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# STUDIES OF THE MITOCHONDRIA FROM *EIMERIA TENELLA* AND INHIBITION OF THE ELECTRON TRANSPORT BY QUINOLONE COCCIDIOSTATS

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## SUMMARY

Intact but fragile mitochondria were isolated from unsporulated oocysts of *Eimeria tenella*. The mitochondria respired in response to succinate, malate plus pyruvate, and L-ascorbate at rates of 1.00, 0.40, and 0.25 $\mu$l $O_2$/min/mg protein, respectively. Spectrophotometric analyses of the cytochromes in mitochondria and whole oocysts revealed *b*-type and *o*-type cytochromes, at roughly similar levels, but no cytochrome *c* could be detected. The mitochondrial respiration was inhibited by cyanide, azide, carbon monoxide, antimycin A, and 2-heptyl-4-hydroxyquinoline-*N*-oxide, but was relatively resistant to rotenone and amytal.

The quinolone coccidiostats buquinolate, amquinate, methyl benzoquate, and decoquinate were identified as very powerful inhibitors of succinate and malate plus pyruvate supported respiration in *E. tenella* mitochondria. None of these four drugs exhibited any inhibitory effect on chicken liver mitochondria. Only 3 pmol of the quinolones per mg mitochondrial protein was needed to achieve 50 % inhibition. The inhibition could not be reversed by coenzymes $Q_6$ or $Q_{10}$. Since the quinolones did not affect L-ascorbate-supported respiration or the activities of submitochondrial succinate dehydrogenase and NADH dehydrogenase, the site of action of the quinolone coccidiostats was tentatively identified as probably near cytochrome *b* in *E. tenella* mitochondria. Mitochondria isolated from an *E. tenella* amquinate-resistant mutant were much less susceptible to quinolone coccidiostats; 50 % inhibition was attained by 300 pmol of the drugs/mg mitochondrial protein. The results suggest that the mechanism of action of quinolone coccidiostats is by inhibiting the cytochrome-mediated electron transport in the mitochondria of coccidia. 2-Hydroxynaphthoquinone coccidiostats were identified as inhibitors of mitochondrial respiration of both *E. tenella* and chicken liver. They inhibited submitochondrial succinate dehydrogenase and NADH dehydrogenase of *E. tenella*, and remained equally active against the mitochondrial function of *E. tenella* amquinolate-resistant mutant.

## INTRODUCTION

The electron transport system in parasitic protozoa are generally recognized as consisting of two different pathways; the orthodox cyanide-sensitive cytochrome

chain and the cyanide-insensitive flavoprotein oxidases linked to some electron sink other than oxygen [1]. Both pathways may exist in any single parasite, but their relative physiological contributions depend on the availability of oxygen [2]. One of the avian coccidia species, *Eimeria tenella*, is known to penetrate the epithelial cells of caeca and grow in an aerobic environment. The parasite has been shown to contain numerous mitochondria of distinctively elongated structure throughout its entire life cycle [3]. During the in vitro sporulation and excystation of *E. tenella*, vigorous respiration was observed and was found to be sensitive to cyanide [4, 5]. Ryley [6] stained *E. tenella* sporozoites with diaminobenzidine and was able to detect cytochrome oxidase in the mitochondria by electron microscopic observation. Our own studies have demonstrated $CO_2$ as the only detectable product excreted by *E. tenella* during sporulation [7]. There are thus strong indications that *E. tenella* respiration is at least partially mediated by the cytochrome system.

Previous studies also revealed that some quinolone coccidiostats, such as buquinolate, amquinate, methyl benzoquate, and decoquinate were strong inhibitors of *E. tenella* respiration in vitro, but without effect on respiration of L-cells or chick embryonic kidney cells [7]. This observation suggested some unusual properties of *E. tenella* mitochondria, and prompted the present investigation which consisted of isolating mitochondria from the parasite and examining the cytochrome content and sensitivity to various inhibitors. A quinolone-resistant mutant of *E. tenella* was utilized to prove the basis of anticoccidial activities of the quinolones. Portions of the work have been previously reported [8].

## MATERIALS AND METHODS

*Isolation of mitochondria from unsporulated oocysts of E. tenella*

The unsporulated oocysts of *E. tenella*, purified and sterilized as previously described [7], were suspended in 0.21 M mannitol, 0.07 M sucrose, 0.01 mM EDTA, and 0.01 M Tris-phosphate (pH 7.6) to a concentration of $2 \cdot 10^7$ oocysts per ml. The oocysts were gently broken in a Teflon pestled tissue homogenizer run at 200 rev./min and 0–4 °C for 10 min. Cell debris was removed by a 10 min centrifugation at $1000 \times g$. Mitrochondria and amylopectin granules [9] were sedimented after centrifugation at $10\,000 \times g$ for 20 min. The pellets were resuspended in the same isotonic buffer, and incubated with α-amylase (20 μg/ml, Worthington Corp.) at room temperature for 1 h to eliminate most of the amylopectin granules. The centrifugations were repeated; the final preparation of *E. tenella* mitochondria was stored at 0–4 °C. There were about 5.0 mg proteins in the extract of $2 \cdot 10^7$ unsporulated oocysts; the mitochondrial fraction derived from it has 0.22 mg proteins.

*Isolation of chicken liver mitochondria and measurement of oxygen uptake.*

Chicken liver mitochondria was isolated immediately before use according to the procedure by Johnson and Lardy [10]. Oxygen uptake by mitochondria was assayed polarographically with a Clark oxygen electrode (YSI Model 53 Biological Oxygen Monitor) at 30 °C. The assay solution consisted of 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 0.01 mM ADP, 50 mM Tris–HCl (pH 7.5) 20 mM phosphate buffer pH 7.5, and 3 to 4 mg mitochondrial protein per ml. The substrate was either 10 mM succinate, 10 mM L-malate+10 mM pyruvate or 5 mM L-ascorbate+0.3 mM *N,N,N′,N′*, tetramethyl-*p*-phenylenediamine.

RESULTS

*The respiratory activity of mitochondria from unsporulated oocysts of E. tenella.* The mitochondria, shown in Fig. 1, had no detectable respiration without added substrate. But when supplemented with succinate, malate plus pyruvate or L-ascorbate, vigorous oxygen uptake ensued at the average rates of 1.00, 0.40 and 0.25 $\mu$l $0_2$/min/mg protein, respectively ($\pm$ 15 %). The activity remained stable for at least 12 h at 0–4 °C, but was destroyed by a 20 min centrifugation at 20 000 $\times g$ or one freezing and thawing of the mitochondrial fraction.

*Analyses of cytochromes from mitochondria and oocysts of E. tenella.* Extracts

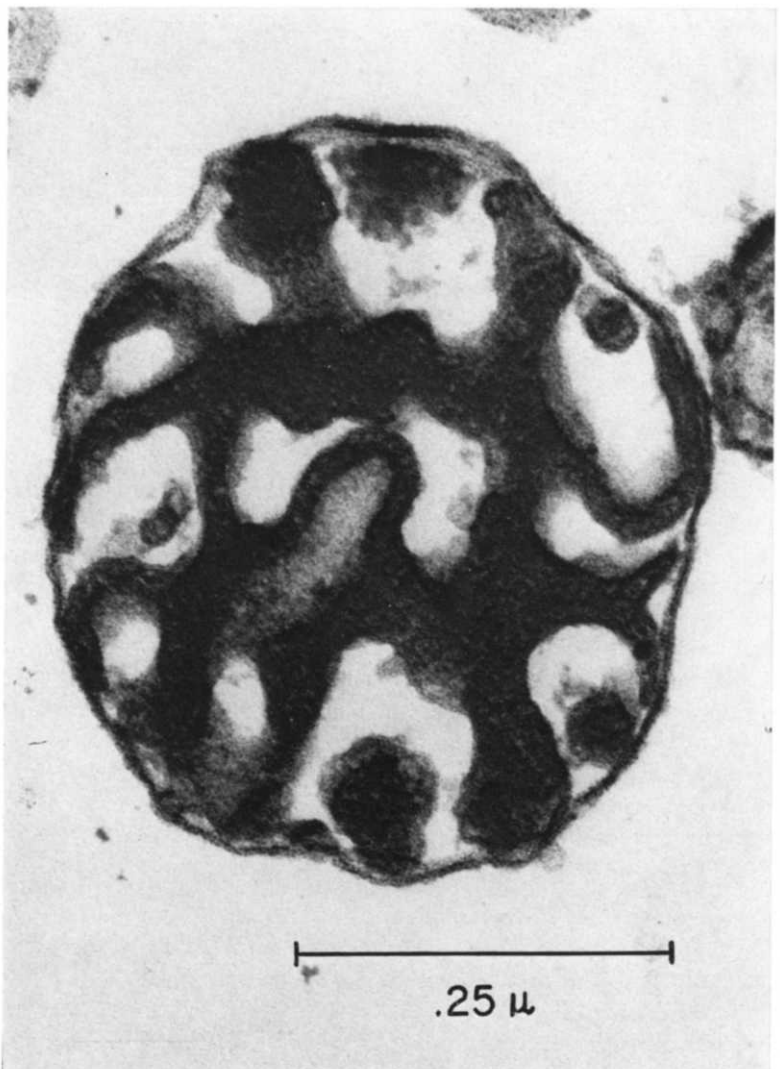


Fig. 1. Electron micrograph of a thin section of isolated mitochondria of *Eimeria tenella* unsporulated oocysts. Sample was suspended in 3 % glutaraldehyde and fixed with 1 % $OsO_4$ at pH 7.2 to 7.4.

of cytochromes from the mitochondria and unsporulated and sporulated oocysts of *E. tenella* were prepared (see Table 1), and analyzed by their reduced vs. oxidized difference spectra recorded in a Cary Model 15 spectrophotometer at 0–4 °C. The data for cytochromes from unsporulated oocysts, represented by Fig. 2, turned out to be similar to those from sporulated oocysts and mitochondria. There were absorption maxima at 605 nm and 563 nm in the dithionite reduced vs. oxidized difference spectra which were probably the $\alpha$-bands of reduced *a*-type and *b*-type cytochromes. However, an absorption maxima at 550 nm, which would correspond to the $\alpha$-band of reduced *c*-type cytochromes, was missing. The information thus suggests the absence of *c*-type cytochromes. The broad band at 524 nm could have been the two $\beta$-bands of reduced cytochromes *a* and *b*, whereas the shoulder at 445 nm and the peak at 428 nm may have been the $\gamma$-bands of reduced cytochromes *a* and *b* (see Fig. 2). In the L-ascorbate reduced vs. oxidized difference spectrum only the $\alpha$, $\beta$ and $\gamma$-bands of reduced *a*-type cytochromes were present, and thus further confirm the previous conclusion that mitochondria of *E. tenella* contain only cytochromes *a* and *b*.

To further characterize the *a*-type cytochromes from mitochondria of *E. tenella*, a difference spectrum between a CO-treated, dithionite-reduced sample and

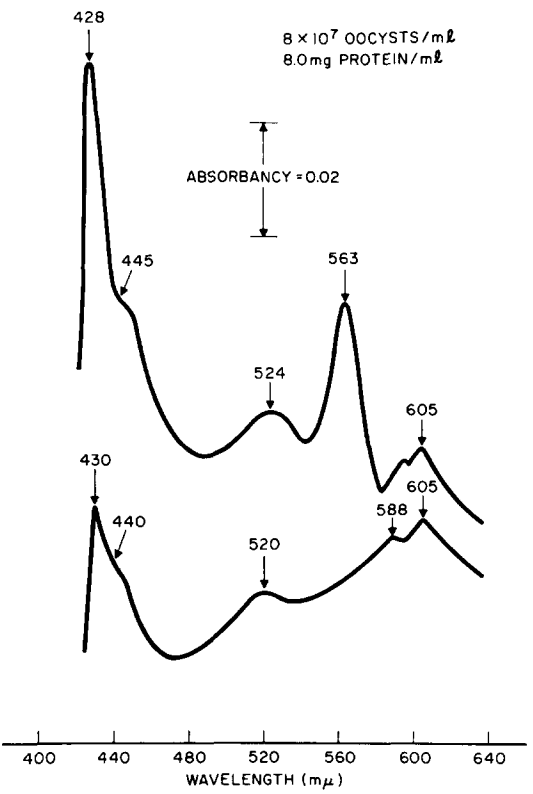


Fig. 2. Difference spectra of the reduced respiratory pigments from *E. tenella* unsporulated oocysts. Upper curve, 1.0 mM dithionite reduced vs. oxidized; lower curve, 1.0 mM L-ascorbate + 0.1 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine + antimycin A (1 $\mu$g/mg protein) reduced vs. oxidized [11].

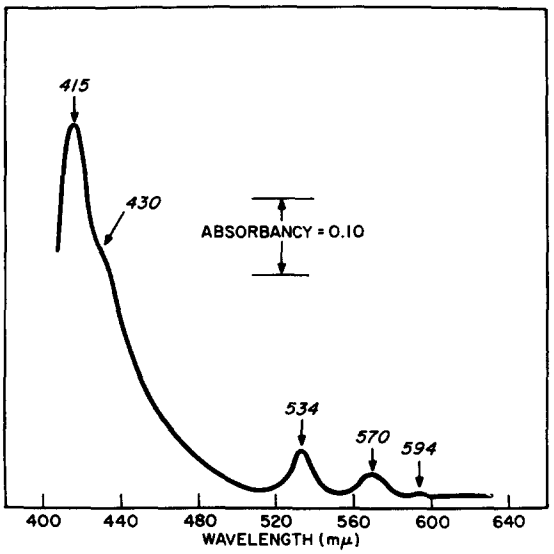


Fig. 3. Difference spectrum of CO-treated, 1.0 mM dithionite reduced vs. 1.0 mM dithionite reduced respiratory pigments from *E. tenella* mitochondria. The extract contained 0.35 mg protein per ml. The sample was bubbled with CO at room temperature for 10 min.

a dithionite-reduced sample is illustrated in Fig. 3. The three bands at 570, 534, and 415 nm coincide well with the $\alpha$, $\beta$ and $\gamma$-bands of CO–cytochrome *o* [13], and thus strongly indicate that the *a*-type cytochrome from *E. tenella* mitochondria may be mostly, if not all, of *o*-type. However, a shoulder at 430 nm in the spectrum (Fig. 3) suggests

TABLE I

CONTENTS OF CYTOCHROMES IN WHOLE OOCYSTS AND MITOCHONDRIA OF *EIMERIA TENELLA*

Cytochromes were extracted by bile salts from mitochondria of *E. tenella* according to Rieske [11]. For extractions of cytochromes from oocysts of *E. tenella*, suspensions of $2 \cdot 10^7$ oocysts per ml were made in 0.10 M Tris-Cl pH 8.0, 0.25 % potassium-deoxycholate, and 0.50 % potassium cholate and sonicated under a Branson solifier Model W185 at 60 watts and 0-4 °C for 2 min. It was incubated at room temperature with frequent shaking for 30 min and centrifuged briefly at 100 000 × *g* to sediment the insoluble material. Protein concentrations of the extracts were determined by the method of Lowry et al. [12]. Extracts from oocysts have about 8 mg protein per ml, whereas extracts from mitochondria contained approximately 0.5 mg protein per ml. Difference spectra of dithionite reduced vs. oxidized, L-ascorbate reduced vs. oxidized and CO-treated, dithionite reduced vs. dithionite reduced were recorded on every sample to provide data for the calculation (ref. Figs 2 and 3). The untreated cytochrome extracts were assumed to be in oxidized form.

| Respiratory components | Concentrations (nmol/mg protein) | | |
|---|---|---|---|
| | Unsporulated oocysts | Sporulated oocysts | Mitochondria |
| *b*-type | 0.072 | 0.075 | 0.720 |
| *c*-type | 0.001 | 0.001 | 0.010 |
| *a*-type | 0.078 | 0.072 | 0.840 |

that some cytochrome $a_3$ may be present [14].

Examination of the difference spectra of many cytochrome extracts enabled us to estimate cytochrome contents in the mitochondria and oocysts of *E. tenella*. Concentrations of cytochrome *b* were calculated using the millimolar extinction coefficients of Chance and Williams [15], whereas those of the *o*-type cytochromes were determined by the extinction coefficients of Taber and Morrison [16]. The results, summarized in Table I, indicate that the cytochromes found in the mitochondrial fraction account for most of the cytochromes in the unsporulated oocysts of *E. tenella*. Little change in the contents of cytochromes was observed during sporulation; the two types of cytochromes are present in about equal quantities in the mitochondria.

*Inhibition of respiration of E. tenella mitochondria; comparison with chicken liver mitochondria.* Cyanide, azide, antimycin A, 2-heptyl-4-hydroxyquinoline-*N*-oxide, and the 2-hydroxynaphthoquinone coccidiostats [7] are all equally potent inhibitors of *E. tenella* and chicken liver mitochondrial respiration supported by malate plus pyruvate or succinate. But the NADH-linked respiration in *E. tenella* mitochondria is about 10 to 100-fold more resistant to rotenone and amytal than that in chicken liver mitochondria (see Table II). Most interestingly, the quinolone coccidiostats, amquinate, buquinolate, methyl benzoquate and decoquinate, are very powerful inhibitors of *E. tenella* mitochondrial respiration linked to either NADH or succinate, but are totally without effect on chicken liver mitochondria (see Table II). 50 % inhibition can be attained by about $10^{-8}$ M of any of the four drugs which corresponds to 3 pmol

TABLE II

INHIBITION OF RESPIRATION IN MITOCHONDRIA FROM *EIMERIA TENELLA* AND CHICKEN LIVERS

The mitochondria samples were assayed at a concentration of about 3.2 mg protein per ml. Inhibitors were added to the assay 1 min after the substrate. Rates of respiration by chicken liver mitochondria assayed under the experimental conditions were: succinate, 1.00; NADH, 0.81; L-ascorbate, 0.50 $\mu$l $O_2$/min/mg protein.

| Inhibitors | Substrates | Concentrations of inhibitors needed for 50 % inhibition of respiration (M) | |
|---|---|---|---|
| | | *E. tenella* mitochondria | Chicken liver mitochondria |
| Rotenone | succinate | $> 1 \cdot 10^{-3}$ | $> 1 \cdot 10^{-3}$ |
| | NADH | $1 \cdot 10^{-5}$ | $1 \cdot 10^{-7}$ |
| | L-ascorbate | $> 1 \cdot 10^{-3}$ | $> 1 \cdot 10^{-3}$ |
| Amytal | succinate | $> 1 \cdot 10^{-3}$ | $> 1 \cdot 10^{-3}$ |
| | NADH | $> 1 \cdot 10^{-3}$ | $1 \cdot 10^{-4}$ |
| | L-ascorbate | $> 1 \cdot 10^{-3}$ | $> 1 \cdot 10^{-3}$ |
| Amquinate | succinate | $1 \cdot 10^{-8}$ | $> 1 \cdot 10^{-3}$ |
| | NADH | $1 \cdot 10^{-8}$ | $> 1 \cdot 10^{-3}$ |
| | L-ascorbate | $> 1 \cdot 10^{-3}$ | $> 1 \cdot 10^{-3}$ |
| 2-Hydroxy-3-(4-phenoxyphenylpropyl)-1,4-napthoquinone | succinate | $1 \cdot 10^{-7}$ | $2 \cdot 10^{-7}$ |
| | NADH | $1 \cdot 10^{-7}$ | $2 \cdot 10^{-7}$ |
| | L-ascorbate | $> 1 \cdot 10^{-3}$ | $> 1 \cdot 10^{-3}$ |

of the drug per milligram of mitochondrial protein. The inhibition cannot be relieved by the presence of $10^{-6}$ M coenzymes $Q_6$ or $Q_{10}$ (gift of Dr A. F. Wagner of Merck and Co., Inc.), but can be bypassed by using L-ascorbate as the substrate. The site of block by the quinolone coccidiostats thus is probably somewhere near cytochrome *b* in the parasite mitochondria.

*Inhibition of succinate dehydrogenase (complex II) and NADH dehydrogenase (complex I) from mitochondria of E. tenella and chicken liver.* Succinate dehydrogenase-coenzyme Q reductase and NADH dehydrogenase-coenzyme Q reductase activities were detected in *E. tenella* and chicken liver mitochondrial preparations (see Table III). But none of the four quinolone coccidiostats, tested at $10^{-4}$ M, showed any appreciable inhibitory effect on any of the four enzymes. It suggests that the quinolones may block electron transport somewhere beyond coenzyme Q in *E. tenella* mitochondria. As has been expected from previous studies by Skelton et al., [19] the 2-hydroxynaphthoquinone coccidiostats exhibited strong inhibition in all the four enzyme assays at $10^{-5}$ M. The inhibition, equally effective against both the *E. tenella* and chicken liver mitochondrial enzymes, could be partially reversed (30–40 %) by the addition of $10^{-4}$ M coenzyme $Q_6$ to the assays.

*Resistance of mitochondria from an E. tenella amquinate-resistant mutant to the*

TABLE III

INHIBITION OF SUCCINATE DEHYDROGENASE-CoQ REDUCTASE (COMPLEX II) AND NADH DEHYDROGENASE-CoQ REDUCTASE (COMPLEX I) FROM MITOCHONDRIA OF *E. TENELLA* AND CHICKEN LIVER

Mitochondria were suspended in 0.1 M phosphate buffer pH 7.4, and 0.1 % potassium cholate to a protein concentration of about 1 mg/ml, and were incubated at 38 °C for 15 min. The succinate dehydrogenase-CoQ reductase activity (Complex II) was assayed by following the reduction of 2,6-dichloroindophenol by succinate through Coenzyme $Q_2$ [17] with about 0.1 mg protein/ml in solution, whereas NADH dehydrogenase-CoQ reductase activity (Complex I) was measured by following the decrease in absorbancy at 340 nm in the presence of NADH, Coenzyme $Q_1$ and 1.0 mg protein per ml [18]. Both assays were run at 38 °C.

| Inhibitors | Concentrations (M) | Specific activities ($\mu$mol · min$^{-1}$ · mg$^{-1}$ protein) | | | |
|---|---|---|---|---|---|
| | | Succinate dehydrogenase-CoQ reductase | | NADH Dehydrogenase-CoQ reductase | |
| | | *E. tenella* | Chicken liver | *E. tenella* | Chicken liver |
| None | — | 0.78 | 0.82 | 0.62 | 1.26 |
| Amquinate | $10^{-4}$ | 0.80 | 0.85 | 0.63 | 1.23 |
| Buquinolate | $10^{-4}$ | 0.75 | 0.84 | 0.60 | 1.20 |
| Methyl benzoquate | $10^{-4}$ | 0.75 | 0.87 | 0.59 | 1.10 |
| Decoquinate | $10^{-4}$ | 0.74 | 0.85 | 0.60 | 1.16 |
| 2-Hydroxy-3-(4-phenoxy-*o*-phenyl)propyl-1,4-naptho-quinone | $10^{-5}$ | 0.05 | 0.06 | 0.07 | 0.10 |
| 2-Hydroxy-3-(4-trans-cyclo-hexyl)cyclohexyl-1,4-naphthoquinone | $10^{-5}$ | 0.04 | 0.06 | 0.06 | 0.12 |

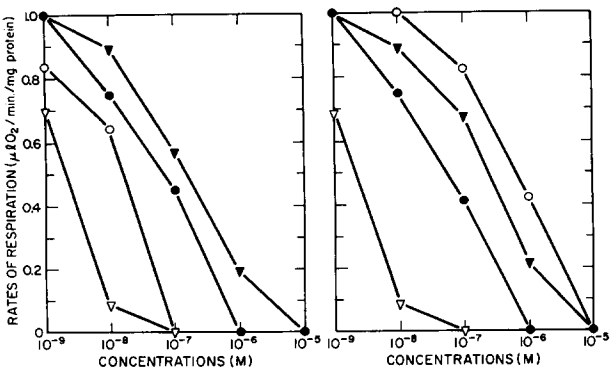


Fig. 4. Inhibition of respiration in mitochondria from (left) *E. tenella* wild type and (right) *E. tenella* amquinate-resistant mutant. 10 mM succinate was the substrate, and each assay contained 4.0 mg protein per ml. For the inhibitors: amquinate, ○—○; 2-hydroxy-3-(4-phenoxyphenyl)propyl-1,4-naphthoquinone, ●—●; antimycin A, ▽—▽; and 2-heptyl-4-hydroxyquinoline-*N*-oxide, ▼—▼.

*quinolone coccidiostats*. Mitochondria from the unsporulated oocysts of an *E. tenella* amquinate-resistant mutant [20] were isolated as previously described. No difference could be detected between the wild type and mutant mitochondria in their physical properties, respiratory activity, total yield or cytochrome contents. They bear similar sensitivities toward cyanide, azide, antimycin A, 2-heptyl-4-hydroxyquinoline-*N*-oxide, and the 2-hydroxynaphthoquinone coccidiostats (see Fig. 4). Rotenone and amytal remain equally ineffective against both wild type and mutant. The only observed difference between the two is the greatly reduced inhibitory potency of the quinolone coccidiostats in the mutant mitochondria. Data in Fig. 4 indicate that succinate-supported respiration in the mutant mitochondria is little affected by amquinate up to $10^{-7}$ M. Only about 50 % of the activity is inhibited by $10^{-6}$ M (300 pmol/mg mitochondrial protein) amquinate, which reflects a 100-fold increase of drug resistance from the wild-type. The magnitude of this resistance corresponds fairly well to that of the mutant *E. tenella* to amquinate during growth in host chickens [20]; the minimal effective dose of amquinate in diets is 30 ppm against wild type and 2000 ppm against amquinate-resistant *E. tenella*. Similar resistance to each of the four quinolones was observed in the mutant mitochondria.

*E. tenella* submitochondrial particles, prepared by sonicating the mitochondria at 60 watts for 30 s (with removal of the intact ones by 20 min centrifugation at $10\,000 \times g$), retained the original specific respiratory activity when succinate was the substrate. Sensitivity to the quinolones in wild type and the lack of it in the mutant were the same in submitochondrial particles, which essentially rules out the possibility of a lower permeability to the quinolones in mutant mitochondria as the basis of resistance.

## DISCUSSION

The cytochrome composition of *E. tenella* parasites is different from that of mammalian mitochondria, as is true for numerous protozoa, e.g., the terminal oxidases of *Trypanosoma rhodesiense* [21] and *Tetrahymena pyriformis* [22] are both

of the *o*-type, whereas *Trypanosoma mega* [23] and *Crithidia fasciculata* [24] contain both $a/a_3$ and *o*-type cytochromes. Ryley [6] recently examined a thick sporozoite suspension of *E. tenella*, and observed two cytochrome bands from 556 nm to 562 nm and from 600 nm to 610 nm. His results are in fairly good agreement with the results in this article.

There can be little doubt that quinolone coccidiostats stop the growth of *E. tenella* by blocking its mitochondrial respiration at a point near cytochrome *b*. The inhibition is so specific that the same quinolones have hardly any effect on chicken liver mitochondria. The inhibition is exceedingly potent; only 3 pmol of the quinolones/mg protein can reduce respiratory activity 50 % in *E. tenella* mitochondria which contain 720 pmol of cytochrome *b*/mg protein (see Table I). There is thus no stoichiometric relationship between the quinolones and cytochome *b*. Other inhibitors like antimycin A and HOQNO, known to act in the $b$-$c_1$ region of cytochrome chain [25, 26], are equally active against mitochondria of *E. tenella* wild-type and mutant, and chicken liver. The two drugs thus must act differently from the quinolone coccidiostats on *E. tenella* mitochondria.

The resistance of *E. tenella* mitochondria toward rotenone and amytal suggest some unusual properties of the NADH dehydrogenase and succinate dehydrogenase portion (Complexes I and II). Beier [27] also observed that nitrofurazone, an anticoccidial agent, inhibited the succinic dehydrogenase activity in *E. intestinalis* without affecting the enzyme in intestinal epithelial cells of rabbits. The inhibition by 2-hydroxynaphthoquinones does not bear specificity to *E. tenella* mitochondria, but rather represents a competition with coenzyme Q observed in other mitochondria as well [19]. This probably explains why these compounds exhibit little therapeutic value.

Understanding the mode of action of quinolone coccidiostats may help to explain the problems of high frequencies of quinolone-resistance development among coccidia [20]. Resistance to the quinolones could be under non-Mendelian genetic control in mitochondria of the parasite. A good example was observed in *Paramecium aurelia* erythromycin-resistant mutants by Beale et al. [28]. The resistance was controlled by mitochondria in the paramecium, and the spontaneous mutation rate was of the order one in $10^7$ mitochondria. But since there were thousands of mitochondria in each cell, the actual frequency of mutation was one in $10^3$ cells.

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