25°C for at least 4 h (Fig. 3). This medium is based on a concentrated duck erythrocyte extract made in a nutrient solution with 6% gelatin. For the best extracellular development of *P.*

lophurae, it must be supplemented with adenosine triphosphate (ATP), pyruvate, and coenzyme A (CoA). To determine whether these supplements had any effect on the sporoplasm of *Nosema*, the following experiments were performed.

MATERIALS AND METHODS

Muscle heavily infected with *N. michaelis* was removed from blue crabs *C. sapidus* and added to 0.1 N KOH and roughly homogenized with a Waring Blendor for 30 s. After 10 min, the suspension was spun at 1,000 rpm for 10 min in an International Clinical Centrifuge. The top material (mostly muscle homogenate and fluid) was decanted and the remainder resuspended in water. This cycle was repeated three to four times with water wash until a pure white sediment of *N. michaelis* spores appeared at the bottom of the tubes. The spores were primed for extrusion by 45–60 min of incubation in freshly made Michaelis Veronal acetate adjusted to pH 10. The buffer was prepared by adding 9.7 g sodium acetate + 14.7 g sodium barbiturate in carbon dioxide-free distilled water to a final volume of 500 ml. After spinning at 2,000 rpm for 10 min, decanting the buffer, the spores were primed for discharge when added to 1 part tissue culture medium 199 and 2 parts Trager's test medium. The test media were prepared in the following ways. Identical small volumes (2 or 3 ml) of freshly prepared duck erythrocyte extract medium (Trager, 1971) were placed in lettered tubes by one of us (W. T.). He then added supplements to

TABLE I

Survival of Sporoplasms of N. michaelis 4 h after Extrusion into Different Media

Exp. no.	Prep.	Duck erythrocyte extract-conc.*	Conc. added			Fine structure of extracellular sporoplasms
			ATP	PYR	CoA	
			mM	*mM*	*mM*	
(1)	A	1 ×	0	0	0	Poor—breaking down
C447	B					
12/7/71		"	2	5	0.1	Good—intact
(2)	A	1 ×	2	5	0	Slightly less good than in B
C448	B	"	2	5	0.1	Best
12/14/71	C	"	0	0	0	Poor—cells broken down, dispersed
(3)	A	1 ×	0	0	0.1	Poor
C450	B	1 ×	0	0	0	Poor
1/4/72	C	1 ×	2	5	0.1	Good
	D	0.25 ×	2	5	0.1	Fair
(4)	A	1 ×	2	5	0.1	Good
C453	B	1 ×	0	0	0	Poor
1/25/72	C	1 ×	2	5	0	Good
	D	1 ×	2	0	0	Good
(5)	A	1 ×	2	5	0.1	Good
C455	B	1 ×	2	5	0.1	Good
4/4/72	C	0.25 ×	0	0	0	Poor—leaky and collapsing
	D	0.25 ×	2	5	0.1	Like B, but outer envelope separating
(6)	A	1 ×	0	0	0.1	Poor
C456	B	"	2	5	0.1	Good
4/11/72	C	"	2	5	0	Not quite as good as B
	D	"	0	5	0	Poor

* 1 × refers to a full strength extract made from 1 vol frozen-thawed duck erythrocytes suspended in 1.3 vol nutrient solution.

0.25 × signifies a mixture prepared from 1 part full strength extract to 3 parts nutrient solution.

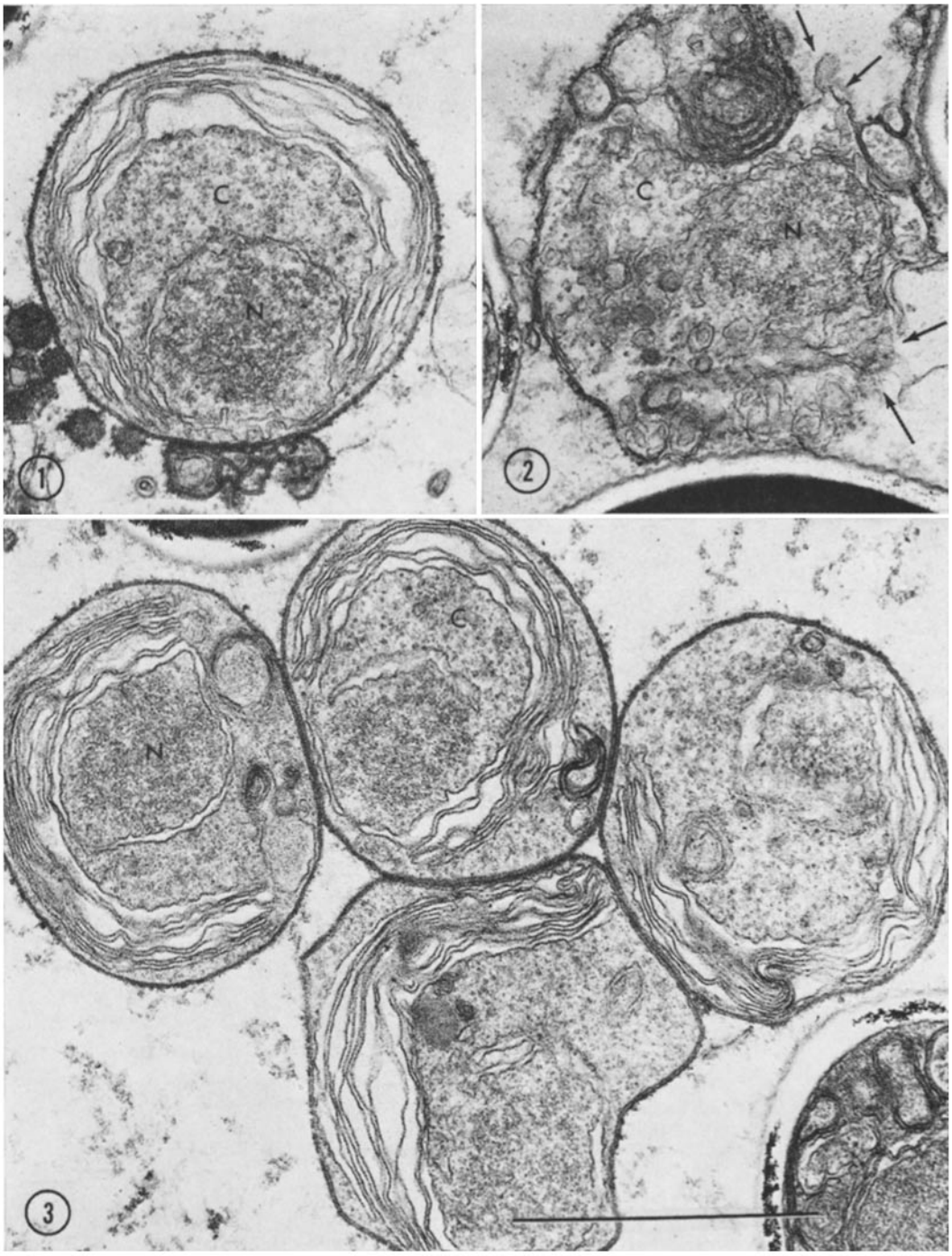

FIGURE 1 *N. michaelis* sporoplasm 10 min after hatching in test medium with pyruvate, CoA, and ATP supplements. Note the loop of cytoplasm (*C*) that extends around the nucleus (*N*) and the characteristic membrane profiles which surround this loop of cytoplasm. × 35,000.

FIGURE 2 Sporoplasm half an hour after hatching in test medium without supplements. The observable cell disintegration included the dissociation of the outer cell membranes (note arrows). Many of the viewed cells in the test medium were more disintegrated than the cell represented here. *C*, cytoplasm; *N*, nucleus. × 35,000.

FIGURE 3 Sporoplasms 4 h after hatching in test medium with pyruvate, CoA, and ATP supplements. Although the cells have about the same degree of structural integrity as newly discharged sporoplasms, there is no indication of growth or differentiation. *C*, cytoplasm; *N*, nucleus. Bar, 1 μm. × 35,000.

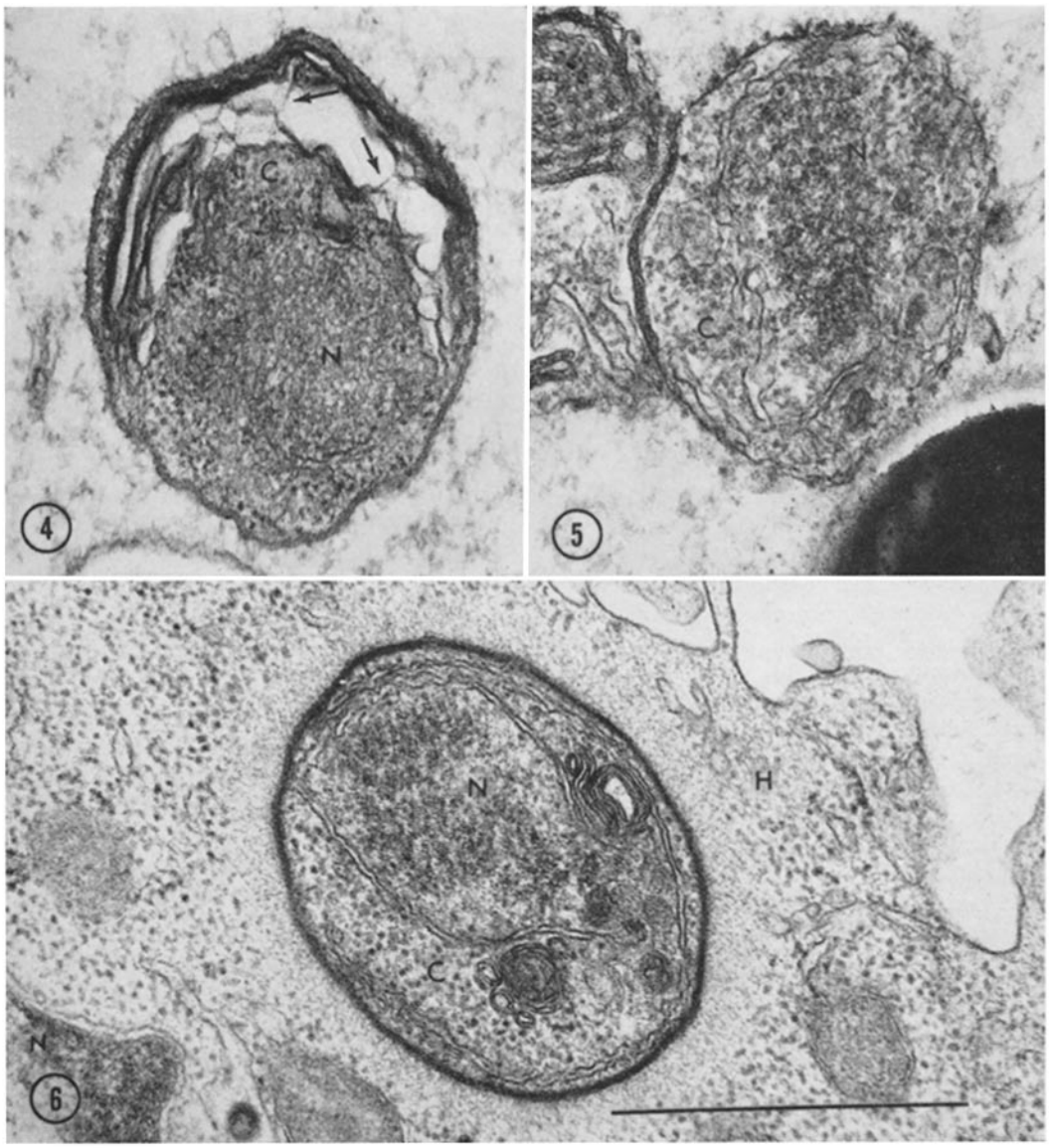

FIGURE 4 Sporoplasm 4 h after hatching in test medium with only ATP. The sporoplasms have a good structural integrity with no breakdown of the outer envelopes; however, unlike sporoplasms found in cells or in medium with all three supplements, the membrane profiles which extend around the loop of cytoplasm (*C*) look abnormal (see arrows). *N*, nucleus. × 35,000.

FIGURE 5 Sporoplasm 4 h after hatching in test medium with only CoA. The outer envelopes of these sporoplasms were frequently collapsed or broken. Although these cells appeared to have some structural integrity, they usually resembled those in test media with no supplements or with only pyruvate. *C*, cytoplasm; *N*, nucleus. × 35,000.

FIGURE 6 Sporoplasm 20 min after invasion of an EL4 ascites tumor cell (C57 BL mouse). The degree of structural order between intracellular sporoplasms and those in test medium with all three supplements is similar. *H*, host cell; *C*, cytoplasm; *N*, nucleus. Bar, 1 μm. × 35,000.

some tubes but not to others and gave the tubes, identified only by their code letter, to E. W. The latter placed aliquots of primed spores from the same batch in each tube, fixed the material after 4 h at 25°C with 2% cacodylate-buffered glutaraldehyde (Polysciences, Inc., Warrington, Pa.). After post-osmium fixation, the cells were stained en bloc for 1 h with 0.5–1% aqueous uranyl acetate and prepared for transmission electron microscopy. Finally, E. W. examined the electron micrographs and graded the condition of the sporoplasms as judged by their integrity and general appearance of their fine structure. Only then was he informed as to the nature of the contents in each tube.

RESULTS AND DISCUSSION

In each of the six experiments (Table I), sporoplasms showed the best appearance in a so-called full strength red cell extract (14% duck hemoglobin and other soluble constituents of the erythrocyte at about half their concentration in the cell) supplemented with ATP, pyruvate, and CoA. Sporoplasms cultivated up to 4 h in this medium bore a close resemblance to those parasites which successfully invade host cells (Fig. 6). When cultured in medium with all three supplements (Figs. 1 and 3), ATP and CoA, or only ATP (Fig. 4), the sporoplasms retained a healthy appearance but showed no observable growth or differentiation. ATP alone provided the most noticeable effect on the structural integrity of the sporoplasms. There were no observable differences when CoA was added with ATP; however, the presence of pyruvate with CoA and ATP seemed to provide a synergistic effect on the structure of the parasites.

In the absence of all three supplements (Fig. 2), with only CoA (Fig. 5), or with only pyruvate, the sporoplasms disintegrated rapidly. This disintegration included the dissociation of the outer membranes and the collapse of the internal structure.

Duck erythrocytes contain ATP at a concentration of about 2.5 mM (see Trager, 1967). It is, however, well known that during the preparation of a cell extract its ATP content falls under the action of membrane-associated ATPase (Zarkowsky and Nathan, 1970). Hence the initial concentration of ATP in the full strength erythrocyte extract would probably be less than the theoretical 1 mM and in the quarter strength extract (exps. 3, 5 of Table I) less than 0.25 mM. We can be certain only of the initial concentration of added ATP (2 mM). This is added after removal of the red cell stroma. It would be of interest to determine whether ADP or 5′-adenylic acid can be substituted for ATP. For *P. lophurae* the former but not the latter is effective (Trager, 1967). It is also important to note that the erythrocyte extract medium in all cases contained adenosine at 0.04 mM, adenosine monophosphate (2′,3′-isomers) at 1.4 mM, and inorganic phosphate at 4 mM. Furthermore, in an additional series of experiments, in which primed spores were incubated for 2 h merely in medium 199, sporoplasms retained a fair degree of integrity only with added ATP at 2 mM. With added inorganic phosphate at 2 mM, or with pyrophosphate at 2 mM, or with 5′-adenylic acid at 2 mM plus inorganic phosphate at 4 mM, the sporoplasms had completely disintegrated. Accordingly it seems likely that the results here reported are attributable to ATP.

The apparent requirement for an external source of ATP could be a normal condition for Microsporidia and it may be one of the reasons for their obligatory dependence on host cells. The intermediate biochemical pathways and endogenous sources of energy have yet to be worked out for these parasites. Nevertheless, it is not a complete surprise that they may utilize an external source of ATP since they have frequently been found suspiciously near host mitochondria during their growth phase of development; furthermore, all pertinent fine structural studies indicate that Microsporidia lack mitochondria.

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