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INHIBITION OF THE RESPIRATION OF *EIMERIA TENELLA* BY QUINOLONE COCCIDIOSTATS

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Abstract—The protozoan parasite *Eimeria tenella* respires vigorously during sporulation and excystation. Sporulation is initiated and sustained by the respiratory activities, and cannot take place under anaerobic conditions. Excystation is not necessarily associated with oxygen consumption and seems to be a passive process solely carried out by the host. Quinolone coccidiostats such as amquinate, buquinolate, methyl benzoquate and decoquinate are reversible inhibitors of *E. tenella* respiration and sporulation. The effective concentrations of the inhibitors for 50 per cent inhibition are 1 to 2×10^{-5} M against respiration during sporulation and 3×10^{-6} M against respiration during excystation. The respiration in sporulation and excystation of an amquinate-resistant *E. tenella* mutant is much less subject to inhibition by the quinolones. The results suggest the inhibition of coccidial respiration as the possible mechanism of anticoccidial activity of the quinolones. Some 2-hydroxynaphthoquinone coccidiostats are also strong inhibitors of *E. tenella* respiration, but they are equally effective against the wild type and the amquinate-resistant mutant.

Coccidia represent a family of sporozoan parasites commonly found in the intestinal epithelial cells of infected animals. The parasites multiply through asexual, binary fissions, in the early phase of development, and eventually differentiate into macrogametocytes and microgametocytes. Union between the two types of cells produces thick-shelled zygotes, otherwise known as unsporulated oocysts which find their way into the feces of infected animals. Once exposed to oxygen, the oocysts sporulate through cytoplasmic contraction and cytokinesis to form four sporocysts each containing two sporozoites. These dormant, sporulated oocysts break open once ingested by an animal, and the sporozoites are released by excystation to invade host intestinal epithelial cells to initiate another cycle of growth. One of the species, *Eimeria tenella*, which specifically parasitizes the caeca of chickens, can be harvested and purified in the form of unsporulated oocysts in sufficient quantities allowing detailed biochemical studies of its sporulation and excystation. Respiratory activities are shown to be associated with these processes [1–3]; these respiratory activities are subject to reversible inhibition by high concentrations of cyanide [2, 3]. The present experiments further characterize the phenomenon of respiration and define the correlation between it and the morphological transformation of the parasite. A family of well known quinolone coccidiostats including buquinolate, amquinate, methyl benzoquate and decoquinate (Fig. 1) are shown to be inhibitors of respiration during both sporulation and excystation of *E. tenella*, but are not effective inhibitors of respiration for an amquinate-resistant mutant. The results strongly suggest the inhibition of coccidial respiration as the probable mechanism of action of the quinolone coccidiostats.

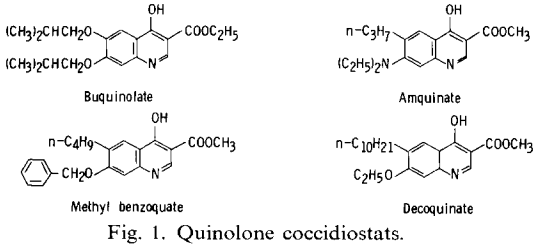

Fig. 1. Quinolone coccidiostats.

MATERIALS AND METHODS

Coccidia. E. tenella (Merck No. 18) oocysts were originally collected from the field and purified through single cell selection. The pure strain was maintained in the laboratory for many years without being exposed to any drugs. An amquinate-resistant mutant of the parasite, *E. tenella* Amq^r, was isolated after four consecutive cycles of cultivation of the parent strain in chickens fed with diets of 15 ppm amquinate [4]. The wild type could be effectively controlled in chickens by 30 ppm amquinate, whereas the mutant was resistant to the drug up to 2000 ppm in the diet. The mutant was also resistant to buquinolate, methyl benzoquate and decoquinate. Both strains of *E. tenella* were stored in the form of sporozoites in a liquid nitrogen tank at −198° [5] for long-term preservation.

Chemicals. 2-Hydroxy-3-(4-trans-cyclohexylcyclohexyl)-1,4-naphthoquinone and 2-hydroxy-3-(4-phenoxyphenyl)-propyl-1,4-naphthoquinone were gifts from Dr. E. F. Rogers of Merck & Co., Inc. Pepsin and trypsin 1-300 were obtained from Nutritional Biochemicals Corp. All the other chemicals were of the highest purity available from commercial sources.

Harvest and purification of unsporulated oocysts. Twelve-day-old White Leghorn chickens were inoculated *per os* with 5×10^4 *E. tenella* sporulated oocysts. The birds were killed 8 days after inoculation and their caeca were removed and stored in an ice-bath. Subsequent operations were carried out at 0–4° unless otherwise specified.

The caeca were opened and the contents collected, suspended in 10^{-4} M dithionite and dispersed by a gentle blending in a Waring blender. The oocysts were sedimented by a 10-min centrifugation at 1000 *g*, resuspended in 10^{-4} M dithionite and the pH of the suspension was adjusted to 2·0 with 1 N HCl. Pepsin was added to a final concentration of 0·1 mg/ml and the suspension was incubated in a 37° water bath for 30 min to permit digestion of tissue materials. The oocysts were sedimented as before, and then purified by a series of stepwise sucrose density differential centrifugations. They remained floating in 1·0, 0·8 and 0·6 M sucrose and were then sedimented by centrifugation in 0·3 M sucrose.

The unsporulated oocysts were then floated and incubated in 5% sodium hypochlorite for 5 min [6]. The precipitates were discarded. The suspension was diluted 6-fold to allow sedimentation of the oocysts by a brief centrifugation. The suspension was tested for bacterial contamination by inoculating agar plates of brain heart infusion, nutrient broth, and yeast extract with 0·1 ml of the suspension containing 2×10^6 oocysts and incubating the plates at 37° for 3 days. Negative results from the plates were used as the criterion of sterility of the suspension. If growth of micro-organisms was observed on the plates, the hypochlorite treatment was repeated until the suspension became sterile in the plate tests. These sterilized, white colored unsporulated oocysts (see Fig. 2) were then repeatedly washed with sterile Ringer's buffer containing 10^{-4} M dithionite until the suspension was freed of chlorine in chlorine paper tests. Such a final preparation of unsporulated oocysts could be preserved in sterile Ringer's buffer plus 10^{-4} M dithionite, 2000 units of penicillin G, and 2000 units of streptomycin/ml under N_2 gas at 0–4° for about 2 weeks without any detectable loss of the ability to sporulate. The average yield was about 10^7 unsporulated oocysts per chicken. Oocysts of *E. tenella* Amq^r were prepared by the same procedure except that the infected chickens were kept on a diet containing 30 ppm amquinate.

Sporulation. The suspension of unsporulated oocysts, washed free of dithionite with sterile Ringer's phosphate buffer, pH 7·4, immediately before sporulation, was contained in a sterile flask up to one-fifth of the flask capacity, and incubated in a 30° water bath with reciprocal shaking at the rate of 100 strokes/min. Cytoplasmic contraction was observed within the first 7–8 hr, and was followed by cytokinesis, sporocyst elongation and maturation [7]. The entire process of sporulation, progressing in about 90 per cent synchrony, was completed within 24 hr with over 90 per cent of the oocysts fully sporulated.

Excystation. The sporulated oocysts of *E. tenella* were stored in Ringer's phosphate buffer plus antibiotics at 0–4° for over 2 months without any appreciable loss of infectivity. They were easily broken in a Teflon pestle tissue homogenizer run at 200 rev/min and 0–4° for 10 min to release sporocysts, which in turn were purified by filtration through a glass bead (Superbrite 100) column [8]. The sporocysts were then incubated in Ringer's phosphate buffer containing 2 per cent sodium taurocholate and 0·5 per cent trypsin 1-300 in a 40° bath. Excystation took place between 30 and 90 min of incubation and freed over 80 per cent of the sporozoites. The sporozoites were purified through a glass bead column and stored in a refrigerator for 2 weeks without losing infectivity in chick embryos [9] or in direct inoculation of chick's duodena.

Measurement of respiratory activities. The respiration of *E. tenella* during sporulation and excystation [1–3] was followed by a YSI model 53 Biological Oxygen Monitor equipped with a Clark electrode. Constant temperatures were controlled by a Haake circulating water bath. The sporulating oocysts were stirred gently because of their fragility, and the output of the Oxygen Monitor was recorded on a Bausch-Lomb VOM 5 recorder.

RESULTS

Biological activities of E. tenella during sporulation. Unsporulated oocysts of *E. tenella* (Fig. 2), suspended in sterile, glass-distilled water, were sporulated (Fig. 3). The supernatant solution in which 2×10^8 oocysts had just completed sporulation was lyophilized to 1·0 ml, a volume about equivalent to that of 2×10^8 packed oocysts. This solution was examined and was found to have little light absorption from 210 to 700 nm, no ninhydrin-positive material, no urea detectable by the Archibald assay [10] and no fatty acid visible by gas–liquid chromatography. The pH of the supernatant solution changed from 6·5 to 6·1 during sporulation, which indicates that little acid or base was produced by the oocysts. In summary, nothing seemed to be excreted by the oocysts during the entire process of sporulation.

Respiration, however, does take place during coccidial sporulation as was first pointed out by Smith and Herrick [1]. *E. tenella* unsporulated oocysts in Ringer's phosphate buffer started vigorous respiration immediately after elevation of the temperature to 30°. The rate of respiration at that temperature remained fairly constant at $0{\cdot}10 \pm 0{\cdot}02$ μl O_2/min/10^6 oocysts for the first 3–4 hr. With *E. tenella* Amq^r mutant, the respiratory activities went much higher: $0{\cdot}25 \pm 0{\cdot}03$ μl O_2/min/10^6 oocysts. Both strains gradually lost respiratory activity upon cold, anaerobic storage, and this activity decline was closely related to the decreases in percentages of oocysts capable of sporulation. CO_2 was identified as an extracellular product of respiration. The rate of CO_2 evolution, measured in a Warburg manometer with 30 per cent KOH in the center well [11], was found to be directly proportional to the rate of oxygen consumption.

Relationship between respiration and sporulation. When *E. tenella* unsporulated oocysts were maintained anaerobic with N_2 gas or dithionite, no sporulation took place after prolonged incubation at elevated temperatures. When the N_2 gas or dithionite was replaced by 10 mM of sodium cyanide or sodium azide, sporulation was also completely stopped. Respiration was restored with resumption of aerobic conditions or removal of the respiratory inhibitors, as was sporulation. It thus seems that respiration is necessary during coccidial sporulation.

Many well known inhibitors of mitochondrial respiration like rotenone and amytal were tested against coccidial respiration during sporulation [12] and

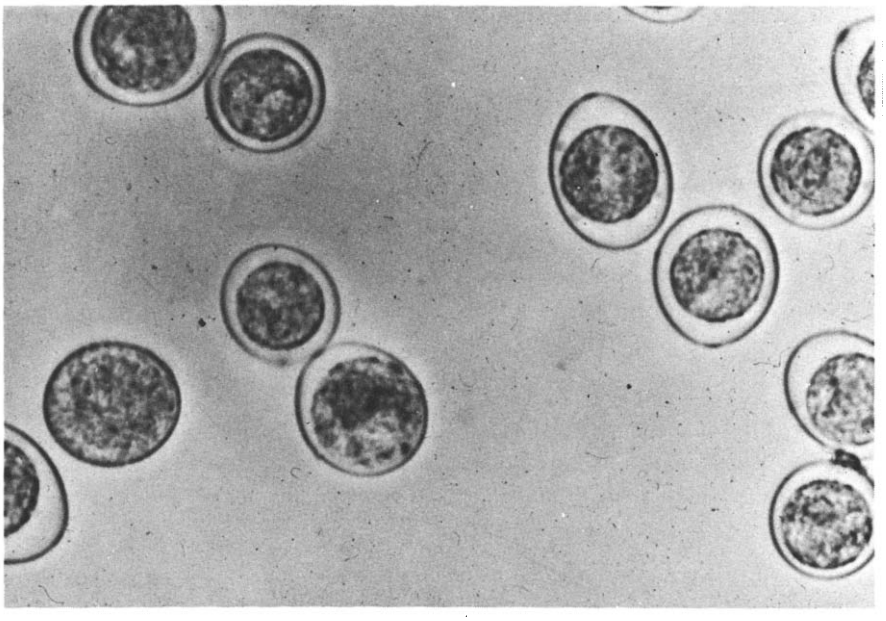

Fig. 2. Unsporulated oocysts of *E. tenella* (1000×).

were found to be ineffective in concentrations up to 10^{-4} M. Many coccidiostats were also tested at or above 10^{-4} M. They included amprolium, clopidol, robenidine, sulfaquinoxaline, pyrimethamine, monensin, nicarbazin, ethopabate and zoaline, but none showed any inhibition of the respiration and sporulation of *E. tenella*.

Inhibition of respiration and sporulation by some coccidiostats. One family of coccidiostats, the quinolones (quinoline carboxylic esters) which included amquinate, buquinolate, methyl benzoquate and decoquinate (see Fig. 1), was found to inhibit both respiration and sporulation of *E. tenella*. Figure 4 shows the effects of amquinate at different concentrations and indicates a transition point at about 1 to 2×10^{-5} M. When the mutant *E. tenella* Amq^r was used, however, amquinate did not inhibit either respiration or sporulation up to 10^{-4} M. This observation strongly suggests the inhibition of respiration as the mechanism of action of amquinate against coccidia. The other quinolone coccidiostats, which share cross-resistance with amquinate [4], exhibited similar potencies in inhibiting respiration and sporulation of *E. tenella*, but were also ineffective against *E. tenella* Amq^r.

2-Hydroxy-3-(4-trans-cyclohexylcyclohexyl)-1,4-naphthoquinone and 2-hydroxy-3-(4-phenoxyphenyl)-propyl-1,4-naphthoquinone, two toxic antimalarial and anticoccidial agents which are inhibitors of succinoxidase and NADH oxidase of chicken liver mitochondria [13, 14], strongly inhibited the respiration and sporulation of *E. tenella*. The inhibition was

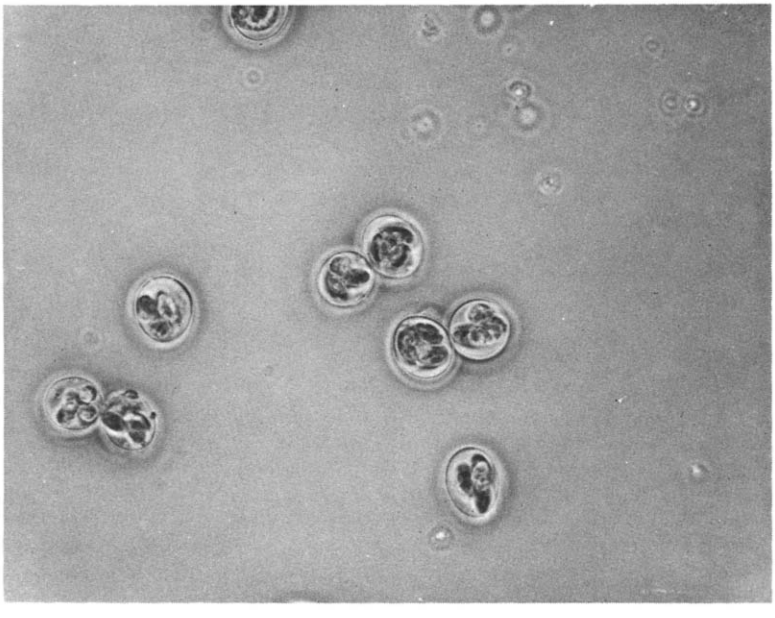

Fig. 3. Sporulated oocysts of *E. tenella* (400×).

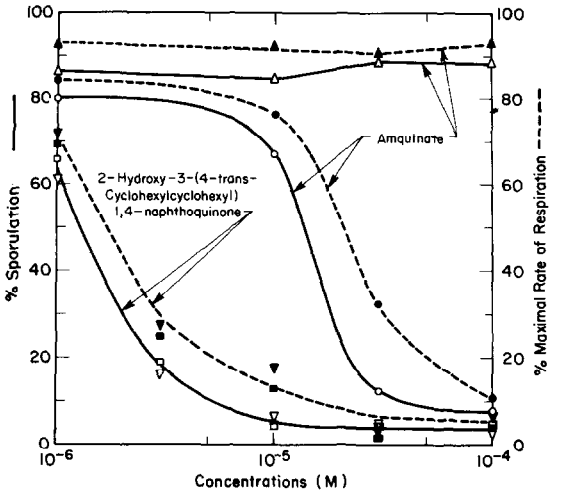

Fig. 4. Inhibition of respiration and sporulation of *E. tenella* oocysts by amquinate and 2-hydroxy-3-(4-trans-cyclohexylcyclohexyl)-1,4-naphthoquinone. *E. tenella* unsporulated oocysts were incubated at a concentration of 10^7 cells/ml at 30°. The rate of respiration was measured 1 hr after the incubation started. The percentage of sporulation was recorded under a microscope 24 hr after the incubation began. *E. tenella* wild type, (○, ●, □, ■); *E. tenella* amquinate-resistant mutant (△, ▲, ▽, ▼). The data represent results of a series of experiments with a variability of ±15 per cent.

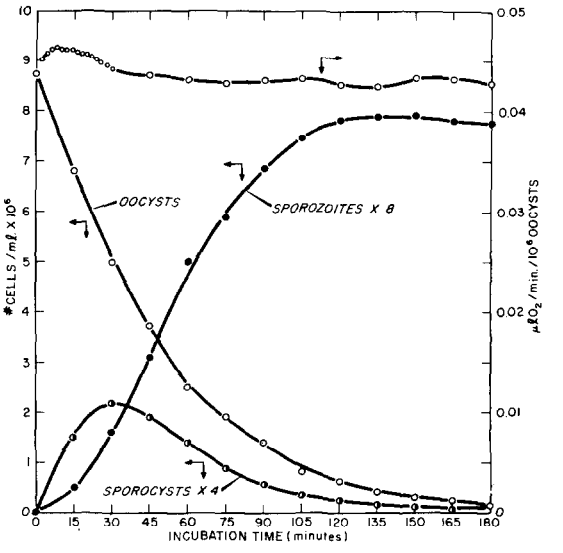

Fig. 6. Excystation *in vitro* of *E. tenella*. *E. tenella*-sporulated oocysts were incubated with taurocholate and crude trypsin at 41·5° in an oxygen monitor. The oocysts were gradually broken by vigorous stirring during the assay. The near horizontal line at the top of the graph indicates the rate of respiration.

about ten times more potent than that by the quinolones, and both the wild type and the Amq^r mutant were equally susceptible to it.

Biological activities during E. tenella *excystation.* While the dormant sporulated oocysts consumed little oxygen even at high temperatures (35–50°), breakage of the oocyst walls by mechanical force immediately initiated vigorous respiratory activities. The respiration of sporocysts (Fig. 5), proceeded at 41·5° at a rate of 0·05 ± 0·01 μl O_2/min/10^6 fresh *E. tenella* oocysts and 0·15 ± 0·03 μl O_2/min/10^6 oocysts in the newly harvested Amq^r mutant. These values were about half of the rates of respiration during sporulation at 30°. They remained constant for the first 3–4 hr after the release of sporocysts and gradually declined to zero during the next few hr (Fig. 6). The rate and duration of sporocyst respiration were reduced by aging of the sporulated oocysts, but were not affected by temperature changes between 35 and 50°. However, at 55°, the respiration was totally stopped (Fig. 7). Excystation was also inhibited at the same high temperature presumably due to inactivation of the needed proteolytic enzymes [15]. How-

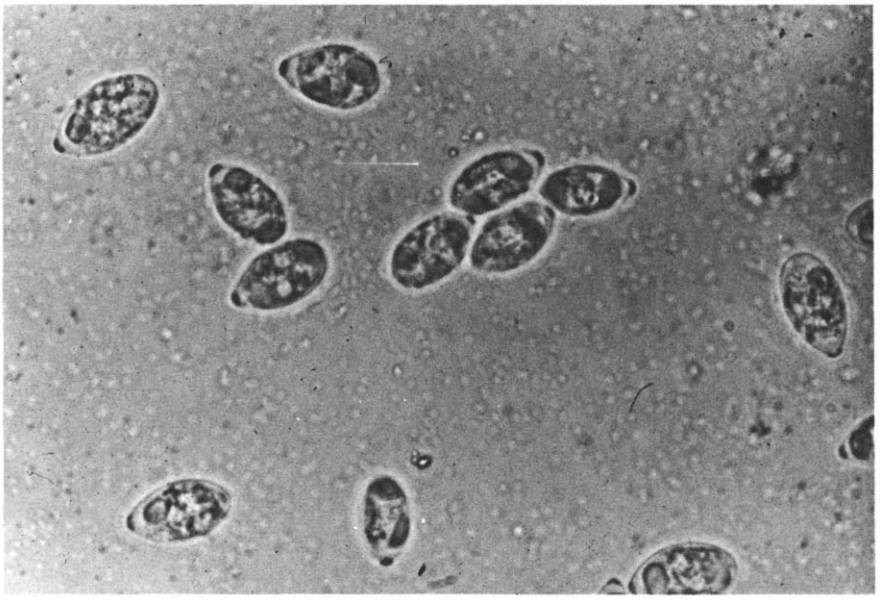

Fig. 5. Sporocysts of *E. tenella* (2000×).

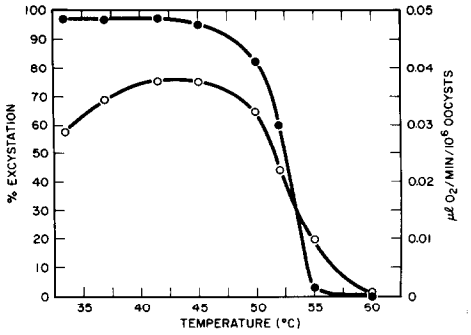

Fig. 7. Effects of temperature changes on the respiration and excystation of *E. tenella* sporocysts. Sporocysts were incubated in the excystation fluid as described in Materials and Methods at different temperatures. Rates of O_2 consumption were recorded at the initial stage of incubation. Numbers of sporocysts and sporozoites were counted under a microscope after 2 hr of incubation. Percentage of excystation, (○——○); rate of respiration (●——●).

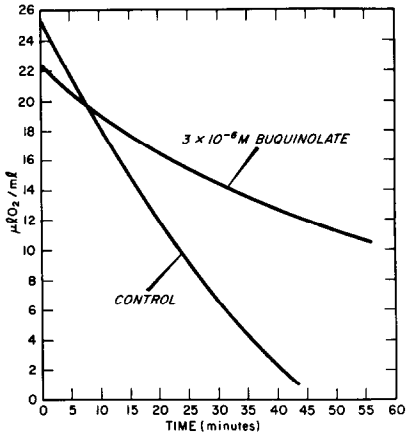

Fig. 9. Polarographic tracing of O_2 consumption vs time by *E. tenella* sporozoites at 41·5°

ever, preincubation of sporocysts at 55° for 5 min resulted in a complete loss of their capabilities to respire and failure of excystation under optimal conditions.

The presence of taurocholate and crude trypsin did not bring about any change in the rate of respiration of the sporocysts. The released sporozoites (Fig. 8) continued to respire for many hr at a slowly declining rate, which was not stimulated appreciably by adding 10^{-3} M succinate, L-malate, pyruvate or acetate. Unlike sporulation, excystation was not dependent on respiratory activity and could proceed normally in the presence of 10^{-4} M dithionite. The sporozoites thus produced resumed respiration immediately after removal of the reducing agent and remained fully infective both *in ovo* and *in vivo*.

Inhibition of respiration of E. tenella *sporocysts and sporozoites.* Although *E. tenella* excystation was not dependent on respiration, the respiration of sporocysts and sporozoites was also suppressed by the same inhibitors of sporulation and sporulation respiration. While rotenone and amytal remained similarly ineffective against respiration, quinolone and 2-hydroxynaphthoquinone coccidiostats seemed to be highly inhibitory against respiration during excystation. A 50 per cent inhibition of respiration was achieved by the presence of 3×10^{-6} M buquinolate (see Fig. 9) during excystation of *E. tenella.*

The inhibition was reversible, and the sporozoites harvested under quinolone coccidiostats retained their infectivities *in ovo* and *in vivo* once the drug was removed.

The effects of quinolones on respiration during excystation of *E. tenella* Amq^r were studied. Figure 10 shows that the mutant was considerably less sensitive to amquinate than was the wild type; only partial inhibition of respiration was attained at 10^{-4} M of the drug, whereas 4×10^{-6} M amquinate totally inhibited the respiration of the wild-type parasites.

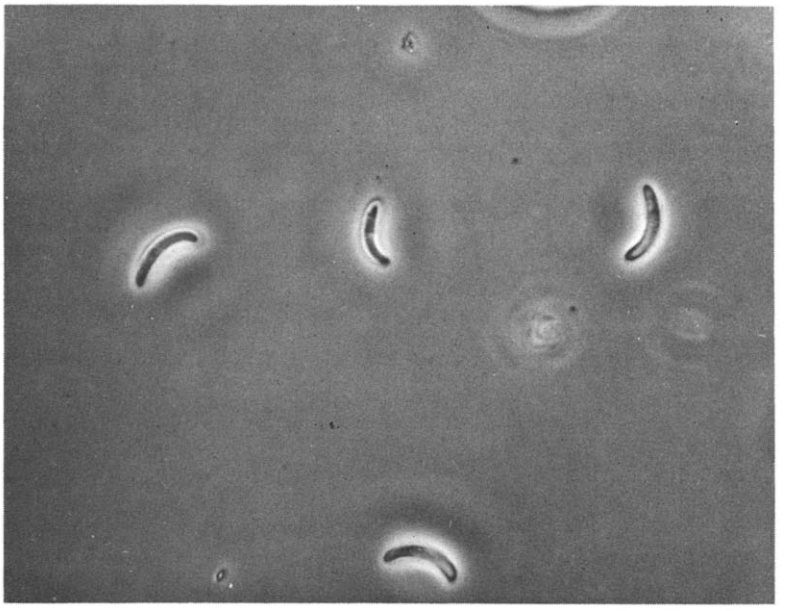

Fig. 8. Sporozoites of *E. tenella* (1000×).

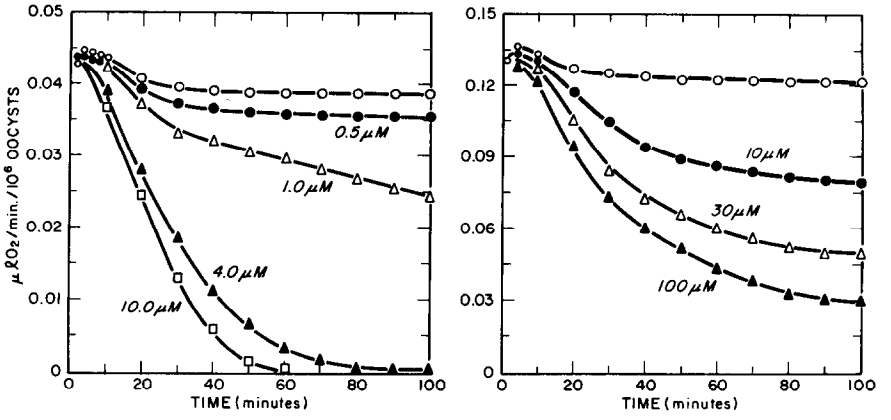

Fig. 10. Effects of amquinate on the respiration of *E. tenella* wild type and amquinate-resistant mutant during excystation. The procedure is described in Materials and Methods. Left, *E. tenella* wild type; right, *E. tenella* amquinate-resistant mutant. The concentrations in the graphs are of amquinate.

2-Hydroxynaphthoquinone coccidiostats were equally effective against *E. tenella* wild type and Amq^r mutant, and they reduced the rates of respiration to 50 per cent of the original level at a concentration of about 10^{-6} M.

DISCUSSION

The experimental results demonstrate quite clearly that respiration is a necessary condition for initiating sporulation of *E. tenella* oocysts. It is, however, not the only requirement, since incubation of *E. tenella* oocysts at a low temperature (0–4°) still permits prolonged respiration at a low rate, but sporulation is stopped at the early stage of cytoplasmic contraction. Further cytokinesis can only take place at elevated temperatures 30° at the optimum [16], with the oocysts respiring simultaneously. A prolonged aerobic incubation at 0–4° followed by an anaerobic incubation at 30° in 10^{-4} M dithionite or vice versa invariably fails to carry *E. tenella* sporulation beyond cytoplasmic contraction. When *E. tenella* unsporulated oocysts were incubated only anaerobically at 0–4° or 30°, not even the cytoplasmic contraction took place.

E. tenella excystation, though also associated with respiration of released sporocysts and sporozoites, is not dependent on it. The release of sporozoites from sporocysts depends only on the presence of bile salts or detergents and crude pancreatic trypsin at neutral pH and 41·5°, a condition which could be adequately provided by a host chicken. Excystation thus may be a process carried out exclusively by the infected host rather than the parasite. In fact, *E. tenella* sporozoites could be freed by the routine process of excystation *in vitro* from sporocysts killed by a brief incubation in chloroform, ethyl acetate or toluene at 20° (unpublished results). Only when the sporocysts are drastically treated with heat or formaldehyde does excystation no longer occur.

The reversible nature of the inhibition of *E. tenella* respiration during sporulation and excystation by the quinolone and 2-hydroxynaphthoquinone coccidiostats agrees with the fact that the quinolones and quinones are true coccidiostats; they inhibit the development of coccidia but do not kill them [17]. The remarkable lack of toxicity among the quinolones in animals [18] has been supported by the observation that respiration in cultured L-cells or embryonic chick kidney epithelial cells is unaffected by the quinolones up to their saturating levels (unpublished observation). On the other hand, respiration of the latter two cells is markedly inhibited by 10^{-6} M of the two highly toxic 2-hydroxynaphthoquinone coccidiostats. Although the two types of anticoccidial drugs appear to share the common mechanism of action by inhibiting the respiration of coccidia, the lack of cross-resistance and the difference in toxicities suggest that they may have different targets in *E. tenella*. The four quinolone drugs exhibit slightly varied potencies against coccidiosis *in vivo* [18], but they all share similar potencies in inhibiting the respiration of *E. tenella*. These differences *in vivo* thus may reflect different efficiencies of absorption by chickens. The effective concentrations of quinolones and 2-hydroxynaphthoquinones to inhibit *E. tenella* respiration also differ between the stages of sporulation and excystation. While the quinolones in the 10^{-5} M range effectively inhibit respiration by *E. tenella* wild-type oocysts, the 10^{-6} M range is sufficient to stop respiration by sporocysts and sporozoites, and only 10^{-8} M is needed to inhibit *E. tenella* mitochondrial respiration [19]. On oocysts, sporocysts and sporozoites, the inhibitory effect sets in gradually (see Fig. 6), whereas immediate inhibition was observed on mitochondria [14]. These discrepancies may reflect different accessibilities of the drug to the parasite mitochondria, which may be the real therapeutic target of the quinolone and 2-hydroxynaphthoquinone coccidiostats.

The site of quinolone action in *E. tenella* mitochondria has been demonstrated to lie between coenzyme Q and cytochrome *b* [19]. Respiration by mitochondria of *E. tenella* Amq^r mutant is 100-fold more resistant to the quinolones than that of the wild type [14]. Although the exact mechanism of resistance is still unknown, it is probably not due to a change in permeability of the mitochondria because mutant submitochondrial particles respire with similarly enhanced resistance to the quinolones [14].

Rotenone and amytal have little inhibitory effect on respiration during sporulation and excystation of *E. tenella* but they do inhibit the respiration of *E.*

tenella mitochondria with a potency of at least 100-fold less than their activity against chicken liver mitochondria [14]. This observation not only reveals another unique feature of *E. tenella* mitochondria but also explains why rotenone and amytal are of little therapeutic value against coccidiosis.

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