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Effect of the Systemic Fungicide Carboxin on Electron Transport Function in Membranes of *Micrococcus denitrificans*

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The systemic fungicide carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) inhibited oxidation of succinate by membranes prepared from *Micrococcus denitrificans*, the K_i being 16 μM. Oxycarboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide-4,4-dioxide), F831 (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide-4-oxide), and another succinate oxidase inhibitor, 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione (TTB) were less effective inhibitors of succinate oxidation by membranes of *M. denitrificans*. Oxidation of other substrates (nicotinamide adenine dinucleotide, reduced form, D-lactate, L-lactate, malate, and D,L-α-hydroxybutyrate) was inhibited to a lesser degree by carboxin, and formate oxidation was entirely resistant. With all substrates tested, oxycarboxin, the dioxide analogue of carboxin, was less effective than carboxin. Carboxin also inhibited dichlorophenol indophenol (DCIP) reductase activities by these membranes in a manner both qualitatively and quantitatively similar to the inhibition of oxidation of the various substrates. The inhibition of DCIP reductase activities by TTB was qualitatively similar to carboxin, but TTB was a less effective inhibitor with all substrates tested. The inhibition of DCIP reductase by carboxin could be relieved by phenazine methosulfate with all substrates except D-lactate. Only slight inhibition of D-lactate-stimulated uptake of [^{14}C]glycine by these membrane vesicles was seen with carboxin. Uptake of [^{14}C]glycine could be stimulated to varying degrees with the other substrates tested, but in no case did carboxin cause significant inhibition. Membranes isolated from *M. denitrificans* are a useful system for investigating the mechanism of inhibition of electron transport function by carboxin, and the use of this system for evaluations of carboxin and its metabolites is suggested.

The potential value of the oxathiins as systemic fungicides was first reported in 1966 (21). The mode of action of carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) was shown to be its ability to act as an inhibitor of succinate oxidation in mitochondria of susceptible fungi (12). The site of action appears to be on an electron carrier component immediate to succinic dehydrogenase (19). The inhibition is more pronounced in intact mitochondria, the soluble form of succinic dehydrogenase being less affected by carboxin (19). Mitochondria from different sources exhibit a wide range of susceptibility to carboxin (12). The K_i was reported to be 0.32 μM in the fungus *Ustilago maydis* and 270 μM in the yeast *Saccharomyces cerevisiae*. Higher plants have a susceptibility similar to yeast, whereas mitochondria isolated from rat liver are somewhat more susceptible.

The present study was undertaken to determine the effect of carboxin on succinate oxidation by membranes from the gram-negative bacterium *Micrococcus denitrificans*. This organism has been shown to have a membrane-bound electron transport system remarkably similar to mammalian mitochondria (16) and a lipid composition more like mammalian mitochondria than bacteria (26). The effects of known electron transport inhibitors on succinate and nicotinamide adenine dinucleotide, reduced form (NADH) oxidation by *M. denitrificans* membranes are similar to the effects of these inhibitors on respiration in mammalian mitochondria (16). This study indicates that succinate oxidation by membranes of *M. denitrificans* has a susceptibility to carboxin very similar to rat liver mitochondria. Although oxidation of succinate is the most susceptible electron transport function tested, oxidation of other substrates is also affected to varying degrees. The results of this study suggest that the various dehydrogenases have different accessibilities to the carboxin-susceptible component.

MATERIALS AND METHODS

Chemicals. Carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) and oxycarboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide-4,4-dioxide), obtained from Uniroyal Chemical as analytical standards, were the gift of R. L. Lester. Compound F831 (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide-4-oxide) was the generous gift of R. A. Davis of Uniroyal Chemical Co., Bethany, Conn. The compound 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione (TTB) was obtained from Eastman Chemicals. Uniformly labeled [^{14}C]glycine was obtained from New England Nuclear Corp. All other chemicals were reagent-grade products from Sigma Chemical Co.

Organisms. *M. denitrificans* (ATCC 13543) was obtained from David C. White and was grown at 30 C with shaking in a semi-defined medium with glucose as carbon source and supplemented with vitamin-free Casamino Acids (Difco) added to 0.5% (wt/vol) final concentration (26).

Preparation of membranes. An overnight culture of cells (2 liters) in late log phase (absorbance at 750 nm of 1.4) was centrifuged, and the cells were washed with 10 mM potassium phosphate buffer (pH 7) and suspended in 1 liter of the same buffer containing 0.5 M sucrose. Following the method of Scholes and Smith (15), 0.3 g of lysozyme was added, and the mixture was stirred at room temperature for 30 min. The initial absorbance at 750 nm of a 1/10 dilution of the cells was 0.28, whereas after the 30-min treatment with lysozyme the absorbance of a 1/10 dilution decreased to 0.12. After harvesting the osmotically susceptible cells by centrifugation, vesicles were prepared by a modification of the method of Kaback (8). The pellet of osmotically susceptible cells was resuspended in 10 ml of 0.1 M potassium phosphate buffer, pH 7, containing 0.5 M sucrose, 20 mM $MgSO_4$, 10 mg of deoxyribonuclease, and 10 mg of ribonuclease. This was added to 2 liters of swirling 50 mM phosphate buffer (pH 7) at 30 C. After 15 min of gentle shaking, ethylenediaminetetraacetic acid was added to 10 mM, and incubation was continued for an additional 15 min. Then $MgSO_4$ was added to bring the concentration to 15 mM and the shaking was continued an additional 15 min, after which the membranes were isolated by centrifugation in the cold for 30 min at 14,000 × *g*. The pellet was washed twice with 0.1 M potassium phosphate buffer, pH 7. The washed pellet was resuspended in the same buffer (about 20 ml) and centrifuged at 2,000 × *g* for 10 min to remove any unlysed cells which remained. The supernatant containing the membrane vesicles was divided into 1-ml samples in plastic tubes and frozen in an acetone-dry ice bath. The vesicles were stored at −90 C for periods up to 2 months. Observation of the membranes by phase-contrast microscopy showed a homogeneous preparation of vesicles of about 1 μm diameter, the same size as the intact cells. The protein content of the preparation was 1.62 mg/ml as measured by the method of Lowry et al. (11).

Enzyme assays. All assays were carried out in 0.1 M potassium phosphate buffer, pH 7, at 30 C. All inhibitors were dissolved in dimethyl sulfoxide and were added so the final concentration of dimethyl sulfoxide in all assays, including the controls, was 0.1%.

Oxygen uptake was measured polarographically with a Clark oxygen electrode (Yellow Springs Instruments) in a 2-ml closed chamber. Substrates were added with a Hamilton syringe through a slit in the chamber to the indicated concentrations, generally 20 mM. The final protein concentration was 0.162 mg/ml for the oxygen uptake assays. Respiratory rates were calculated on the basis of 220 μM oxygen in the air-saturated buffer at 30 C.

Dichlorophenol indophenol (DCIP) reductase activity was assayed by following the decrease in absorbance at 600 nm, using a Gilford 2400 spectrophotometer. Specific activity was calculated using an extinction coefficient of 21 × 10^3 M^{-1} cm^1 for DCIP. The 2-ml reaction mixture contained 32 μg of membrane protein in 0.1 M phosphate buffer (pH 7), 0.05 mM DCIP, and 2 mM KCN. Reactions were started by the addition of substrate to 15 mM concentration. In some experiments phenazine methosulfate (PMS) was added to the indicated concentrations.

Uptake of [^{14}C]glycine (U) was measured as follows: Membrane vesicles were suspended in 0.1 M phosphate buffer (0.8 mg of protein/ml) and allowed to equilibrate at 30 C in a volume of 0.1 ml. The indicated oxidizable substrate was added to 20 mM, followed by the addition of 2 nmol of [^{14}C]glycine (specific activity of 157,000 counts/min per nm) at zero time. After the appropriate time, the reaction was stopped by dilution with 2 ml of buffer and filtered rapidly on a membrane filter (Millipore Corp.). The tube and filter were washed with an additional 2 ml of buffer, and the filters were counted, after drying, in a liquid scintillation spectrometer. All values were corrected for an appropriate blank containing [^{14}C]glycine but no oxidizable substrate.

RESULTS

Oxidation of succinate. Carboxin was an effective inhibitor of oxygen utilization by membranes of *M. denitrificans* using succinate as substrate (Table 1). Appreciable inhibition (55%) was reached at a concentration of 10 μM carboxin. A Dixon (5) plot of data obtained in a similar experiment is shown in Fig. 1. The K_i was 16 μM and the inhibition was of a noncompetitive nature. For purposes of comparison,

TABLE 1. *Effect of carboxin on the oxidation of succinate by membranes of M. dentrificans*

Carboxin (μM)	Oxygen uptake[a]	% Inhibition
0	221	0
0.1	210	5
1.0	218	1
10.0	100	55
100.0	35	84
1,000.0	10	95

[a] In nanomoles of oxygen per minute per milligram of protein in the presence of 20 mM succinate.

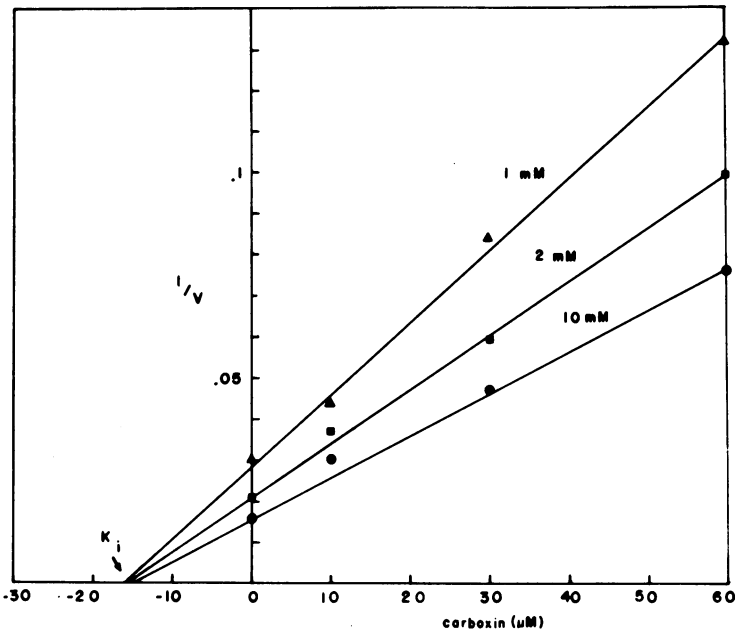

FIG. 1. *Effect of varying concentrations of carboxin on the oxidation of succinate by membranes of M. denitrificans. Succinate concentrations used were 1 mM (▲), 2 mM (■), and 10 mM (●). Oxygen uptake was measured with a Clark type electrode as described in Materials and Methods. V, nanomoles of O_2 per minute per milligram of protein.*

Table 2 shows the effects of carboxin, oxycarboxin (the dioxide analogue of carboxin), compound F831 (the monoxide analogue of carboxin), and TTB, an inhibitor of succinate oxidation. At 1 mM concentrations, carboxin was clearly the most potent inhibitor (96% inhibition), whereas oxycarboxin had only half the effect (54% inhibition) and F831 was the least effective (6% inhibition). The inhibition by TTB (46%) was similar to that caused by oxycarboxin.

Oxidation of other substrates. The membranes of *M. denitrificans* are capable of oxidizing a number of substrates (Table 3). As can be seen from this data, 1 mM carboxin was most effective inhibiting succinate oxidation (96% inhibition), had less effect on oxidation of NADH, D-lactate, L-lactate, D,L-α-hydroxybutyrate, and malate, and had no effect on formate oxidation. For each substrate, the degree of inhibition by oxycarboxin was less. No oxidation of α-glycerol phosphate or D,L-β-hydroxybutyrate could be detected in these membranes.

TABLE 2. *Comparison of inhibitors of succinate oxidation by membranes of M. denitrificans*

Inhibitor (1 mM)	Oxygen uptake[a]	% Inhibition
None	221	0
Carboxin	9	96
Oxycarboxin	102	54
F831	196	6
TTB	119	46

[a] In nanomoles of oxygen per minute per milligram of protein in the presence of 20 mM succinate.

TABLE 3. *Effects of carboxin and oxycarboxin on substrate oxidation by membranes of M. denitrificans*

Substrate[a]	Oxygen uptake[b]	% Inhibition	
		Carboxin (1 mM)	Oxycarboxin (1 mM)
Succinate	238	96	54
NADH	421	47	0
D-Lactate	97	36	13
L-Lactate	94	13	3
D,L-α-hydroxybutyrate	107	46	4
Malate	102	51	23
Formate	135	0	0

[a] The final concentration of all substrates was 20 mM, except NADH which was 1 mM.

[b] Control rates of oxygen uptake are expressed as nanomoles of oxygen per minute per milligram of protein.

DCIP reductase activities. When DCIP was

used as the terminal acceptor of electrons from the oxidation of various substrates by the *M. denitrificans* membranes (Table 4), the relative activities with respect to the substrate oxidized were similar to those seen in Table 3 using oxygen as terminal acceptor. Table 4 also shows that the pattern of inhibition of DCIP reductase by 1 mM carboxin was similar to that observed with oxygen as acceptor. Again succinate oxidation by DCIP was almost completely inhibited, whereas activities using other substrates were less affected. A 48% stimulation was seen with formate as oxidized substrate. The last column in Table 4 shows the percent inhibition by 1 mM TTB, in all cases less than inhibition by carboxin.

The addition of the artificial electron acceptor PMS to the DCIP assays relieved the inhibition by carboxin in most cases (Table 5). With succinate or malate as substrates, only half the inhibition was seen in the presence of PMS as was observed in the absence of PMS (Table 4). This effect was also observed with TTB inhibition. D-Lactate, however, was an exception. In the presence of PMS, carboxin had a slightly greater effect on D-lactate oxidation, whereas the effect of TTB was the same (10% inhibition with or without PMS). Figure 2 is a double reciprocal plot of data obtained in a similar experiment, varying the concentration of PMS. The inhibition of DCIP reduction by carboxin could be relieved in a competitive manner by PMS, using succinate (Fig. 2A) or malate (Fig. 2B) as substrate. If D-lactate was used (Fig. 2C), no relief by PMS of inhibition by carboxin was observed.

Transport of glycine. Membrane vesicles of *M. denitrificans* have been shown to carry out active transport of amino acids, which is dependent on oxidation of D-lactate (8). Figure 3 shows the kinetics of uptake of [^{14}C]glycine stimulated by D-lactate. The presence of 2 mM carboxin had very little effect on accumulation of glycine by these vesicles. This was the highest concentration of carboxin that could be used without formation of a precipitate. Uptake of [^{14}C]glycine can be stimulated by oxidation of substrates other than D-lactate (Table 6). Carboxin at 1 mM inhibited glycine uptake slightly when D-lactate or malate were oxidized substrates, but otherwise it stimulated uptake of glycine.

TABLE 4. *Effects of carboxin and TTB on DCIP reductase activity of Membranes of M. denitrificans*

Substrate[a]	Control rate[b]	% Inhibition[c]	
		Carboxin (1 mM)	TTB (1 mM)
Succinate	0.115	94	50
NADH	0.500	40	6
D-Lactate	0.023	26	10
L-Lactate	0.037	14	30
D,L-α-hydroxybutyrate	0.028	10	0
Malate	0.025	72	14
Formate	0.021	(48)	0

[a] The final concentration of all substrates with 15 mM, except NADH which was 1 mM.

[b] Control rates of DCIP reduction are expressed as micromoles of DCIP reduced per minute per milligram of protein.

[c] Parentheses indicate stimulation.

TABLE 5. *Effects of carboxin and TTB on PMS-DCIP reducatase activity of membranes of M. denitrificans*

Substrate[a]	Control rate[b]	% Inhibition[c]	
		Carboxin (1 mM)	TTB (1 mM)
Succinate	0.240	50	26
NADH	0.180	[c]	—
D-Lactate	0.034	38	10
L-Lactate	0.043	10	0
D,L-α-hydroxybutyrate	0.027	0	0
Malate	0.038	39	0
Formate	0.010	0	0

[a] The final concentration of all substrates was 15 mM, except NADH which was 1 mM.

[b] Control rates of DCIP reduction are expressed as micromoles of DCIP reduced per minute per milligram of protein. All assays contained 1.3 mM PMS.

[c] Addition of carboxin to the assay containing NADH resulted in a precipitate.

DISCUSSION

Membranes can be isolated from *M. denitrificans* easily and are able to retain electron transport function for several months if stored at −90 C. Since the K_i observed for carboxin inhibition of succinate oxidation in this system (16 μM; Fig. 1) is similar to that reported for rat liver mitochondria; (40 μM; ref. 12), this would be a useful system for toxicological evaluations of carboxin and its metabolites. The dioxide, oxycarboxin, was a less effective inhibitor of succinate oxidation (Table 2). This would be expected since this compound is less toxic to mitochondria from many sources (12, 13, 19). Compound F831, the oxidation product of carboxin in plants and animals (2), was a poor inhibitor of succinate oxidation (Table 2). This compound is less toxic to mitochondria from fungi (2, 12, 13, 19) and rat liver (12).

The inhibition of electron transport function by carboxin in membranes prepared from *M.*

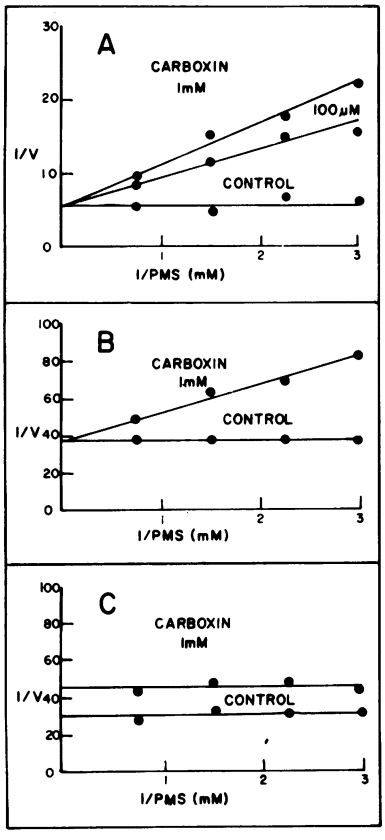

FIG. 2. *Double reciprocal plots of the effect of varying concentrations of PMS on reduction of DCIP by membranes of M. denitrificans in the presence and absence of carboxin. DCIP reduction was assayed spectrophotometrically as described in Materials and Methods. (A) shows data obtained with succinate as oxidized substrate, (B) with malate, and (C) with* D-*lactate. The concentration of all substrates was 15 mM. V, micromoles of DCIP reduced per minute per milligram of protein.*

denitrificans was variable, depending on the substrate oxidized. This was observed using oxygen (Table 3) or DCIP (Table 4) as terminal electron acceptor. In both assays, oxidation of succinate was most susceptible to carboxin, whereas formate oxidation was entirely resistant. These results suggest that the dehydrogenases are affected differently by carboxin or that the various dehydrogenases do not have the same access to the carboxin-susceptible component of the electron transport chain. This, of course, assumes that there is only one susceptible component. Since DCIP is thought to accept electrons from cytochrome *b* (6), the carboxin-susceptible site must lie between the dehydrogenase and cytochrome *b*, perhaps on cytochrome *b* itself.

The data in Table 4 indicate that the action of carboxin on DCIP reductase activities was qualitatively similar to TTB, although TTB

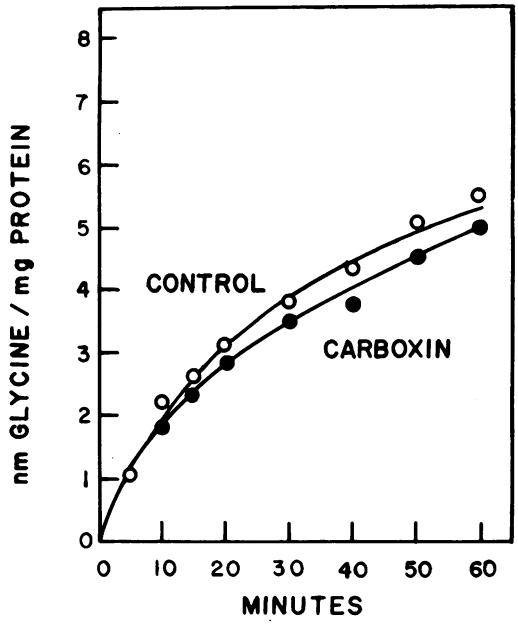

FIG. 3. *Kinetics of* D-*lactate stimulated glycine uptake by membranes of M. denitrificans in the presence (●) and absence (○) of 2 mM carboxin. After the appropriate incubation in the presence of 20 mM* D-*lactate, [^{14}C]glycine uptake was determined by liquid scintillation counting after collecting the membrane vesicles on membrane filters (Millipore Corp.,) as described in Materials and Methods. All values were corrected for blanks containing no* D-*lactate (<0.01 nmol of glycine/min per mg).*

TABLE 6. *Effect of carboxin on glycine transport by membranes of M. denitrificans*

Substrate[a]	Control[b]	% Inhibition[c]
		Carboxin (1 mM)
Succinate	0.198	(3)
NADH	0.190	(83)
D-Lactate	4.145	5
L-Lactate	4.179	(1)
D,L-α-hydroxybutyrate	1.170	(24)
Malate	0.200	10
Formate	3.734	(19)

[a] The final concentration of all substrates was 20 mM, except NADH which was 1 mM.

[b] Control rates of glycine uptake are expressed as nanomoles [^{14}C]glycine transported in 15 min per mg of protein after correction for blanks with no substrate added.

[c] Parentheses indicate stimulation.

was less potent. Bruni and Racker (1) showed that cytochrome *b* was necessary for TTB susceptibility and activity in the reconstituted succinate-ubiquinone reductase system in mitochondria. However, cytochrome *b* did not appear to undergo oxidation reduction, leading them to the conclusion that it had a structural role which involved phospholipids. Carboxin was previously postulated to have an action similar to TTB (20).

The participation of cytochrome *b* both before and after coenzyme Q in the transfer of electrons between succinate and cytochrome *c* was demonstrated by Gibson in mammalian mitochondria (6). The idea that different species of cytochrome *b* exist in mammalian mitochondria and have variable access to other electron transport components is also supported by other studies (7, 18, 22). There is recent evidence in *Mycobacterium phlei* for at least three active forms of cytochrome *b* (3). The scheme of electron transfer components presented in Fig. 4 is based primarily on observations made on *M. phlei* (3). These workers in Brodie's laboratory found that one species of cytochrome *b* was not reduced by succinate, whereas NADH reduced all of the enzymatically reducible cytochrome *b*. The three species of cytochrome *b* are arbitrarily designated in the scheme as 1, 2, and 3, cytochrome b_1 being the one lying exclusively on the NADH pathway. The components in the scheme, including coenzyme Q, have been observed to participate in electron transfer reactions in *M. denitrificans* membranes (16).

Based on the data presented in this paper, one can envision that the carboxin-susceptible component is cytochrome b_2 or a non-heme-iron protein closely associated with it. This would explain how succinate oxidation can be completely inhibited by carboxin, whereas some oxidation of NADH can proceed via the branch containing b_1. In heart muscle mitochondria, it was found that no cytochrome *b* could be reduced by succinate in the presence of TTB, whereas one-third of the cytochrome *b* was not reduced by NADH in the presence of this inhibitor (22). The varying susceptibilities to carboxin of oxidation of other substrates by *M. denitrificans* membranes can also be explained on the basis of varying accessibility to the carboxin-susceptible component. The resistance of formate oxidation is in keeping with the idea that formate utilizes a unique segment of the respiratory chain in other bacteria (14, 23, 24).

This study also shows that PMS can relieve the inhibition of DCIP reduction by carboxin with most substrates (Table 5), D-lactate being the notable exception. Double reciprocal plots in Fig. 2 show that the relief by PMS is competitive with respect to carboxin using succinate or malate, but, again, there is no alteration of the carboxin inhibition where D-lactate is the oxidized substrate. That carboxin inhibition of succinate oxidation in mitochondria could be relieved by PMS or by solubilizing the succinic dehydrogenase has been reported (20). In fact, White (25) reported that PMS could relieve carboxin inhibition in a competitive manner. PMS is thought to accept electrons directly from the dehydrogenase and pass them directly to DCIP (17). Relief of carboxin inhibition by PMS would, therefore, seem to suggest that the site of inhibition is the same site as the physiological electron acceptor from the dehydrogenase. The true acceptor is not known with certainty; in fact the path of electron flow may vary according to conditions (18). Inhibition by TTB is also relieved by PMS (Table 5), again with the exception of D-lactate. The interaction of carboxin with the D-lactate segment of the respiratory chain certainly seems to be different.

The D-lactate dehydrogenase may be unique in some respects. This dehydrogenase in yeast was found to contain zinc, rather than nonheme-iron as found in the other dehydrogenases (4). Kaback (9, 10) showed that uptake of amino acids and valinomycin-induced uptake of rubidium by membrane vesicles prepared from *M. denitrificans* was stimulated by oxidation of D-lactate. As can be seen in Table 6, uptake of glycine can be stimulated by other substrates as well. L-Lactate and formate are both as effective as D-lactate. However, in no case is there significant inhibition of glycine uptake by carboxin at 1 mM. This concentration of carboxin was able to inhibit oxidation of D-lactate by 36%. Concentrations higher than 2 mM (as used in Fig. 3) resulted in a precipitate. With the exception of trace inhibition when D-lactate or malate are used, there is a stimulation of glycine uptake by carboxin in all other cases.

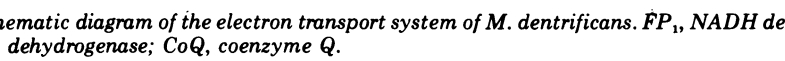

FIG. 4. *Schematic diagram of the electron transport system of M. dentrificans. FP_1, NADH dehydrogenase; FP_2, succinic dehydrogenase; CoQ, coenzyme Q.*

Perhaps complete inhibition of oxidation of D-lactate or L-lactate would be required before an effect on glycine uptake would be observed.

In addition to its value as a toxicological tool, this system utilizing membranes of *M. denitrificans* has great potential in determining the site and mechanism of inhibition of electron transport by carboxin. Furthermore, carboxin could be a valuable tool in elucidating electron flow in the segments of the electron transport chain immediate to the dehydrogenases.

ACKNOWLEDGMENTS

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