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EFFECTS OF DECOQUINATE AND CLOPIDOL ON ELECTRON TRANSPORT IN MITOCHONDRIA OF *EIMERIA TENELLA* (APICOMPLEXA: COCCIDIA)

MITCHELL FRY*‡ and RAYMOND B. WILLIAMS†

* Department of Biochemical Parasitology, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, U.K.; † Coccidiosis Section, Wellcome Research Laboratories, Berkhamsted, Herts. HP4 2QE, U.K.

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Abstract—Resistance of the chicken coccidium *Eimeria tenella* to the anticoccidial agents decoquinate and clopidol, and the synergistic activity of mixtures of these compounds have been confirmed *in vivo*. Inhibition of electron transport by decoquinate and clopidol has been studied in mitochondria isolated from unsporulated oocysts of the same lines of *E. tenella* as those used in the *in vivo* studies. Electron transport in mitochondria of a drug sensitive line was susceptible to inhibition by both decoquinate and clopidol, and mitochondria isolated from lines made resistant to one or the other of these compounds showed a corresponding resistance at the level of electron transport. Combinations of low concentrations of decoquinate and clopidol caused a greater inhibition of electron transport than expected from summation of their individual actions. Isobolograms showed that decoquinate and clopidol in fact potentiated each other's effect on electron transport. Induced resistance to either decoquinate or clopidol resulted in an increased sensitivity of electron transport to inhibition by the other drug. Cytochrome spectra of *E. tenella* mitochondria and a biphasic response of NADH-oxidase and terminal oxidase activity to inhibition by cyanide or azide suggest the presence of two functional terminal oxidases. There is a correlation between the resistance of electron transport to inhibition by decoquinate or clopidol and the susceptibility to inhibition by cyanide or azide. Mitochondrial electron transport that is more resistant to inhibition by decoquinate exhibits greater sensitivity to cyanide and azide; electron transport that is more resistant to inhibition by clopidol exhibits a decreased sensitivity to cyanide and azide. Resistance to decoquinate and clopidol is discussed in view of a possibly branched electron transport chain in mitochondria of *E. tenella*.

The primary action of quinolone and pyridone anticoccidial drugs is to prevent the growth of, rather than to kill, coccidia (Apicomplexa: Coccidia). In the case of *Eimeria tenella*, which parasitizes the chicken *Gallus gallus*, the sporozoite when it has first invaded a host cell is susceptible to this static action. Lack of such susceptibility of later stages has been suggested to be due to failure of the drugs to penetrate deep into the gut wall [1]. In fact, stages up to second schizonts are susceptible to a secondary cidal action of quinolones (R. B. Williams, unpublished results).

Challey and Jeffers [2] demonstrated that drug combinations containing a 4-hydroxyquinolone plus the pyridine clopidol (= meticlorpindol) were synergistic in their action. Further investigation revealed collateral sensitivity between decoquinate and clopidol [3]. Pre-existing resistance of a line of *Eimeria acervulina* to decoquinate was reversed through the acquisition of resistance to clopidol. Furthermore, it was shown that induction of resistance to clopidol served to enhance the sensitivity to decoquinate of a line previously unexposed to the latter drug. These authors [3] hypothesized that populations resistant to clopidol contain individuals capable of employing an alternative metabolic pathway not affected by clopidol but which is readily blocked by decoquinate and related 4-hydroxyquinolones. Synergism between clopidol and the quinolone methyl benzoquate (= nequinate) has been confirmed against *E. acervulina* [4] and *E. tenella* [5]; a synergistic mixture of methyl benzoquate and clopidol produced a partially coccidiocidal effect [5].

The studies of Wang [6–8] have shown quinolones to be potent inhibitors of mitochondrial respiration in *E. tenella* as well as showing that resistance to quinolones is manifest at the level of mitochondrial electron transport [7].

The present studies have concentrated on the effects of decoquinate and clopidol on mitochondrial respiration in *E. tenella*, on synergism between these compounds in chickens and at the level of electron transport, and on the correlation between *in vitro* and *in vivo* drug resistance and terminal oxidase activity.

MATERIALS AND METHODS

Chemicals. Clopidol (meticlorpindol) was obtained from the Dow Chemical Co. (Midland, MI) and decoquinate from May and Baker Ltd. (Dagenham, Essex, U.K.) (Fig. 1). Respiratory substrates and inhibitors were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.); other compounds were from BDH Chemicals Ltd. (Poole, Dorset, U.K.).

Biological materials. We have followed the ter-

‡ To whom correspondence should be addressed.

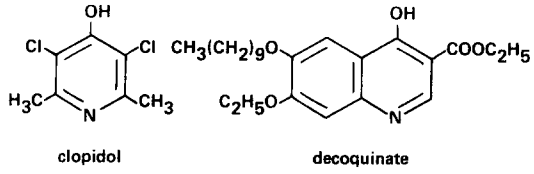

Fig. 1. Chemical formulae of decoquinate and clopidol.

minology for the description of populations of coccidia and the guidelines for the designation of strains and lines recommended by the British Coccidiosis Discussion Group [9]. All three lines of *E. tenella* used in this work were derived from the Weybridge strain, the derivation, maintenance and characterization of which have previously been published [10]. The Weybridge strain was derived from a field sample prior to 1947 (before the commercial use of prophylactic anticoccidial agents) and has been maintained ever since as a laboratory strain, i.e. without exposure to any drugs. The control line used (the Berkhamsted line) has been maintained in untreated chickens at the Wellcome Department of Veterinary Research, first at Frant (Kent) and since 1973 at Berkhamsted (Herts.), since it was derived from a specimen of the Weybridge strain received in 1968. Maintenance was by merozoite passage [10], 51 passages in 13 years. The decoquinate resistant line (Berkhamsted decR) was selected in 1981 from the Berkhamsted line of the Weybridge strain by three consecutive passages of oocysts through chickens fed diets medicated with 8, 12, then 16 mg/kg of decoquinate, and has been subsequently maintained by 6-monthly merozoite passages through untreated chickens. The clopidol resistant line (Berkhamsted cloR) was selected in 1982 from the Berkhamsted line of the Weybridge strain by two consecutive passages of oocysts through chickens fed diets medicated with 120 then 130 mg/kg of clopidol, and has since been multiplied up once by merozoite passage through untreated chickens. Oocysts of the same lines were used for both *in vivo* and *in vitro* studies.

For *in vivo* studies, sporulated oocysts for sensitivity and synergism tests were multiplied up by infecting chickens, collecting the droppings containing unsporulated oocysts into 2% potassium dichromate solution (to prevent bacterial and fungal growth); sieving the resulting slurry into centrifuge bottles; centrifuging and discarding the supernatant; mixing the deposit with saturated sodium chloride solution and centrifuging again; siphoning off the floating layer of oocysts into water and centrifuging to obtain a deposit; finally discarding the supernatant and adding 2% potassium dichromate to the oocyst deposit after which the suspension was incubated at 28° for 3 days to sporulate the oocysts. They were then stored at 4° until needed. For *in vitro* studies, unsporulated oocysts were produced as above except that the chicken droppings were initially collected into 10^{-4} M sodium dithionite (to inhibit sporulation) and kept at 4° until sieving and further treatment as above. Finally, instead of placing the last deposit of oocysts into potassium dichromate for incubation, the unsporulated oocysts were treated with sodium hypochlorite solution (containing 0.625% w/v available chlorine), washed thoroughly and placed in Ringer solution with 100 units/ml nystatin B.P., 100 i.u./ml benzyl-penicillin B.P. and 100 i.u./ml streptomycin sulphate B.P., and stored at 4°.

In vivo *sensitivity tests*. One-week-old cockerels (Ross Ranger breed) were housed in wire-floored cages at 27° with continuous lighting. Drugs were incorporated in the food, which was available *ad libitum*. The diet was chick mash (LD5), vitamin K-deficient, with no other added growth promoters or anticoccidial agents. Chicks were infected, 24 hr after medication commenced, with the appropriate line of *E. tenella* (Weybridge strain) by administration of 1 ml aqueous suspension of 50,000 sporulated oocysts into the crop of each bird. All birds were observed for mortality due to coccidiosis up to 1 week after infection, when they were killed and the caeca were examined for lesions, which were recorded as present or absent. Drug doses are expressed as mg/kg in the diet, since it has been shown that the concentration of a drug constantly passing along the intestinal tract is more relevant to its efficacy against coccidiosis *in vivo* than the concentration in mg/mg chicken body weight [11].

Isolation of mitochondria. Mitochondria were isolated essentially according to Wang [7]. All preparative procedures were carried out at 0–4°. Mitochondria from unsporulated oocysts were isolated and assayed in medium of the following composition: 0.21 M mannitol; 0.07 M sucrose; 5 mM KH_2PO_4; 0.5 mM EGTA; 2.5 mM $MgCl_2$; and 2 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES), pH 7.2. Approximately 2×10^9 unsporulated oocysts were washed twice in the above medium, and resuspended in 5 ml of medium to give approximately 4×10^8 oocysts per ml. Oocysts were broken in a Teflon pestled tissue homogenizer run at 1000 rpm; sixty passes were sufficient to cause 70–80% oocyst breakage.

Broken oocysts were diluted in medium to 100 ml and centrifuged at 1000 *g* for 10 min in an 8×50 angle rotor of an MSE HS18 ultracentrifuge. Cell debris and unbroken oocysts were discarded and the supernatant was then centrifuged at 10,000 *g* for 20 min. The pellet consisted of a dark brown protein layer overlaying a dense white pellet of amylopectin. The protein layer was carefully dislodged using a spatula, resuspended by gentle homogenization in isolation medium, and centrifuged again at 10,000 *g* for 20 min. The resulting mitochondrial pellet was resuspended to 10 mg protein per ml and stored on ice prior to use. Respiratory activity of mitochondrial preparations remained stable for up to 6 hr of storage. About 15 mg of mitochondrial protein was obtained from 2×10^9 oocysts. Mitochondrial preparations were not characterized enzymically, but were examined by electron microscopy. Although the 10,000 *g* pellet was concentrated in mitochondria, 5–10% of mitochondria appeared damaged; principal contamination was by amylopectin granules and a small number of morphologically empty single-membraned vesicles of variable size. 'Mitochondrial' preparation is therefore used as an operational term throughout this text.

Measurement of respiration. Oxygen uptake by mitochondria was assayed polarographically using

Table 1. Respiratory rates of *E. tenella* mitochondria with different substrates

Substrate	Respiratory rate (natoms O/min/mg protein)
NADH	98.7 ± 9.7
NADPH	56.2 ± 6.6
Succinate	49.8 ± 4.1
α-Glycerophosphate	26.8 ± 4.2
Pyruvate + malate	23.6 ± 3.1
Ascorbate + TMPD	114.6 ± 8.6
Ascorbate + cytochrome *c*	79.7 ± 7.5

Results are the mean of two separate determinations on four different mitochondrial preparations, ±S.D. Mitochondria isolated from a drug sensitive (Berkhamsted) line of *E. tenella*.

a closed system Clark-type oxygen electrode (YS1 Model 53 Oxygen Monitor) at 37°. A final assay volume of 3 ml was used containing approximately 1 mg of mitochondrial protein. The assay procedure was standardized as follows: 2.9 ml assay medium (of the same composition as isolation medium; see above), pre-equilibrated to 37° and bubbled with air, was added to the cell followed by 100 μl of mitochondrial protein (~1 mg protein). Then 100 sec was allowed for the oxygen trace to equilibrate, at which time substrate was added by microsyringe (10 μl in assay medium). A further 100 sec was allowed for the oxygen uptake rate to become established, at which time inhibitors could be added; oxygen uptake remained linear for at least 8 min. Decoquinate or clopidol was added as solutions in 'AnalaR' dimethyl formamide. Final concentrations of substrates used were as follows: 0.3 mM NADH; 3.3 mM succinate; 1 mM L-malate plus 1 mM pyruvate; 3.3 mM α-glycerophosphate; 0.3 mM NADPH; and 3.3 mM ascorbate plus 0.8 mM *N,N,N′,N′* tetramethyl-*p*-phenylenediamine (TMPD) or 0.03 mM cytochrome *c*. These substrate concentrations were optimal for maximum respiratory rates. The effect of inhibitors is expressed as a % inhibition of respiratory rate and as an EC_{50} (that concentration required to give 50% inhibition of respiration).

The oxygen electrode was calibrated by the *N*-methylphenazonium methosulphate mediated production of hydrogen peroxide from NADH [12].

Measurement of spectra. A mitochondrial suspension was adjusted to 10 mg protein per ml and made 1% v/v in both cholate and Triton X-100. After gentle stirring for 1 hr at room temperature, the solution was centrifuged at 10,000 *g* for 30 min to sediment insoluble material, the supernatant being used for spectral analysis. Cytochrome spectra were measured at 4° on a Beckman DU-8 spectrophotometer using quartz cuvettes containing 200 μl each of protein solution.

Protein estimation. Protein was estimated using the Bio-Rad Coomassie blue protein assay kit (Bio-Rad Laboratories Ltd.), using bovine gamma-globulin as a standard.

RESULTS

Respiration of E. tenella *mitochondria*

Using the sensitive Berkhamsted line, relatively high rates of respiration were obtained with NADH as substrate (Table 1), although pyruvate + malate supported only low respiratory activity. Terminal oxidase activity with ascorbate + TMPD could more than account for the overall respiratory rate of NADH oxidase. Given the high rate of respiration with NADH, this substrate was preferentially employed throughout these studies.

Although the 10,000 *g* pellet from the broken, unsporulated oocysts consisted mainly of mitochondria, probable contamination should be borne in mind when interpreting the results of this study. Isolated mitochondria showed no respiratory response to ADP with either NADH or succinate as substrates, nor was there any effect on respiration by the uncoupler carbonyl *m*-chlorophenylhydrazone. Although electron microscopic examination of mitochondrial pellets revealed a small number of

Table 2. The effect of classical electron transport inhibitors on *E. tenella* mitochondrial respiration

Inhibitor	EC_{50} (M)	
Rotenone		4.0×10^{-5} ($2.2–6.3 \times 10^{-5}$)
Thenoyltrifluoroacetone	*8.5×10^{-5} ($7.6–9.5 \times 10^{-5}$)	9.2×10^{-5} ($8.3–10.4 \times 10^{-5}$)
Antimycin A		3.5×10^{-9} ($2.8–4.2 \times 10^{-9}$)
2-Heptyl-4-hydroxyquinoline *N*-oxide		9.5×10^{-8} ($8.9–10.2 \times 10^{-8}$)
Cyanide		2.6×10^{-6} ($2.4–2.9 \times 10^{-6}$)
Azide		1.0×10^{-3} ($0.7–1.4 \times 10^{-3}$)

Results are given for inhibition of NADH-oxidase, with NADH as substrate, and represent mean values of two separate determinations on four different mitochondrial preparations. Figures in parentheses represent 95% fiducial limits. Mitochondria isolated from a drug sensitive Berkhamsted line of *E. tenella*.

* With succinate as substrate.

Table 3. Responses of drug resistant or sensitive lines of *E. tenella* in chickens to decoquinate

Decoquinate concentration (mg/kg in diet)	Chickens protected*		
	Berkhamsted (sensitive)	Berkhamsted decR (decoquinate resistant)	Berkhamsted cloR (clopidol resistant)
6400	–	0	–
3200	–	0	–
1600	–	0	–
800	–	0	–
400	–	0	–
200	–	0	–
40	10	–	10
20	10	–	10
10	3	–	7
5	0	–	3
2.5	0	–	0
1.25	0	–	0
0.625	–	–	0

—, Not tested.
* No. of chickens out of ten surviving the infection without having caecal lesions.

obviously broken mitochondria, the majority of mitochondria appeared intact. *E. tenella* isolated mitochondria are therefore apparently uncoupled with respect to oxidative phosphorylation, either inherently or as a result of the necessarily rather vigorous isolation procedure adopted.

Table 2 shows the effect of some classical electron transport inhibitors on *E. tenella* mitochondrial respiration. Respiration was particularly susceptible to antimycin A and 2-heptyl-4-hydroxyquinoline *N*-oxide. However, an unusually high concentration of rotenone was required to inhibit NADH oxidase, and thenoyltrifluoroacetone, generally regarded as a specific inhibitor of mammalian succinate oxidase [13], inhibited both NADH and succinate oxidase similarly. The above results are consistent with those reported by Wang [7] using mitochondria isolated from unsporulated oocysts of *E. tenella*.

In vivo *sensitivity tests*

Table 3 shows the responses of the Berkhamsted, Berkhamsted decR and Berkhamsted cloR lines of the Weybridge strain of *E. tenella* to decoquinate administered continuously in the diet. The respective EC_{50}s with their 95% fiducial limits (estimated by probit analysis) were 11.4 mg/kg (9.0–14.5 mg/kg), >6400 mg/kg (fiducial limits not calculable) and 7.1 mg/kg (5.6–9.0 mg/kg).

Table 4 shows the responses of the Berkhamsted, Berkhamsted cloR and Berkhamsted decR lines to clopidol. Their respective EC_{50}s and 95% fiducial limits were 133.9 mg/kg (110.5–161.5 mg/kg), 433.4 mg/kg (381.9–493.9 mg/kg) and 133.1 mg/kg (108.7–160.2 mg/kg).

In vivo *synergism of decoquinate and clopidol*

Combinations of decoquinate and clopidol were

Table 4. Responses of drug resistant or sensitive lines of *E. tenella* in chickens to clopidol

Clopidol concentration (mg/kg in diet)	Chickens protected*		
	Berkhamsted (sensitive)	Berkhamsted cloR (clopidol resistant)	Berkhamsted decR (decoquinate resistant)
600	–	7	–
550	–	8	–
500	–	7	–
450	–	3	–
400	–	7	–
350	–	3	–
300	–	0	–
250	10	0	9
200	8	0	9
150	5	–	9
100	4	–	0
50	0	–	0

–, not tested.
* No. of chickens out of ten surviving the infection without having caecal lesions.

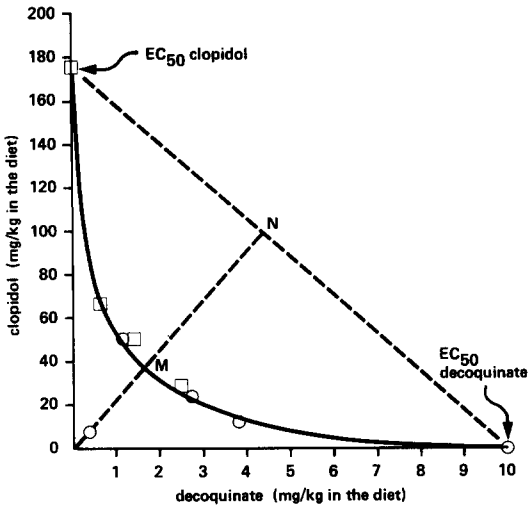

Fig. 2. Control of caecal lesions in chicks by decoquinate–clopidol mixtures; EC_{50} isobologram. Clopidol EC_{50}s, □; decoquinate EC_{50}s, ○. *E. tenella* Weybridge strain, Berkhamsted line.

found to produce inhibition of mitochondrial respiration that was significantly greater than that expected from a summation of the inhibition observed when these two compounds were used alone. This synergistic effect was studied in detail against the Berkhamsted line of *E. tenella* using the graphical method of Hewlett [14]. Figure 2 is an isobologram of the responses (EC_{50}s) of the Berkhamsted line to decoquinate or clopidol, alone or as mixtures. A synergistic effect is clearly indicated; $R = 2.48$(R, the joint action ratio, is defined as ON/OM in Fig. 2 [14]).

Respiration of sensitive and drug resistant E. tenella

Activity of NADH oxidase was compared in mitochondria isolated from sensitive and drug resistant lines derived from the Weybridge strain of *E. tenella*. Table 5 shows that consistently higher respiratory rates were given by mitochondria from a decoquinate resistant line (Berkhamsted decR) compared to a sensitive line (Berkhamsted), and that consistently lower respiratory rates were given by mitochondria from a clopidol resistant line (Berkhamsted cloR). In all cases the uptake of oxygen was linear for at least 10 min.

Inhibition of respiration by decoquinate and clopidol

Decoquinate was a potent inhibitor of *E. tenella* mitochondrial respiration, with an EC_{50} of 2.9×10^{-10} M (95% fiducial limits of $2.2–3.8 \times 10^{-10}$ M) against a decoquinate sensitive line. Statistical treatment was by regression analysis of truncated data. Respiration was inhibited by 70% over a relatively narrow change in concentration of decoquinate, between 7×10^{-11} and 6×10^{-10} M (Fig. 3). However, inhibition of respiration was not complete even at a decoquinate concentration of 10^{-7} M. Mitochondria isolated from decoquinate resistant *E. tenella* showed an almost 80-fold resistance to this anticoccidial agent (Fig. 3), with an EC_{50} of 2.3×10^{-8} M (95% limits $1.3–4.0 \times 10^{-8}$ M). Thus, not only is *E. tenella* mitochondrial respiration highly susceptible to inhibition by decoquinate, but resistance to this drug is also manifest at the level of electron transport.

The present findings have also shown clopidol to be a relatively potent inhibitor of *E. tenella* mitochondrial respiration, with an EC_{50} of 2.1×10^{-6} M (95% limits $1.3–3.4 \times 10^{-6}$ M) against a clopidol sensitive line (Fig. 4). Mitochondria isolated from clopidol resistant *E. tenella* showed an almost 3-fold resistance to this drug; a clopidol EC_{50} of 6.1×10^{-6} M (95% limits $5.8–7.1 \times 10^{-6}$ M) was obtained against a clopidol resistant line (Fig. 4).

Further examination of Figs. 3 and 4 indicates that mitochondria isolated from decoquinate resistant *E. tenella* were rather more sensitive to inhibition by clopidol; EC_{50} (Berkhamsted decR line) = 8.4×10^{-7} M (95% limits $7.9–8.9 \times 10^{-7}$ M) compared with EC_{50} (Berkhamsted line) = 2.1×10^{-6} M (Fig. 4). Although less marked, there was also an increased sensitivity to decoquinate in mitochondria isolated from clopidol resistant *E. tenella*; EC_{50} (Berkhamsted cloR line) = 1.5×10^{-10} M (95% limits $1.1–2.1 \times 10^{-10}$ M) compared with EC_{50} (Berkhamsted line) = 2.9×10^{-10} M (Fig. 3).

In vitro *synergistic inhibition of respiration by decoquinate and clopidol*

Using the graphical method of Hewlett [14], Figs. 5 and 6 are the EC_{20} and EC_{50} isobolograms of the respiratory inhibitions of the Berkhamsted line of *E. tenella* by decoquinate or clopidol, alone or as mixtures. A synergistic effect is indicated. R for Fig. 5 is 1.78, and for Fig. 6 is 1.19.

Inhibition of mitochondrial respiration by cyanide and azide

Although the terminal oxidase inhibitors cyanide and azide were able to inhibit respiration completely in *E. tenella* mitochondria, their effects on sensitive,

Table 5. Mitochondrial respiratory rates of sensitive, decoquinate resistant and clopidol resistant *E. tenella*

Line	Respiratory rate* (natoms 0/min/mg protein)
Sensitive (Berkhamsted)	98.1 (10) ± 10.2
Decoquinate resistant (Berkhamsted decR)	108.2 (7) ± 9.8
Clopidol resistant (Berkhamsted cloR)	72.4 (4) ± 5.6

Results are mean values, the figures in parentheses denoting the number of different mitochondrial preparations studied, ±S.D.

* Respiratory rate with NADH as substrate.

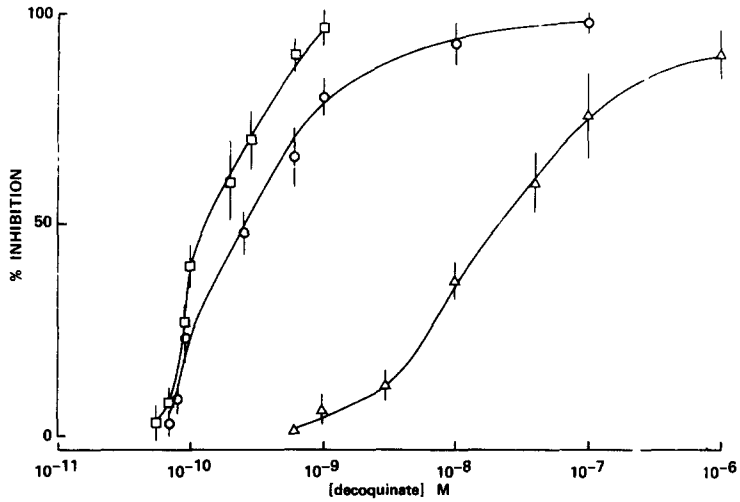

Fig. 3. Semi-log plot of inhibition of mitochondrial respiration by decoquinate. Points were calculated from the mean values of three determinations per mitochondrial preparation, averaged over a number of different mitochondrial preparations (four for sensitive and decoquinate resistant and two for clopidol resistant preparations). Sensitive line, ○—○; decoquinate resistant line, △—△; clopidol resistant line, □—□. Bars represent S.D.

and decoquinate and clopidol resistant mitochondria were quite different (Fig. 7). Statistical analyses carried out by non-linear regression showed that mitochondria isolated from sensitive *E. tenella* responded to low concentrations of cyanide with an EC_{50} of 2.9×10^{-6} M cyanide (95% fiducial limits 2.8–3.1×10^{-6} M) although a cyanide concentration in excess of 4×10^{-5} M was required for complete inhibition of respiration (Fig. 7). Decoquinate resistant mitochondria were extremely sensitive to cyanide inhibition, and 3×10^{-7} M cyanide was sufficient to give 50% inhibition of such lines (95% limits 2.9–3.2×10^{-7} M); in contrast, clopidol resistant mitochondria were less susceptible to cyanide inhibition and 9.6×10^{-6} M cyanide was necessary to give 50% inhibition of respiration (95% limits 8.8–10.7×10^{-6} M). In both drug sensitive and resistant lines, a relatively high level of cyanide was required to give complete inhibition ($>3 \times 10^{-5}$ M). A similar picture was observed with azide (Fig. 8). Decoquinate resistant mitochondria were more sensitive to azide than were sensitive lines, whereas clopidol resistant mitochondria were less susceptible to azide inhibition than were sensitive lines. Fifty per cent inhibition of respiration was achieved at azide concentrations of 9.1×10^{-4} M (95% limits 8.7–9.6×10^{-4} M) (Berkhamsted line), 3.1×10^{-4} M

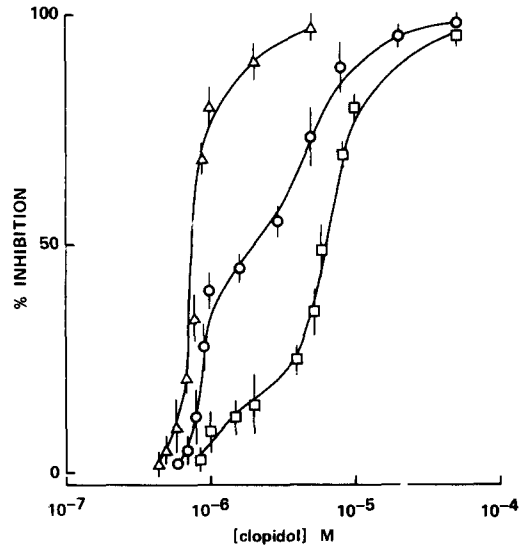

Fig. 4. Semi-log plot of inhibition of mitochondrial respiration of clopidol. Details as in legend to Fig. 3. Sensitive strain, ○—○; decoquinate resistant strain, △—△; clopidol resistant strain, □—□.

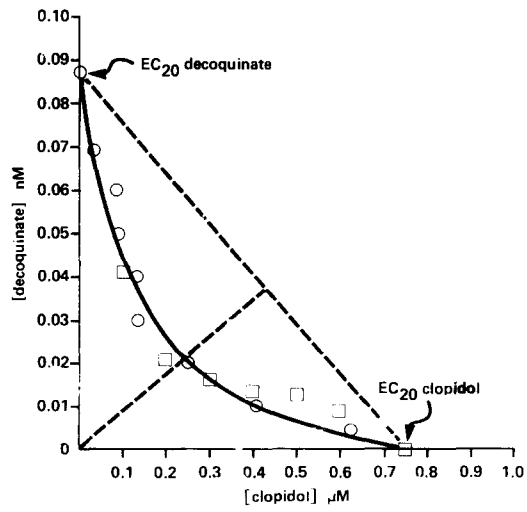

Fig. 5. Inhibition of mitochondrial respiration by a decoquinate–clopidol combination; EC_{20} isobologram. Clopidol EC_{20}s, □; decoquinate EC_{20}s, ○.

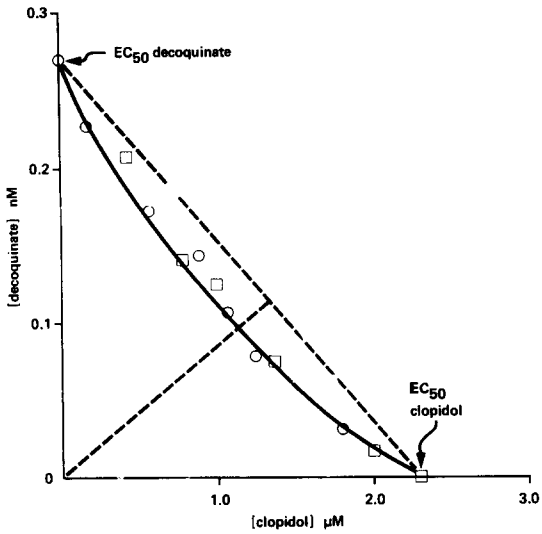

Fig. 6. Inhibition of mitochondrial respiration by a decoquinate–clopidol combination; EC_{50} isobologram. Clopidol EC_{50}s, □; decoquinate EC_{50}s, ○.

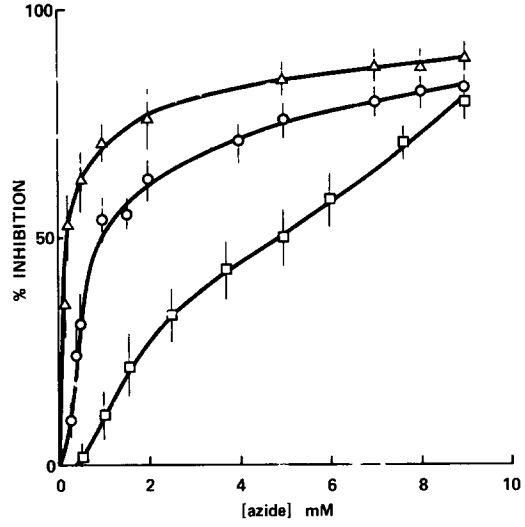

Fig. 8. Inhibition of mitochondrial respiration by azide. All details as in legend to Fig. 7.

(95% limits 2.9–3.3 × 10^{-4} M) (Berkhamsted decR line) and 5.1 × 10^{-3} M (95% limits 4.7–5.5 × 10^{-3} M) (Berkhamsted cloR line).

Cytochrome spectra of E. tenella *mitochondria*

Cytochrome spectra of *E. tenella* mitochondria showed prominent absorption maxima at 605, 560, 445 and 428 nm, with a partially resolved component at 555 nm (Fig. 9). Absorption maxima at 605 and 445 nm were characteristic of cytochromes aa_3 of cytochrome oxidase. Interaction with carbon monoxide produced the difference spectrum shown in Fig. 10; absorption maxima at 570, 534 and 415 nm coincided well with the α, β and γ-bands of CO-cytochrome *o* [15], and the shoulder at 427 nm suggested the presence of cytochrome a_3 [16]. These spectra are in agreement with those reported by Wang [7]. Cytochrome spectra were also taken of mitochondria from decoquinate and clopidol resistant lines of *E. tenella*, and were found to be qualitatively and quantitatively similar to those shown in Figs. 9 and 10.

Terminal oxidases of E. tenella *mitochondria*

Cytochrome spectra of *E. tenella* mitochondria thus indicate the presence of two cytochromes that are capable of reacting with carbon monoxide, cytochrome a_3 of cytochrome oxidase and a cytochrome

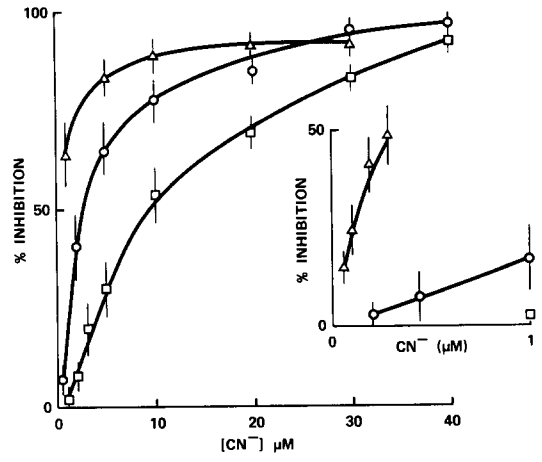

Fig. 7. Inhibition of mitochondrial respiration by cyanide. Inhibition by cyanide was determined against mitochondria isolated from sensitive (○—○), decoquinate resistant (△—△) and clopidol resistant (□—□) lines of *E. tenella*. Results are the average of two determinations on each of six (sensitive line), five (decoquinate resistant line) and two (clopidol resistant line) mitochondrial preparations. Bars represent S.D.

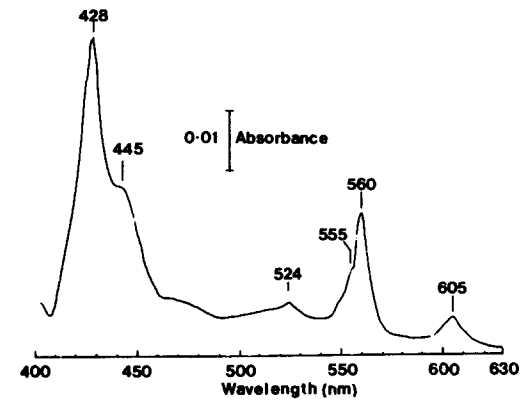

Fig. 9. Reduced minus oxidized difference spectrum of *E. tenella* mitochondria. Protein concentration = 0.9 mg/ml. Reference sample was oxidized by the addition of hydrogen peroxide (final concentration = 0.025%, v/v). Sample reduced by addition of sodium dithionite to 1 mM. Temperature = 4°, slit width = 0.1 nm, scan speed = 20 nm/min. Spectrum shown is that for mitochondria isolated from a sensitive line of *E. tenella*.

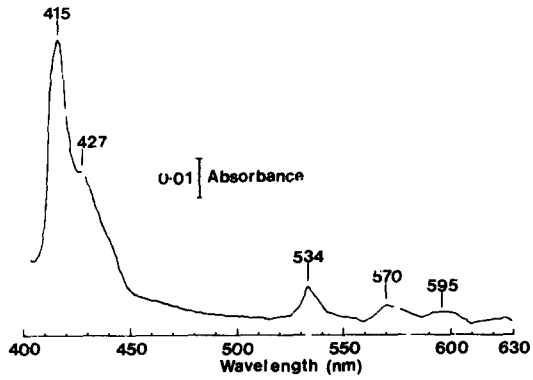

Fig. 10. Carbon monoxide reduced minus reduced difference spectrum of *E. tenella* mitochondria. Protein concentration = 0.85 mg/ml. Sample was bubbled with CO for 20 min at 4°. Other details as in legend to Fig. 9.

possibly analogous to cytochrome *o* [15], suggesting the possibility of two terminal oxidases in such mitochondria. This possibility was strengthened by consideration of the cyanide and azide inhibition of mitochondrial respiration of *E. tenella*. When the data of Figs. 7 and 8 were plotted in the form of a Dixon plot (Figs. 11 and 12), the biphasic character of cyanide and azide inhibition of *E. tenella* mitochondria became apparent. Two apparent K_i values for cyanide were obtained against sensitive *E. tenella* mitochondria, the one equal to 1×10^{-6} M corresponding to the inhibition of a small proportion (<30%) of the total respiration while the other of 9.2×10^{-6} M corresponded to inhibition of the major portion of respiration (>70%) that was more resistant to inhibition by cyanide (Fig. 11). Similarly, two apparent K_i values were obtained for azide, equal to 2.5×10^{-4} and 4.4×10^{-3} M (Fig. 12). Similar K_i values were given for cyanide inhibition of decoquinate resistant mitochondria (1×10^{-6} and 7.1×10^{-6} M) and clopidol resistant mitochondria (7.1×10^{-6} M), as well as for azide inhibition of decoquinate resistant mitochondria (2.5×10^{-4} and 4.5×10^{-3} M) and clopidol resistant mitochondria (3.8×10^{-3} M). Low K_i values for cyanide and azide inhibition of clopidol resistant mitochondria were not readily apparent from the data in Figs. 11 and 12.

In mitochondria isolated from sensitive *E. tenella*, inhibition of up to 70% of the respiration with 6×10^{-10} M decoquinate resulted in the remainder of the respiration being fully inhibited by 1.5×10^{-6} M cyanide and 5×10^{-4} M azide. In decoquinate resistant mitochondria, even that small portion of respiration (about 10%) which was insensitive to 10^{-6} M decoquinate was fully inhibited by 1.5×10^{-6} M cyanide. However, in sensitive mitochondria, that portion of the respiration remaining after addition of low concentrations of clopidol (up to 10^{-6} M) was inhibited completely only by higher concentrations of cyanide (>3×10^{-5} M) or azide (>9×10^{-3} M).

Terminal respiration of *E. tenella* mitochondria (Berkhamsted line) was assayed using ascorbate + TMPD or ascorbate + cytochrome *c*.

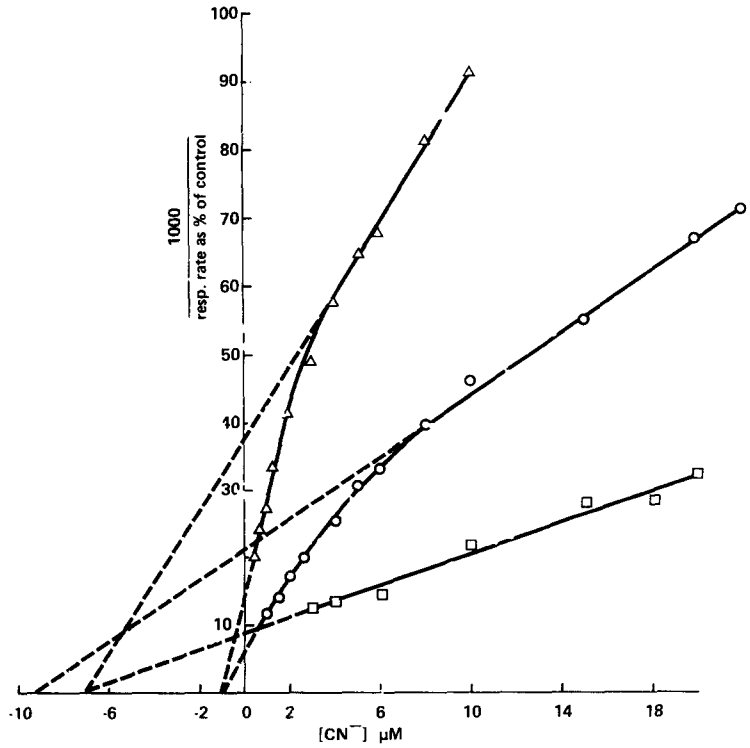

Fig. 11. Dixon plot for inhibition of mitochondrial respiration by cyanide. Sensitive line, ○—○; decoquinate resistant line, △—△; clopidol resistant line, □—□. Data replotted from Fig. 7.

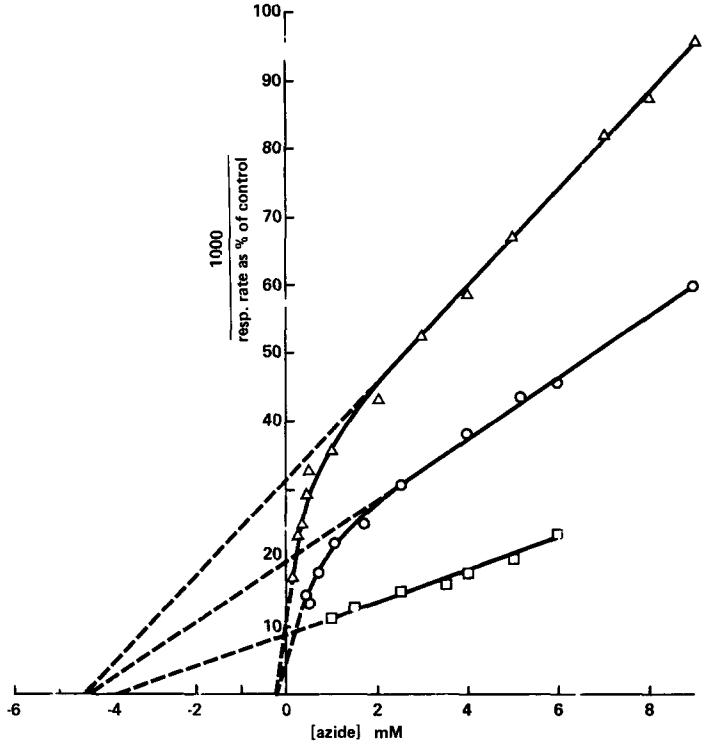

Fig. 12. Dixon plot for inhibition of mitochondrial respiration by azide. Details as in legend to Fig. 11. Data replotted from Fig. 8.

Little or no respiratory activity was obtained with ascorbate alone, but addition of TMPD or cytochrome *c* ensured a relatively high rate of oxygen utilization (see Table 1); examples are given in the oxygen traces of Fig. 13 and show the effect of cyanide and azide on respiration. Ascorbate + TMPD-supported respiration was more than 60% inhibited by 2.3×10^{-6} M cyanide or 3×10^{-4} M

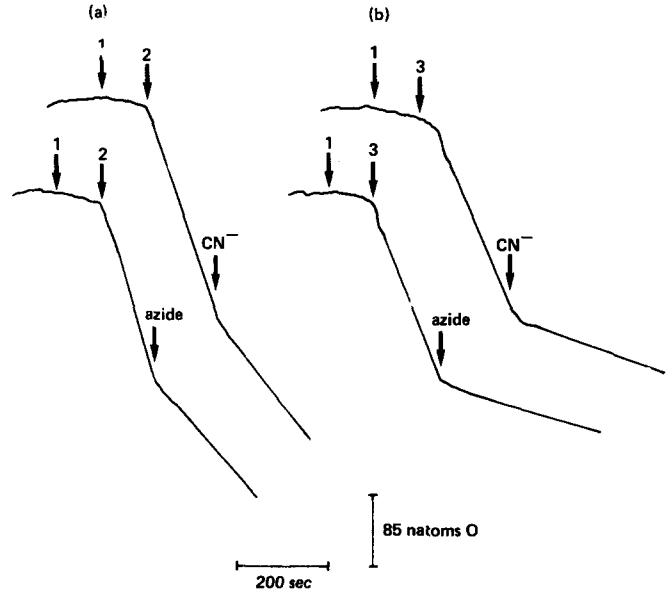

Fig. 13. Polarographic traces for *E. tenella* mitochondria respiring with (a) ascorbate + TMPD and (b) ascorbate + cytochrome *c*. Arrows denote point of addition of cyanide (to 2.3×10^{-6} M) or azide (to 3×10^{-4} M). 1 = Addition of ascorbate, 2 = addition of TMPD, 3 = addition of cytochrome *c*. Mitochondria were isolated from a drug sensitive line of *E. tenella*. Other details as in Materials and Methods.

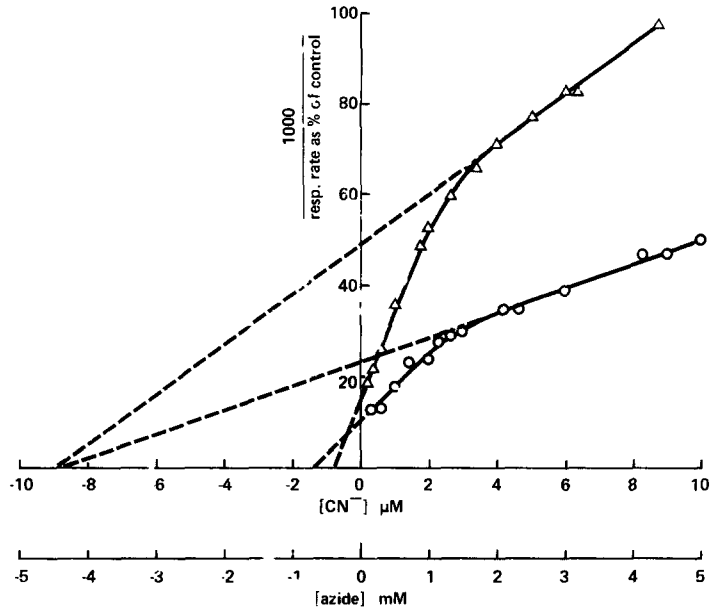

Fig. 14. Dixon plots for cyanide and azide inhibition of ascorbate + TMPD respiration in *E. tenella* mitochondria. Inhibition by cyanide, O—O; inhibition by azide, △—△. Mitochondria were isolated from a drug sensitive line of *E. tenella*. Results are the mean of two determinations on three different mitochondrial preparations.

azide; these concentrations of cyanide and azide produced greater than 85% inhibition of ascorbate + cytochrome *c* respiration. Inhibition of ascorbate + TMPD-supported respiration by cyanide and azide is shown in the form of a Dixon plot in Fig. 14 and clearly indicates a biphasic response of terminal respiration to these inhibitors. Apparent K_i values for cyanide of 1.4×10^{-6} and 8.7×10^{-6} M and for azide of 4×10^{-4} and 4.4×10^{-3} M were obtained.

DISCUSSION

The present studies support and expand those reported by Wang [7, 8]. Mitochondrial electron transport in *E. tenella* is susceptible to inhibition by the anticoccidial agents decoquinate and clopidol. Further, mitochondria isolated from decoquinate and clopidol resistant strains of *E. tenella* are also resistant to the corresponding drug at the level of electron transport. These results support the notion that the mode of action of the quinolone and pyridone anticoccidials is at the level of mitochondrial electron transport. Furthermore, the synergistic nature of quinolone and pyridone drug combinations observed *in vivo* against coccidia [2] has been confirmed and also shown to be operative at the level of inhibition of mitochondrial respiration.

The results of the *in vitro* and *in vivo* sensitivity tests provide some interesting and useful comparisons. The *in vivo* EC_{50}s of decoquinate and clopidol on the Berkhamsted line of *E. tenella* (Weybridge) differ by nearly 12-fold but the equivalent *in vitro* EC_{50}s differ by over 7000-fold. It may be that the *in vitro* results represent the true relative values and that the discrepancies between the *in vivo* results reflect differences in the pharmacokinetics of the drugs, but for the present this must remain as speculation only. Mitochondria isolated from the clopidol resistant line of *E. tenella* showed a 2.9-fold resistance to clopidol, a result confirmed in the *in vivo* test (3.2-fold). *In vitro*, the decoquinate resistant line showed an almost 80-fold resistance to decoquinate, but *in vivo* resistance appeared to be >560-fold. This difference also might be explained by consideration of the pharmacokinetics of decoquinate. When decoquinate is fed continuously at different concentrations to chickens, tissue levels are not increased when diet concentration exceeds a certain critical level. From published data this concentration is ≤30 mg/kg [17]. Hence, parasites resistant to the tissue concentration resulting from this critical diet concentration will not be controlled by administering more drug in the diet, and resistance will thus appear to be absolute although in reality it is not. Apparently, over the dose range of clopidol used, this phenomenon does not occur. (We were unable to trace any tissue residue data over a range of doses of clopidol in the literature.)

In vivo, mixtures of decoquinate and clopidol are highly synergistic, although, at the level of electron transport, synergism is most marked at low drug concentrations. Some caution should be exercised in comparing directly the synergism observed *in vivo* with that observed at the level of electron transport; the basis of the synergistic effect might be quite different in the two cases. Synergism at the level of electron transport may fundamentally be most important in determining the site and mode of respiratory inhibition by these two drugs.

Collateral sensitivity to quinolones has been observed in coccidia resistant to clopidol [3]. In the present study, collateral sensitivity *in vivo* was demonstrated only in the increased sensitivity of a clo-

pidol resistant line of *E. tenella* to decoquinate. The obverse was not shown. However, the *in vitro* results of this study do suggest that collateral sensitivity in both directions is manifest at the level of electron transport, although it was more evident in the sensitivity towards clopidol of decoquinate resistant mitochondria than vice versa.

There is a definite relationship between the susceptibility of mitochondrial respiration to inhibition by decoquinate or clopidol on the one hand, and to inhibition by cyanide and azide on the other. Resistance of respiration to decoquinate results in an increased sensitivity to cyanide and azide, whereas resistance to clopidol results in a decreased sensitivity to cyanide and azide. That portion of the total respiration most sensitive to cyanide and azide is most sensitive to clopidol, but relatively insensitive to decoquinate. Cyanide and azide inhibition of drug sensitive and resistant lines of *E. tenella* mitochondria, expressed in the form of Dixon plots, supports spectral data in suggesting the presence of two terminal oxidases in such mitochondria. Respiration mediated by that terminal oxidase sensitive to low concentrations of cyanide and azide (K_i of 1×10^{-6} and 2.5×10^{-4} M, respectively) is detectable in sensitive and decoquinate resistant mitochondria, although it accounts for a greater proportion of the respiration in the resistant strain; in clopidol resistant mitochondria this terminal oxidase apparently contributes only a minor amount to the respiratory activity.

Ascorbate + TMPD or ascorbate + cytochrome *c* are generally accepted substrates for the assay of cytochrome oxidase. Nevertheless, artificial electron donors, such as *p*-phenylenediamine, have been widely used to study bacterial oxidases, and the assay of a cytochrome *o* oxidase from *Pseudomonas aeruginosa* using ascorbate + TMPD has recently been described [18]. It is probably significant therefore that the assay of terminal respiration in *E. tenella* mitochondria with ascorbate + TMPD produces two K_i values for cyanide or azide inhibition. A comparison with those concentrations of cyanide and azide that are necessary to inhibit mammalian cytochrome oxidase would suggest that the major contributing oxidase is probably of the cytochrome aa_3 type (low K_i for cyanide and azide), while spectral evidence would suggest that a cytochrome *o* oxidase may also be operative (higher K_i for cyanide and azide). In the experiments described here, K_i values obtained for cyanide and azide inhibition of ascorbate + TMPD respiration agree closely with those obtained for NADH-oxidase.

The possibility of two functional oxidases in *E. tenella* mitochondria, and consequently of a branched or parallel electron transport chain, provides an interesting though speculative basis for the observed resistance to decoquinate and clopidol. Although Wang [7] had showed clopidol to have no obvious effect on mitochondrial respiration in *E. tenella*, it was later proposed [19] that there might be an alternative pathway of electron transport in coccidial mitochondria susceptible to inhibition specifically by clopidol. The present studies appear to at least partly support that proposal. Respiration in mitochondria isolated from sensitive *E. tenella* is presumably mainly via a dominant pathway fully sensitive to decoquinate and culminating in a terminal oxidase inhibited fully only by relatively high concentrations of cyanide and azide (possibly an *o*-type cytochrome oxidase). However, resistance to decoquinate could be the result of electron flow being diverted mainly through a pathway that is inhibited only by relatively high concentrations of decoquinate and terminating in a cytochrome aa_3 oxidase that is sensitive to very low concentrations of cyanide and azide. Similarly, resistance to clopidol could result from electron transport being diverted through that pathway inhibited by rather higher concentrations of clopidol, and terminating in that terminal oxidase inhibited by relatively high concentrations of cyanide and azide. The pathway resistant to decoquinate is nevertheless sensitive to inhibition by low concentrations of clopidol, in the same way that the pathway more resistant to clopidol is still sensitive to low concentrations of decoquinate. Both pathways are presumably inhibited by decoquinate and clopidol, but one pathway (terminating in the cytochrome aa_3 oxidase) contains a low affinity binding site for decoquinate and a high affinity binding site for clopidol, whereas the other pathway (terminating in an alternative oxidase) contains a high affinity binding site for decoquinate and a lower affinity binding site for clopidol. High and low affinity binding sites may be more readily identifiable in the case of decoquinate (compare on EC_{50} for decoquinate of 2.9×10^{-10} M against the Berkhamsted line, with an EC_{50} of 2.3×10^{-8} M against the Berkhamsted decR line) than in the case of clopidol (compare an EC_{50} for clopidol of 2.1×10^{-6} M against the Berkhamsted line, with an EC_{50} of 6.1×10^{-6} M against the Berkhamsted cloR line).

In summary, the inhibition of *E. tenella* mitochondrial electron transport by decoquinate and clopidol, and the resistance to these anticoccidial agents observed at the level of electron transport supports the notion that these drugs exert their effect on electron transport in *E. tenella*. These results also correlate favourably with observations made *in vivo* pertaining to the synergistic effects of quinolone and pyridone coccidiostats and to the collateral sensitivity to decoquinate observed in clopidol resistant lines of coccidia. In addition, the results of this study suggest the existence of a branched or parallel electron transport chain in *E. tenella* mitochondria that could provide the basis for resistance to these anticoccidial agents and may also explain their synergistic behaviour.

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