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The Effects of the Antiparasitic Drugs Levamisole, Thiabendazole, Praziquantel, and Chloroquine on Mitochondrial Electron Transport in Muscle Tissue from *Ascaris suum*

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SUMMARY

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The effects of the antiparasitic drugs levamisole, thiabendazole, chloroquine, and praziquantel on electron transport in *Ascaris* muscle submitochondrial particles were compared. These compounds were found to inhibit various mitochondrial NADH-oxidizing enzyme activities, including NADH oxidase and NADH-fumarate, NADH-duroquinone, and NADH-cytochrome *c* reductases. The inhibitory effects of these drugs were the same toward both NADH oxidase and NADH-fumarate reductase activities, indicating a common inhibitory site for both enzyme systems. Evidence for the location of this inhibition site has been obtained from kinetic analyses. Inhibition of NADH oxidase activity by the drugs was found to be noncompetitive toward NADH, whereas a competitive relationship resulted in the inhibition of the enzyme-catalyzed reduction of duroquinone by NADH with respect to the quinone. This suggests that the primary sensitive site is located where the NADH dehydrogenase reduces the quinone. The failure to observe appreciable inhibition by the drugs of the mitochondrial oxidation of NADH by ferricyanide demonstrates that the drugs do not act between substrate and flavoprotein of the NADH dehydrogenase segment. With the exception of thiabendazole, the drugs did not significantly affect either succinate oxidase or succinate-cytochrome *c* reductase activities. Nor did the compounds significantly affect the inhibition of succinate dehydrogenase. Again with the exception of thiabendazole, reduced coenzyme Q_2-cytochrome *c* reductase activity of the submitochondrial particles was essentially insensitive to concentrations of inhibitors that highly diminished NADH oxidase and NADH-fumarate and NADH-duroquinone reductase activities. These results clearly assign a locus of inhibition by various anthelmintics and by chloroquine to the quinone-reducing site of the mitochondrial NADH dehydrogenase segment. Comparative studies with thiabendazole indicate that this compound is inhibitory at additional sites of the electron transport system of the parasite.

INTRODUCTION

Comparison of the electron transport system of the mitochondria of *Ascaris* and other parasitic helminths with that of higher animals has revealed some remark-

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able differences (for reviews, see refs. 1–3). One basic characteristic is that the reoxidation of mitochondrial reduced pyridine nucleotide may be accomplished mainly by a fumarate-dependent pathway (4–6). Since the role of this system in the mitochondrial energy metabolism of many helminth parasites appears to be as vitally important as the classical respiratory chain in higher animals (4–9), this system has been considered a particularly vulnerable site for chemotherapeutic attack. Broad-spectrum anthelmintics, such as levamisole[1] or thiabendazole and some of its chemically related compounds, have been reported to act by inhibiting this enzymatic system and its associated ATP production in a number of helminth parasites (10–15).

Evidence of the last few years indicates that the mode of chemotherapeutic action of those drugs which affect the fumarate reductase reaction in parasites may be far more complex than originally thought (13, 16–20). This large amount of published material still does not provide a comprehensive mechanism of lethal action of these drugs. Recent pharmacological studies with levamisole (17–20) suggest that the initial action of this drug may be on the neuromuscular system rather than on mitochondrial energy-generating processes.

The present study was undertaken to understand more fully the mechanism of electron transport inhibition induced by particular drugs. We have therefore investigated the kinetics of various NADH- and succinate-oxidizing activities of the muscle mitochondria from the intestinal nematode *Ascaris suum* and the effects of levamisole, thiabendazole, and praziquantel on these reactions. The last of these compounds has only recently been discovered to be chemotherapeutically active against cestode and schistosome infections (21, 22).

In addition, the effect of the conventional antimalarial drug chloroquine on the mitochondrial electron transport system of *Ascaris* was investigated. This seemed of particular interest because chloroquine shows some structural similarity to praziquantel and has been reported to inhibit electron transport in mammalian mitochondria in the span from NADH to duroquinone and coenzyme Q_1 competitively with respect to the quinone (23).

A preliminary report of this study has appeared (24), and a detailed study on the membrane-bound NADH- and succinate-oxidizing system of *Ascaris* muscle mitochondria is to be reported elsewhere.

METHODS

Preparation of Ascaris submitochondrial particles. Muscle mitochondria of adult female *A. suum* were prepared as described recently (25). The mitochondria were resuspended in 1.5 ml of 2 mM EDTA (pH 8.5) per 10 g of initial muscle fresh weight and subjected to sonic irradiation for 2 min at 0°, employing a Branson Sonifier with a microtip at a power setting of 50 W. The sonicated suspension was centrifuged for 10 min at 10,000 × *g*. The turbid supernatant was removed and recentrifuged at 105,000 × g for 30 min. The supernatant was discarded, and the pellet was resuspended in a medium consisting of 220 mM mannitol, 70 mM sucrose, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate, and 0.1 mM EDTA (pH 7.4) and centrifuged as before. The resultant pellet was resuspended in the mannitol–sucrose medium (final protein concentration, approximately 20 mg/ml) and used as such or stored frozen at −20°.

Analytical procedures. Unless otherwise specified, electron transport enzyme activities were determined spectrophotometrically in the presence of 0.1 M Tris-HCl (pH 8.0), 1.0 mM EDTA[2], and the appropriate amount of mitochondrial par-

[1] Levamisole is the levorotatory isomer of 2,3,5,6-tetrahydro-6-phenyl imidazo[2,1-*b*]thiazole (the racemic mixture is known as tetramisole); thiabendazole is 2-(thiazol-4'-yl)benzimidazole; praziquantel is 2-cyclohexylcarbonyl-1,3,4,6,7,11b-hexahydro-2H-pyrazino[2,1-*a*]isoquinoline-4-one; chloroquine is 7-chloro-4-(4-diethylamino-1-methylbutylamino)-quinoline.

[2] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; DQ, duroquinone; Q, coenzyme Q or ubiquinone, with the subscript referring to the number of isoprene side chains.

ticle preparation. Unless otherwise indicated, all enzyme assays were conducted at 25° in a final volume of 1.0 ml.

NADH and succinate dehydrogenase assays also contained 1.0 mM ferricyanide and 0.1 mM NADH or 10 mM potassium succinate, respectively. Reduction of ferricyanide was followed at 420 nm (ϵ = 1.03 M^{-1} cm^{-1}). NADH-fumarate, NADH-DQ, and NADH-Q_2 reductase activities were measured anaerobically in a final volume of 2 ml. In addition to Tris-HCl and EGTA, assay mixtures contained 0.1 mM NADH and 10 mM fumarate or quinone derivative as indicated in the figures. Oxidation of pyridine nucleotide was followed at 340 nm (ϵ = 6.22 M^{-1} cm^{-1}). The assays for NADH–cytochrome *c* and succinate–cytochrome *c* reductase activities contained, in addition to buffer and EGTA, 0.1 mM cytochrome *c* and 0.1 mM NADH or 10 mM potassium succinate, respectively, and the increase in absorbance at 550 nm (ϵ = 18.5 M^{-1} cm^{-1}) was followed.

NADH oxidase activity was assayed in the presence of 0.1 M Tris-HCl (pH 8.0) and 1.0 mM EGTA, either spectrophotometrically at 340 nm or polarographically using a Clark electrode for measuring oxygen consumption. The NADH concentration was 0.1 mM or 0.5 mM, respectively.

Succinate oxidase activity was measured polarographically, utilizing the Clark electrode. The conditions were otherwise the same as those described for the NADH oxidase assay, except that NADH was replaced by 10 mM potassium succinate.

Reduced Q_2–cytochrome *c* reductase activity was assayed by measuring spectrophotometrically the reduction of cytochrome *c* with the reduced quinone as donor at 550 nm. The assay medium consisted of 1.0 ml (final volume) of 0.1 M potassium phosphate (pH 7.4), 1.0 mM EGTA, 0.10 mM cytochrome *c*, 0.15 mM reduced Q_2, 10 mM KCN, and the appropriate amount of particle preparation. Q_2 was reduced with sodium dithionite according to Rieske (26). An identical sample of the assay system, but with no enzyme present, was used as the reference.

Protein was estimated by the method of Lowry *et al.* (27). Other experimental conditions are described in detail in the figure and table legends.

Chemicals. Chloroquine and duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone) were purchased from Sigma Chemical Company. Coenzyme Q_2 (2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone) was a gift from Dr. A. Wagner of the Merck Institute for Chemotherapeutic Research. The anthelmintics were kindly supplied by the companies shown: levamisole, Janssen Pharmaceutica; thiabendazole, Merck Sharp & Dohme; praziquantel, Bayer AG.

RESULTS

The inhibition of NADH oxidase and NADH-fumarate reductase activities of helminth mitochondria by levamisole and thiabendazole has been reported (10–16), but the mechanism of the inhibition was not studied. The effects of various drugs, including levamisole and thiabendazole, on NADH oxidation in *Ascaris* submitochondrial particles appeared to be independent of whether oxygen or fumarate was used as the terminal electron acceptor (Table 1). The drug-induced inhibition of both alternate NADH-oxidizing pathways was similar, suggesting a common inhibitory site for both pathways. The apparent K_i values derived from Dixon plots were found to decrease in the order levamisole thiabendazole praziquantel chloroquine. The effects of the chemotherapeutic agents on NADH–cytochrome *c* reductase activity were also studied (Table 1). The apparent K_i values resembled those of NADH oxidase and NADH-fumarate reductase, again indicating a common site of drug attack for NADH oxidation following different terminal pathways.

The Lineweaver-Burk plots of Fig. 1 show that the inhibition of NADH oxidase activity by the various drugs was noncompetitive with respect to its donor substrate NADH. These experiments suggest that the substrate site of the NADH-oxidizing pathway does not appear to be the main point of interaction of all these agents. This can also be seen from the data of Table 2. Employing the ferricyanide assay

TABLE 1

Effects of drugs on NADH oxidation of Ascaris muscle submitochondrial particles employing various electron acceptors

Apparent inhibition constants (K_i) were calculated from Dixon plots of variation of activity with inhibitor concentration. Enzyme activities, in the presence and absence of the indicated drugs, were assayed spectrophotometrically in Tris-HCl at a final pH of 8.0 and 25°. The assay systems were incubated with the inhibitors for 5 min prior to the initiation of the reaction. Enzyme activities in the absence of inhibitors were 75, 58, and 157 nmoles of NADH oxidized per minute per milligram of protein for NADH oxidase, NADH-fumarate reductase, and NADH–cytochrome *c* reductase, respectively. Average concentrations of submitochondrial particle protein in the reaction mixtures were 124, 106, and 66 μg/ml, respectively. Other experimental details are described in the legend to Fig. 2 and under METHODS.

Drug	Apparent inhibition constant (K_i) for drug			
	NADH → O_2	NADH → fumarate	NADH → cytochrome *c*	NADH → DQ
	μM	μM	μM	μM
Levamisole	510	550	600	100
Thiabendazole	303	290	490	70
Praziquantel	232	240	360	70
Chloroquine	2.3	3.0	2.6	1.2

for measuring electron transport-linked NADH dehydrogenase, levamisole, thiabendazole, and chloroquine were essentially without effect on this activity, and only a low level of inhibition was noted with praziquantel. However, the same drug concentrations were markedly inhibitory toward NADH oxidase activity.

As the inhibition of NADH oxidase and NADH-fumarate reductase activities by the drugs was obviously attributable to a common inhibitory site at a point before the branch of both alternate pathways (see Fig. 3), it was of interest to examine the effects of these compounds on the acceptor (probably quinone) site of the NADH dehydrogenase segment. The latter enzyme complex is expected to be the common primary dehydrogenase for both alternate electron transport pathways, as shown for mammalian mitochondria (4, 28). Our results indicate that certain substituted benzoquinones, such as duroquinone (DQ) or ubiquinone 2 (Q_2), can function as electron acceptors in the mitochondrial NADH dehydrogenase segment of the parasite. These compounds were shown to be reduced at a site after the rotenone site[3] and thus resemble the reduction of endogenous ubiquinone (Q_{10}) in the corresponding mammalian enzyme complex in this regard.

Levamisole, thiabendazole, praziquantel, and chloroquine inhibited mitochondrial reduction of DQ by NADH competitively with respect to the quinone (Fig. 2). The corresponding apparent K_i values were found to be considerably lower than those for NADH oxidase, NADH-fumarate reductase, and NADH–cytochrome *c* reductase activities, which indicates that the DQ reductase reaction is the most sensitive site of the entire electron transport system. However, much higher concentrations of drugs were required to produce similar inhibition rates with Q_2 as acceptor than with DQ.

The results of Table 2 also demonstrate that (with the exception of thiabendazole) the drugs did not significantly affect the oxidation of reduced Q_2 by submitochondrial particles with cytochrome *c* as acceptor under conditions in which NADH oxidase activity was markedly inhibited.

Concentrations of the drugs affecting NADH oxidation were generally without effect on the succinate-oxidizing activities of *Ascaris* mitochondrial particles (Table 3). As with the NADH counterpart, none of the drugs significantly inhibited the substrate site of the succinate dehydrogenase segment in assays with ferricyanide as oxidant. Marked sensitivity of succinate-cytochrome *c* reductase and succinate oxidase activities was found only with thiabendazole, which caused approximately 50% inhibition at 1 mM. Interestingly, the inhibition of NADH oxidase by the drugs investigated was found to be dependent on pH over the range 7.0–9.0 (not shown). This effect is probably connected with increased dissociation of protonated ring atoms of the drugs with increasing pH.

[3] Unpublished observations.

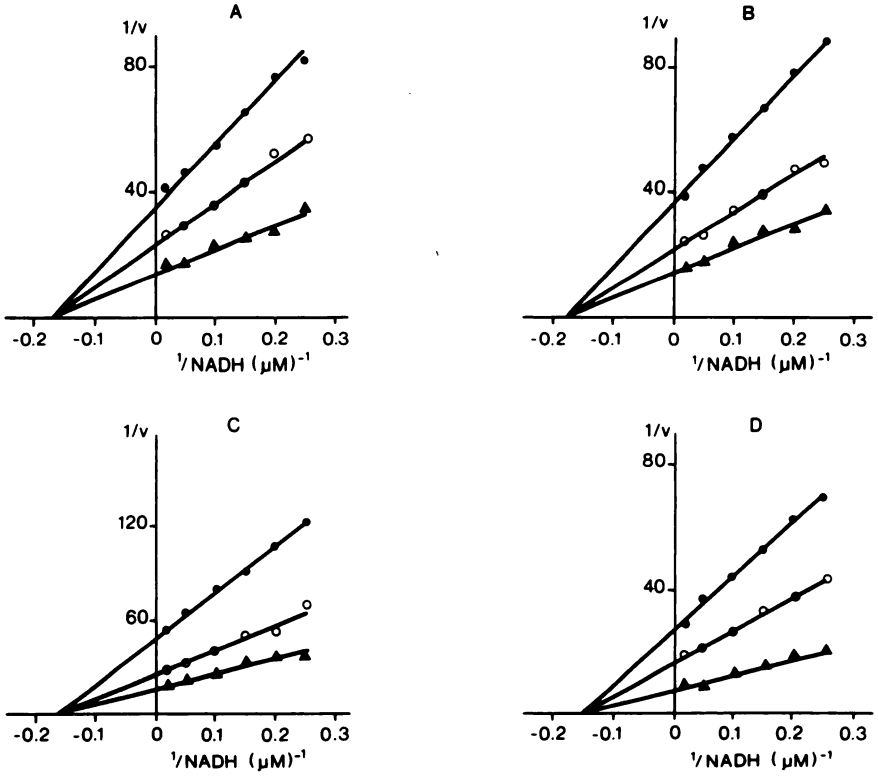

FIG. 1. *Noncompetitive inhibition of NADH oxidase activity in Ascaris muscle submitochondrial particles by various antiparasitic drugs: Lineweaver-Burk plots of variation of activity with NADH concentration*

Details of the conditions for the measurement of NADH oxidase activity are given in Table 1 and under METHODS. Enzyme assays contained an average of 175 μg of submitochondrial particle protein per milliliter. A. ▲——▲, no levamisole; ○——○, 0.3 mM levamisole; ●——●, 0.8 mM levamisole. B. ▲——▲, no thiabendazole; ○——○, 015 mM thiabendazole; ●——●, 0.6 mM thiabendazole. C. ▲——▲, no praziquantel; ○——○, 0.15 mM praziquantel; ●——●, 0.6 mM praziquantel. D. ▲——▲, no chloroquine; ○——○, 2 μM chloroquine; ●——●, 4 μM chloroquine.

DISCUSSION

The inhibitory effects of levamisole and anthelmintics with benzimidazole structures on electron transport of mitochondrial preparations from helminth parasites has already been reported, although the site of action of these drugs has remained undetermined. Prichard (12) found that the NADH-fumarate reductase system of *Haemonchus contortus* mitochondria is much more susceptible to thiabendazole than is NADH oxidase, indicating two different sites of drug action. Other studies (11, 13) have interpreted the inhibitory effect of levamisole on electron transport of mitochondrial preparations derived from various nematode parasites as an effect on the terminal fumarate-reducing reaction, which is believed to proceed by reversal of succinate dehydrogenation (5).

The present paper confirms the observed inhibitory effects of levamisole and thiabendazole on NADH oxidation, together with the finding that in *Ascaris* submitochondrial particles the oxidase and NADH-fumarate reductase activities were similarly affected by these drugs. This supports the idea that the drugs investigated interrupt electron transport at one site common to both NADH-oxidizing pathways.

Additional studies on the influence of these drugs on NADH-duroquinone reductase activity assign a locus of drug inhibition on the NADH dehydrogenase segment in the region that mediates the reaction

TABLE 2

Effects of drugs on NADH dehydrogenase, NADH oxidase, and reduced Q_2-cytochrome c reductase activities of Ascaris muscle submitochondrial particles

NADH dehydrogenase, NADH oxidase, and reduced Q_2-cytochrome *c* reductase activities in the absence of inhibitors were 3.52, 0.054, and 0.092 μmoles/min/mg of protein. Average concentrations of submitochondrial particle protein in the reaction mixtures were 5, 114, and 162 μg/ml, respectively. Other experimental conditions are described in Table 1 and under METHODS.

Drug	Concentration	Inhibition of		
		NADH dehydrogenase	NADH oxidase	Reduced Q_2-cytochrome *c* reductase
	mM	%	%	%
Levamisole	1.0	3	62	4
	5.0	0	85	
Thiabendazole	1.0	0	66	78
	2.0	0	87	
Praziquantel	1.0	18	81	8
	5.0	27	97	
Chloroquine	0.05	0	92	0
	0.25	0	100	

between the enzyme and the quinone derivative. A possible explanation for the discrepancies of our results from those described by other authors could lie in the different particle preparations employed. However, it is difficult to accept the idea that structural alterations of the electron transport chain can shift the specific binding site of an inhibitor from one locus to another.

Ascaris is known to contain rhodoquinone (29) in place of ubiquinone (Q_{10}) in mammalian mitochondria. Evidence has been presented by Sato *et al.* (30) that rhodoquinone participates in the *Ascaris* mitochondrial succinate oxidase system as ubiquinone does in the corresponding mammalian system. This may suggest that quinone also functions as a natural acceptor for reducing equivalents from the iron-sulfur proteins of the parasitic mitochondrial NADH dehydrogenase in the same way as Q_{10} does in mammalian mitochondria (31, 32). Therefore the observed inhibition of NADH oxidase and NADH-fumarate reductase reactions by the drugs

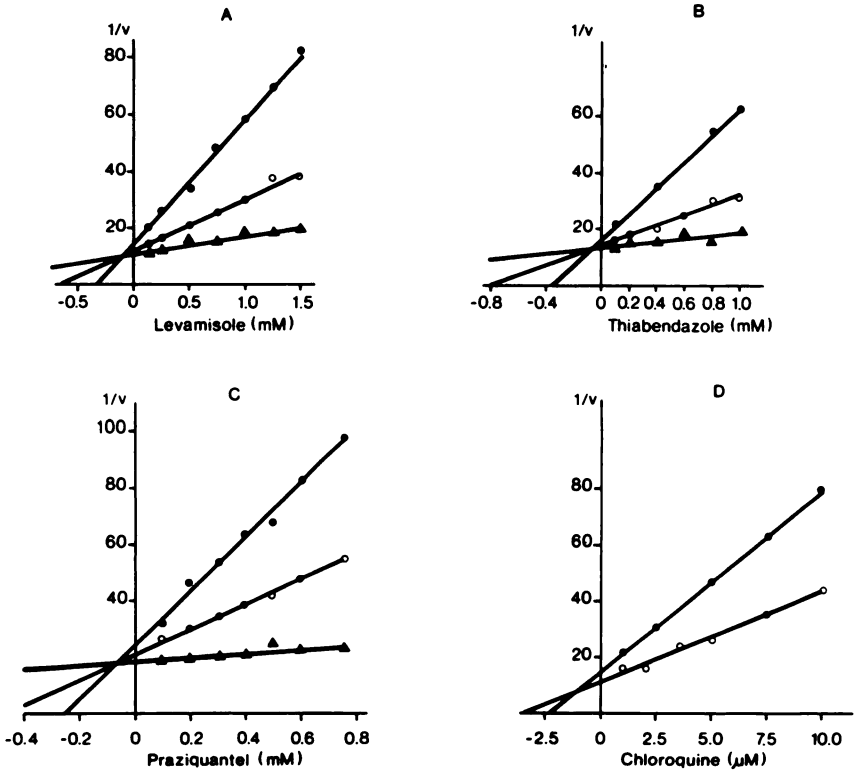

TABLE 3

Effects of drugs on various succinate-oxidizing activities of Ascaris muscle submitochondrial particles

Incubations contained 1.0 mM (final concentration) inhibitors except for chloroquine, which was 0.1 mM in the succinate dehydrogenase and succinate cytochrome *c* reductase assays and 0.04 mM in the succinate oxidase assay. Other experimental conditions are described in Table 1 and under METHODS. Succinate oxidation activities in the absence of drugs were 655, 79, and 23 nmoles of succinate oxidized per minute per milligram of protein. Average concentrations of submitochondrial particle protein in the reaction mixtures were 76, 14, and 304 μg/ml, respectively.

Drug	Inhibition of		
	Succinate dehydrogenase	Succinate-cytochrome *c* reductase	Succinate oxidase
	%	%	%
Levamisole	0	0	0
Thiabendazole	0	53	52
Praziquantel	0	10	0
Chloroquine	12	2	9

is expected to occur through an interaction with the endogenous quinone function. Further support for this conclusion derives from the finding of similar behavior for the various anthelmintics and the antimalarial drug chloroquine. Skelton *et al.* (33) have suggested that chloroquine inhibits NADH oxidase in the region of ubiquinone function. More recently (23, 34) this compound has been described as a competitive inhibitor of the respiratory chain NADH-DQ and NADH-Q_1 reductase activities, with a K_i of 2.8 μM for the NADH-DQ reductase reaction. From these results it has been concluded by Lawford and Garland (23) that the chloroquine-sensitive site can be equated with the site at which the enzyme reduces the quinone. In concurrence with these results, the *Ascaris* system shows the NADH-DQ reductase to be competitively inhibited by chloroquine with a K_i value of 2.6 μM, which is close to the 2.8 μM value for the mammalian enzyme.

Our observation that Q_2 reduction by chloroquine and the other drugs was inhibited much less than duroquinone reduction is also in accord with the literature. Ruzicka and Crane (34) have suggested from inhibitor studies with chloroquine that Q_2 accepts electrons from a carrier site on NADH dehydrogenase that is located before the site of the duroquinone reaction and thus is less influenced by the drug. However, the suggestion made by the same authors that chloroquine may inhibit the oxidation of reduced Q_2 rather than the reduction of Q_2 is not consistent with our findings. With the exception of thiabendazole, neither the anthelmintics nor chloroquine significantly affected reduced Q_2–cytochrome *c* reductase activity. The possibility that the substrate site of NADH dehydrogenase could have been affected by the drugs has been excluded by the finding that NADH-ferricyanide reductase activity was reduced only slightly or not at all under conditions that markedly inhibited electron transport to the fumarate reductase or terminal oxidase, respectively.

In contrast to the drug responses of the various NADH-oxidizing pathways, succinate oxidase and succinate–cytochrome *c* reductase activities were significantly inhibited only by thiabendazole. This would indicate at least an additional site of inhibition by the benzimidazole compound. As outlined in Fig. 3, the primary site of

FIG. 2. *Competitive inhibition of NADH-DQ reductase activity in Ascaris muscle submitochondrial particles by various antiparasitic drugs*

Experimental details are described under METHODS. Assay mixtures contained an average of 51 μg of submitochondrial particle protein per milliliter. A. Dixon plot of variation of activity with levamisole concentration: ●——●, 6.25 μM DQ; ○——○, 50 μM DQ; ▲——▲, 190 μM DQ. B. Dixon plot of variation of activity with thiabendazole concentration: ●——●, 5 μM DQ; ○——○, 100 μM DQ; ▲——▲, 300 μM DQ. C. Dixon plot of variation of activity with praziquantel concentration: ●——●, 10 μM DQ; ○——○, 50 μM DQ; ▲——▲, 200 μM DQ. D. Dixon plot of variation of activity with chloroquine concentration: ●——●, 6.25 μM DQ; ○——○, 50 μM DQ.

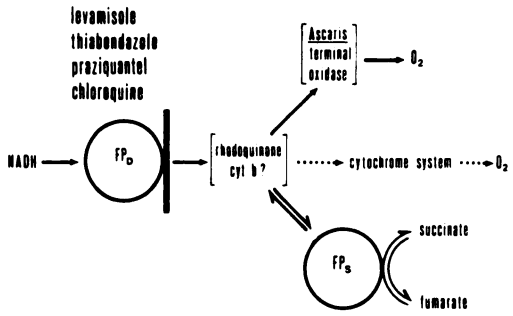

FIG. 3. *Schematic representation of NADH and succinate oxidase systems in Ascaris muscle mitochondria and possible site of inhibition by antiparasitic drugs*

The solid bar represents this possible inhibition site. The terms FB_D and FP_S refer to the flavoproteins of NADH dehydrogenase and succinate dehydrogenase, respectively. The dotted arrows represent reactions of minor or doubtful significance.

attack would reside between NADH dehydrogenase and quinone. A second inhibitory site may exist in the region between succinate dehydrogenase and the terminal alternate oxidase. Our experiments show that the substrate site of the succinate dehydrogenase is not affected by any of the drugs investigated at concentrations that strongly affected various NADH-dependent oxidizing activities.

In spite of the fact that the precise organization and function of mitochondrial electron transport in *Ascaris* muscle are still far from clear (1–3), a scheme is proposed (based on current knowledge and data to be reported elsewhere) indicating the presumed sites of inhibition by the drugs investigated (Fig. 3). Whether or not the anthelmintic agents employed act chemotherapeutically by inhibiting mitochondrial electron transport, and thus its associated phosphorylation system, cannot be determined from these studies alone. However, there is much evidence indicating that inhibition of the fumurate reductase system may be not relevant to the mode of action of these drugs *in vivo*. Experiments *in vitro* have shown that concentrations of levamisole necessary to induce muscular contraction of adult *A. suum* are far below those required for inhibition of electron transport and thus the synthesis of ATP (18, 20). Wang and Saz (35) found that low concentrations of levamisole caused complete cessation of motility of various filarial parasites, which apparently do not possess a functional fumarate reductase system. Similarly, thiabendazole showed ovicidal activity in eggs of *Nematodirus spathiger* which also have no demonstrable fumarate reductase (19). Praziquantel, which exhibits high cestocidal and schistosomicidal activity, was found to be without effect against *Ascaris*[4] although this drug was readily taken up by the worm in a system *in vitro*[5]. In addition, electron transport in submitochondrial particles from mammalian tissues was found to be affected by the drugs investigated, apparently by a mechanism similar to that in the parasite system[5] (23), i.e., inhibition of the NADH dehydrogenase segment at the quinone reducing site.

These observations make it difficult to visualize drug effects on mitochondrial electron transport as the primary site of chemotherapeutic attack. On the other hand, it cannot be completely excluded at the moment that the effects observed may contribute to the over-all chemotherapeutic activity, as previously suggested for levamisole (13).

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