Inhibition of Respiration in Pythium Species by Ethazol

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The trivial name ethazol was first proposed by A. H. McCain (20), however, the name ethazole frequently encountered in the literature refers to the same fungicide.

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ABSTRACT

Ethazol [5-ethoxy-3-(trichloromethyl)-1,2,4-thiadiazole] concentrations that inhibited growth also proportionately decreased respiration in *Pythium debaryanum*, *P. ultimum*, *P. sylvaticum*, and *P. vexans*. Time-course studies revealed that 18 μM of the chemical inhibited growth in culture by 50% after 4 hours. While glucose and acetate oxidations were relatively insensitive, those of succinate and malate were inhibited 50% after only 3 hours of treatment.

Since the uncoupler dinitrophenol did not reverse ethazol inhibition, the fungicide does not interfere with oxidative phosphorylation. Evidence suggested a block in the electron transport system. Tetramethyl-*p*-phenylenediamine reversed the inhibition indicating that the site of ethazol inhibition is between cytochromes b and c. Cysteine and ascorbic acid also reversed ethazol inhibition of oxygen uptake.

The site of ethazol action was verified in isolated mitochondria from *Pythium* species. In *P. ultimum*, ethazol (18 μM) reduced NADH and succinate oxidations by 50% after 1 minute. The proximity or identity with the antimycin-inhibited site was demonstrated in *Pythium* species and beef heart mitochondria.

Mitochondrial respiration in *Fusarium oxysporum* f. sp. *vasinfectum*, pinto bean, and beef heart was much more resistant to ethazol inhibition than that in *Pythium* species. Differential sensitivity to respiratory inhibition may therefore account for selectivity of ethazol.

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Additional key words: polarographic measurement, fungicides.

Ethazol [5-ethoxy-3-(trichloromethyl)-1,2,4-thiadiazole] was introduced in 1966 for seed and soil treatments against a number of plant diseases (1). Since then, numerous reports proved its value for controlling pre- and postemergence damping-off, being particularly effective against *Pythium* and *Phytophthora* spp.

Little is known about the mode of ethazol action. The research reported here was directed at determining the biochemical action of this fungicide.

MATERIALS AND METHODS.—*Culture and growth conditions.*—The isolates used in this study were *Pythium debaryanum* Hesse (P-6), *P. ultimum* Trow (67-1), *P. sylvaticum* Campbell & Hendrix (P-22), and *P. vexans* de Bary (P-18). These isolates were chosen as representative of 11 *Pythium* and *Phytophthora* spp. initially screened for the response to ethazol. The *P. ultimum* isolate was obtained from J. G. Hancock, Jr. while the other three *Pythium* isolates were from R. D. Raabe. *Fusarium oxysporum* Schlecht f. sp. *vasinfectum* (Atk.) Snyd. & Hans. was provided by S. N. Smith. The isolates were grown in shake culture on potato-dextrose broth or in petri dishes on potato-dextrose agar (PDA). For toxicity tests, mycelial fragments (10-12 mg, dry weight) were seeded in 100 ml of broth containing 0, 6, 18, 60, 180, 600, 1,800, and 6,000 μM ethazol and grown at 26 C for 4 days. Mycelium of each isolate was filtered in a Büchner funnel, rinsed with distilled water, and dried to constant weights in a 45 C oven. Shake cultures in broth grown at 26 C for 3-4 days were used for measurements of mycelial respiration. Dosage response based on sporangial and zoospore germination of rolled-oat cultures were examined in hanging-drop slides. On PDA, 18 μM ethazol was introduced with pipettes near the periphery of hyphae in logarithmic growth phase. Inhibition of linear growth was determined for 12 hours by measuring the increments in colony diameters.

Respiratory measurements.—Oxygen uptake by mycelial fragments and mitochondria was measured polarographically in a 1.5-ml vessel with a Clark recording oxygen electrode (6, 18) installed on a Gilson Model KM Oxygraph. Mycelium of each isolate from shake culture was rinsed thoroughly with water, filtered, and suspended in sterile deionized water. Then it was divided into tiny (8-10 mg dry weight) fragments with forceps, and allowed to stand for 1 hour to reduce endogenous respiration. The assay solution consisted of 50 mM potassium phosphate buffer, pH 7.4, plus 50 mM potassium chloride. Whenever N,N,N'N'-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) was used, 5 mM $MgCl_2$ was added to the solution to minimize uncoupling of oxidative phosphorylation (18). Respiratory rates were measured at room temperature (27

± 1 C) and expressed in nmoles O_2 utilized per minute per milligram mycelial dry weight or per milligram protein.

Preparation of a mitochondrial fraction.—Fungal mitochondria were isolated by the modified procedures of Wong et al. (38), and Mathre (22). Mycelium of each *Pythium* sp. and mycelium and conidia of *Fusarium oxysporum* f. sp. *vasinfectum* were grown in potato-dextrose broth shake cultures for 72 hours. The mycelia were filtered, rinsed with deionized water, and suspended in a medium containing 500 mM sucrose, 10mM phosphate buffer, pH 7.2, 10 mM KCl, and 5 mM $MgCl_2$ (22) at 5 C. Cells were homogenized in a Sorvall Omnimixer and finally disrupted with a Branson Sonifier. The suspensions were centrifuged at 500 *g* (*Pythium* spp.) and 270 *g* (*F. oxysporum* f. sp. *vasinfectum*) for 10 minutes to remove cell debris. The supernatant fractions were then centrifuged at 10,000 *g* for 15 minutes. Mitochondrial pellets were washed once, and suspended with the aid of a Vortex mixer in the same medium.

Pinto bean (*Phaseolus vulgaris* L.) mitochondria were isolated from dark-grown hypocotyls by the method of Ikuma and Bonner (13). Heart mitochondria were obtained from newly slaughtered beef according to the procedure of Smith (29).

Dry weight and protein determination.—Mycelial fragments used in the respiratory measurements were dried to constant weights in a 45 C oven. Mitochondrial protein was measured by the method of Hartree (11). Equal volumes of mitochondrial suspensions from their respective preparations were used in the analyses.

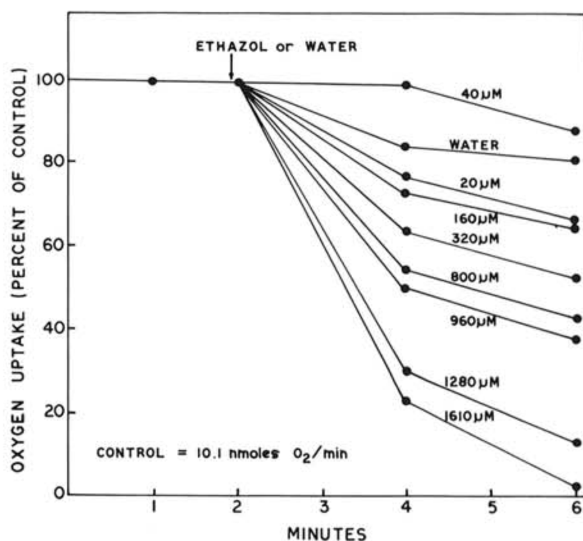

Fig. 2. Respiratory responses of *Pythium debaryanum* to varying ethazol concentrations. Oxygen uptake by 3-day-old mycelial fragments was measured polarographically with a recording oxygen electrode at 27 C. The assay solution consisted of 50 mM potassium phosphate buffer, pH 7.4, plus 50 mM potassium chloride. The arrow indicates the time when water or ethazol (200 µl) was added to attain the indicated final concentrations. Glucose (100 mM) was used as the substrate.

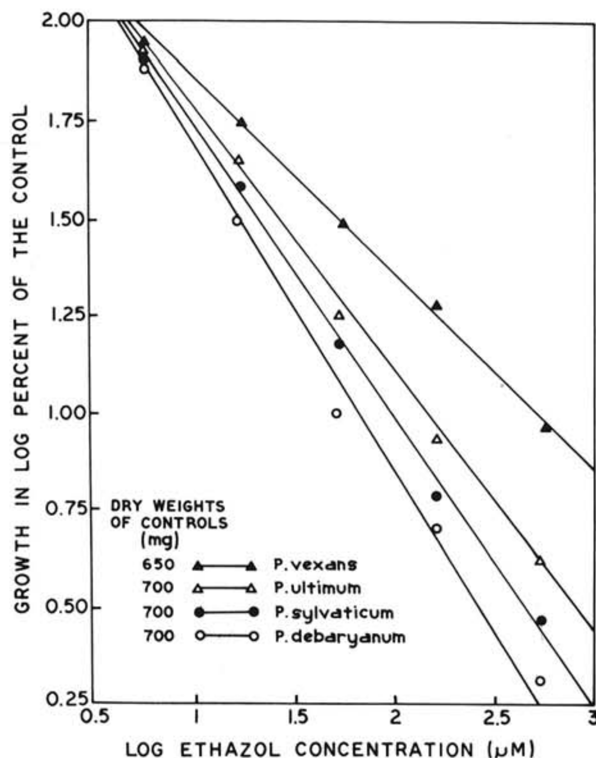

Fig. 1. Dosage response of *Pythium* spp. to ethazol. Mycelium of each isolate (10-12 mg dry weight) was seeded in 100 ml potato dextrose broth containing 0, 6, 18, 60, 180, 600, 1,800, and 6,000 µM ethazol, grown as shake cultures at 26 C and mycelial dry weight determined after 4 days.

Chemicals.—Technical ethazol was supplied by Olin Corporation. Compounds with low water solubility were initially dissolved in 95% ethanol, and the solution subsequently diluted with water to attain the desired concentrations.

RESULTS.—*Growth and germination response of isolates to ethazol.*—Isolates were exposed to increasing concentrations of filter-sterilized ethazol in potato dextrose broth to establish the dosage response of mycelial growth (Fig. 1). The sensitivity of the isolates tested varied. *Pythium vexans* with an ED_{50} of 20 µM ethazol was consistently more tolerant than *P. sylvaticum, P. ultimum,* and *P. debaryanum* with ED_{50} values of 14, 16, and 13 µM, respectively. Responses based on direct sporangial germination and zoospore release, and inactivation by different ethazol concentrations were similar to the above ED_{50} values.

Actively growing colonies of four *Pythium* spp. on PDA showed response to ethazol within hours. Drastic reduction in growth occurred within 6 hours after introducing 18 µM ethazol near the mycelial tips. Mycelial growth was retarded by about 50% within 4 hours. *Pythium vexans* was the most tolerant isolate; responses of *P. sylvaticum* and *P. ultimum* were almost similar, while *P. debaryanum* was the most sensitive. Ethazol inhibited growth to a maximum in *P. vexans* within 4 hours. Later, the isolate appeared to reverse ethazol inhibition. In the case of the other *Pythium* spp., the chemical attained maximum inhibition in 8 hours.

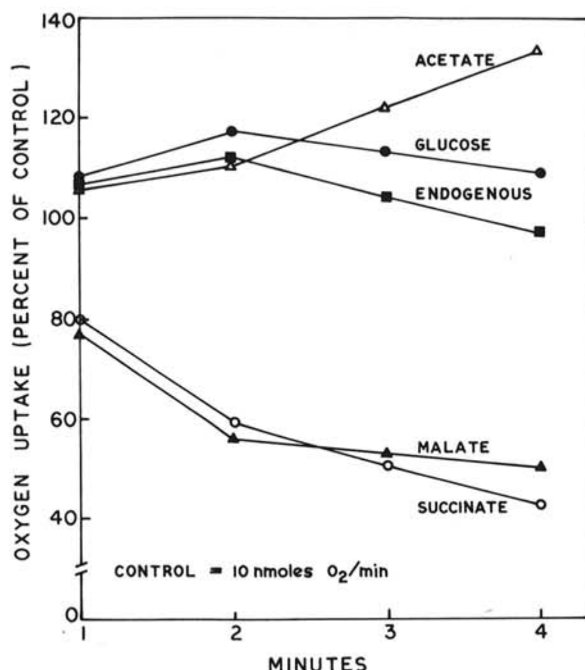

Fig. 3. Utilization of some respiratory intermediates by *Pythium debaryanum* pretreated with 18 μM ethazol for 3 hours. Mycelial fragments of 3-day-old cultures were pretreated for 3 hours with water or 18 μM ethazol prior to polarographic measurements of oxygen uptake. Substrates used were 100 mM glucose, acetate, malate, and succinate.

Respiratory assay and responses of Pythium debaryanum to varying ethazol concentrations.—Various substrates were tested for oxidation by *P. debaryanum*. Glucose, fructose, galactose, pyruvate, α-ketoglutarate, succinate, and malate were equally utilized by the mycelium. Comparable utilization of the substrates eliminated the possibility that fungal glucose oxidase was responsible for oxygen uptake. With 100 mM glucose as the substrate, ethazol either stimulated or inhibited respiration, depending on concentration (Fig. 2). Low concentrations of the fungicide had little effect or were stimulatory, whereas higher concentrations proportionately decreased oxygen uptake. With concentrations of about 1.0, 1.3, and 1.6 mM ethazol, oxygen uptake was correspondingly reduced by 50, 70, and 78% after 2 minutes. Generally, 1.8 mM or higher completely inhibited mycelial respiration in 8 to 10 minutes of treatment.

Substrate oxidation by ethazol-treated Pythium debaryanum.—To delimit the effect of ethazol on respiration, the utilization of several respiratory intermediates by treated and nontreated mycelial fragments was determined. *Pythium debaryanum* mycelium pretreated 3 hours with 18 μM ethazol stimulated endogenous respiration, as well as acetate (100 mM) and glucose (100 mM) oxidations (Fig. 3). However, malate (100 mM) and succinate (100 mM) oxidations were drastically inhibited. After 4 minutes, utilization of the latter intermediates was reduced by about 50%.

Effect of dinitrophenol on respiration by ethazol-treated Pythium debaryanum.—A known uncoupler, dinitrophenol (DNP), was used to determine if ethazol inhibits oxidative phosphorylation in whole cells. Ethazol concentrations of 80 μM, and 1,400 μM, respectively, reduced respiration after 2 minutes to about 65% and 30% of the control with succinate, and 78% and 25% with malate as the substrate. Addition of 1.33 mM DNP did not release ethazol inhibition at either of the ethazole concentrations with either substrate. Nontreated mycelium consumed oxygen at an almost constant rate within the period of observation. However, addition of DNP (1.3 mM) to untreated mycelium immediately increased oxygen uptake. Higher concentrations of DNP decreased the rate of oxygen consumption.

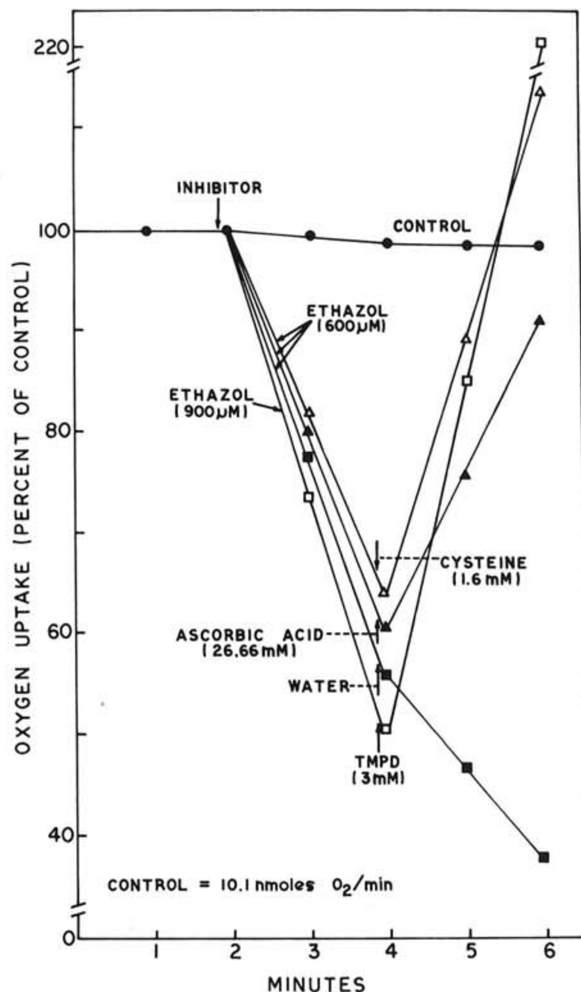

Fig. 4. Ethazol inhibition on respiration (oxygen uptake) of *Pythium debaryanum* reversed by TMPD (a dye material electron donor), ascorbic acid, and cysteine. Oxygen uptake by 3-day-old mycelial fragments was measured polarographically with a recording oxygen electrode at 27 C. The vertical arrows indicate the time when ethazol, cysteine, ascorbic acid, TMPD, and water (250 μl) were added to attain the desired concentrations. Succinate (100 mM) was used as the substrate. Results with malate as substrate were essentially similar.

TABLE 1. Effect of ethazol on succinate and NADH oxidations by mitochondrial preparations from various sources. Oxygen uptake by partially purified mitochondria was measured polarographically with a recording oxygen electrode at 27 C. Mitochondria were suspended in a medium containing 500 mM sucrose, 10 mM potassium phosphate buffer, pH 7.2, 10 mM KCl, and 5 mM $MgCl_2$ and comprised 250 μliters of the reaction mixture

Mitochondrial source[b]	Ethazol (μM)	Oxygen uptake after 1 min[a] (% of control) With succinate (13.3 mM)	With NADH (2.35 mM)
P. ultimum	0 (H_2O)	95	87
	18	50	48
	180	46	51
	400	54	47
	800	13	10
P. debaryanum	18	77	77
	180	55	46
F. oxysporum f. sp. *vasinfectum*	18	109	99
	180	116	111
	400	100	105
	800	100	98
	1400	91	95
Beef heart	18	107	92
	180	108	41
	400	101	13
	800	51	. . .
Pinto bean	18	109	104
	180	121	107
	400	145	82

[a]The values reported are based on the respiration rates prior to the addition of ethazol or water. These rates ranged from 10 to 20 nmoles O_2 per minute per mg protein.

[b]Mitochondria were isolated from 3-day-old shake cultures of *Pythium debaryanum*, *P. ultimum*, and *Fusarium oxysporum* f. sp. *vasinfectum*, from heart of newly slaughtered beef, and from dark-grown hypocotyls of pinto bean.

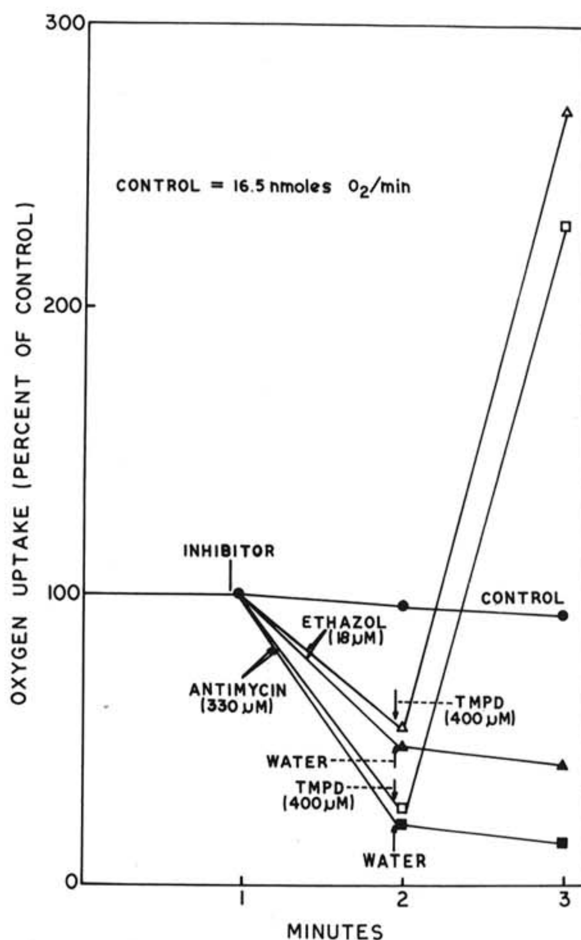

Fig. 5. Ethazol and antimycin A inhibitions of NADH-driven electron transport in mitochondria from *Pythium ultimum* and released by TMPD. Oxygen uptake by partially purified mitochondria from 3-day-old shake cultures of *P. ultimum* was measured polarographically with a recording oxygen electrode at 27 C. Mitochondria were suspended in a medium containing 500 mM sucrose, 10 mM potassium phosphate buffer, pH 7.2, 10 mM KCl, and 5 mM $MgCl_2$ and comprised 250 μliters of the 1.5 ml reaction mixture. The vertical arrows indicate the time when ethazol, antimycin A, TMPD, or water (50 μl), were added to attain the desired concentrations. NADH (2.35 mM) was used as the substrate. Results with succinate as substrate were similar except that oxygen uptake for the control was 8.5 nmoles O_2 per minute.

Ethazol inhibition of the electron transport system in intact cells.—TMPD was used in further studies to delimit the site inhibited by ethazol in the electron transport system. TMPD is a dye capable of acting as an electron donor, and can mediate a bypass of the electron transport chain at the cytochrome b-c site (17, 18). *Pythium debaryanum* treated with 900 μM ethazol showed 50 and 70% inhibition of succinate and malate oxidation, respectively. TMPD (3 mM) immediately reversed oxygen uptake and after 2 minutes resulted in respiration rates of about 220% of the control with succinate-driven electron transfer, and about 220% with malate as substrate (Fig. 4). These results indicated release of ethazol inhibition by TMPD. An equivalent amount of water had no effect on ethazol inhibition of oxygen uptake. Oxidation rates of both substrates in nontreated cells were essentially constant within the period of observation. Introduction of TMPD to nontreated cells increased oxygen uptake about 40% with both succinate and malate, consistent with the dye's ability to donate electrons to the transport chain (18). The other isolates exhibited similar responses to these treatments.

Ethazol inhibition released by cysteine and ascorbic acid.—Other compounds capable of acting as alternative electron donors to the electron transport chain also reversed ethazol inhibition. Cysteine (2) and ascorbic acid can be suitable mediators like TMPD (14), and can donate electrons to cytochrome c. When cysteine (1.6 mM) and ascorbic acid (26.66 mM) were introduced, the rate of oxygen uptake by ethazol-treated mycelial fragments was restored from 64% to 115% of the control, and from 60% to 95%, respectively, (Fig. 4) after 2 minutes. Nontreated mycelium respired at a constant rate for at least 6 minutes, while treated mycelium showed

steadily declining rates. When ascorbic acid (26.66 mM) was used as a substrate with TMPD (3 mM), ethazol (800 μM) either did not, or only slightly, altered the rate of oxygen consumption. These results were confirmed in mitochondrial preparations.

Ethazol versus antimycin and KCN inhibitions of mycelial respiration.—Inhibition by ethazol was very similar to that found for antimycin. Whole cells of *P. debaryanum* exhibited the same type of responses to antimycin (3.75 mM) and TMPD (3 mM) treatments. With malate as substrate, antimycin A reduced oxygen uptake after 2 minutes to 17% of the control, and the addition of TMPD reversed the inhibition giving respiration rates of 160% of the control after 2 minutes. With succinate, the respective values were 30% and 180%. KCN (13.3 mM) induced the expected inhibition (90%), and was only slightly reversed by TMPD.

Ethazol inhibition of the electron transport system in isolated mitochondria.—The site inhibited by ethazol in the respiratory chain was also studied in cell-free systems containing partially purified mitochondria. Since malate was not utilized by mitochondria of *Pythium* spp., NADH (2.35 mM) and succinate (13.3 mM) were used as the substrates. Respiration by isolated mitochondria was much more sensitive to ethazol inhibition than that in mycelial fragments. Ethazol at 18 μM reduced succinate oxidation by 50% (Table 1), and that of NADH by 52% (Fig. 5, Table 1) within 1 minute in mitochondria from *P. ultimum*. Treatment with 180 and 400 μM did not appreciably change the degree of inhibition of oxygen uptake (Table 1). TMPD again was very effective in reversing the ethazol inhibition of mitochondrial respiration (Fig. 5). Mitochondrial preparations from other *Pythium* spp. responded similarly to these treatments.

Comparison of ethazol inhibition with other respiratory inhibitors—The similarity between ethazol and antimycin inhibition was also observed with isolated mitochondria. Antimycin (330 μM) reduced succinate and NADH oxidations by 82 and 81%, respectively, within 1 minute in *P. ultimum* mitochondria (Fig. 5). As in the case with ethazol, TMPD also reversed antimycin A inhibition of oxygen uptake. Succinate and NADH oxidation by beef heart mitochondria also were inhibited by both ethazol and antimycin A, and were subsequently released by TMPD. Both the NADH and succinate-generated electron transport in beef heart mitochondria were stimulated slightly by the addition of 45 μM ADP, an indication of a coupled respiration. KCN also inhibited respiration by mitochondria from *P. ultimum* (95%), and beef heart (94%). In contrast with ethazol and antimycin, TMPD did not reverse KCN inhibition of oxygen uptake.

Sensitivity by mitochondrial preparations from other organisms to ethazol.—For comparative purposes, responses of mitochondria from other organisms to ethazol also were investigated. Mitochondria from *F. oxysporum* f. sp. *vasinfectum*, beef heart, and pinto beans were much more resistant to respiratory inhibition by ethazol (Table 1) than those from *Pythium* species. The concentration that reduced oxygen uptake by about 50% in *P. ultimum* mitochondria (18 μM) either stimulated, or only slightly affected, the succinate and NADH oxidations in those of other species tested. Respiratory responses varied not only between the species, but also with respect to the substrates. NADH oxidation was much more susceptible to ethazol than succinate oxidation with beef heart or pinto bean mitochondria.

Fusarium oxysporum f. sp. *vasinfectum* mitochondrial respiration was very resistant to ethazol. Concentrations as high as 1,400 μM had little effect on oxygen uptake by this fungus (Table 1). Dosage response based on mycelial growth in cultures revealed that *F. oxysporum* f. sp. *vasinfectum* is indeed very resistant to the fungicide.

The succinate-generated electron transport in beef heart mitochondria was not inhibited by 400 μM ethazol. Treatment with 800 μM fungicide reduced oxygen uptake by 49% after 2 minutes. On the other hand, NADH oxidation was more sensitive to ethazol. Thus, 18, 180, and 400 μM decreased oxygen uptake by 8, 59, and 87%, respectively.

Succinate oxidation by pinto bean mitochondria was stimulated by as high as 400 μM ethazol. This concentration reduced oxygen uptake in NADH-driven electron transport by 18%. TMPD readily reversed ethazol inhibition of both oxidations as in mitochondria of *Pythium* spp. mitochondria.

DISCUSSION.—The results of this investigation show that concentrations of ethazol which were inhibitory to growth of *Pythium* spp. (Fig. 1) also inhibited respiration (Fig. 2, 3, 4, and 5). Ethazol, at ED_{50} values for mycelial growth causes little immediate effect on respiration by mycelial fragments. However, at higher concentrations, inhibition occurred immediately after the additions of ethazol (Fig. 2). Moreover, the degree of reduction in oxygen uptake was proportional to the amount of chemical that was introduced. This is a common attribute of respiratory inhibitors (19). Growth of *Pythium* spp. were inhibited by about 50% after 4 hours of treatment with 18 μM ethazol. The oxidation rates of glucose, acetate, malate, and succinate revealed their relative proximities to the site of action in the respiratory chain (Fig. 3). The magnitude of inhibition in oxidations of malate and succinate can account for the 50% inhibition in mycelial growth by 4 hours of exposure to 18 μM ethazol.

Since DNP did not reverse ethazol inhibition of malate and succinate oxidations, the fungicide is probably not an inhibitor of oxidative phosphorylation. Dinitrophenol is known to reverse the oligomycin inhibition of coupled phosphorylations in mitochondrial respiration (12, 15, 16, 28). The lack of increase in oxygen uptake by ethazol-treated mycelium upon addition of DNP also indicated a possible ethazol-induced block in the main pathway of electron transfer.

The site inhibited by ethazol in the electron transfer chain of whole cells and isolated mitochondria was localized with TMPD dye. Since the dye released ethazol inhibition, the site of action must be between cytochromes b and c. TMPD-mediated electron transfer circumvents the antimycin-sensitive site of the respiratory chain (17, 18). The ethazol-sensitive site is very near, if not identical with, the site inhibited by antimycin A (8, 18, 25). However, ethazol is not analogous with antimycin A in chemical structure. It would, therefore, be interesting to determine the precise nature of inhibition by these two chemicals at a similar site in the electron transport system.

Both the succinate and internally generated NADH

oxidations were considerably reduced by ethazol. The possibility that the fungicide interferes with succinic and NADH dehydrogenases was eliminated, since the TMPD shunt specifically bypass only the block occurring between cytochromes b and c. Inhibition of the succinate and NADH dehydrogenases with malonate and rotenone, respectively, prevents the TMPD-induced respiration of the blocked succinate-driven and NADH-driven electron transport. The oxidation of reduced cytochrome b upon the addition of TMPD to the antimycin A-blocked respiratory chain was demonstrated spectrophotometrically (18).

In contrast to responses by mycelial fragments, the ED_{50} concentrations of ethazol (Fig. 1) also caused an immediate 50% inhibition of succinate and NADH oxidations (Fig. 5, Table 1) in isolated mitochondria of *P. ultimum*. The alteration in respiration followed immediately after the fungicide was introduced. This response in the cell-free system is strong evidence that ethazol is indeed a respiratory inhibitor. The release of inhibition by TMPD (Fig. 4 and 5) strengthened the claim that the block in the respiratory chain is between cytochromes b and c. Since electron transfer here is easily inhibited, it is believed to be the primary site of ethazol action. The almost equal degree of inhibition by 180 and 400 μM fungicide (Table 1) seem to confirm the fungistatic activity even at relatively higher concentrations. The length of time required to attain 50% respiratory inhibition by ED_{50} concentrations of ethazol varied between whole cells and isolated mitochondria. This suggested that the cytoplasmic, and possibly the mitochondrial, membranes act as permeation barriers to the influx of ethazol. Thus, it takes time to localize and act at the primary target of inhibition in intact mycelium.

The mitochondrial preparations from various organisms had differential sensitivity to the fungicide. Oxygen uptake in *Pythium* spp. was more sensitive to ethazol inhibition than those in *F. oxysporum* f. sp. *vasinfectum*, beef heart, and pinto beans (Table 1). The specificity of fungicides for certain groups of organisms have been correlated with uptake and binding (4, 21, 27, 35), biochemical selectivity (3), and detoxification (24). The enzymes of the electron transport system are bound to the mitochondrial membranes (9, 30). The penetration of the cytoplasmic membrane, and possibly also that of the mitochondria, may, therefore, be important in conditioning sensitivity and tolerance to this fungicide. The insensitivity of *F. oxysporum* f. sp. *vasinfectum* mitochondria to ethazol (Table 1) would indicate that the cytoplasmic permeability would not be an important factor with this fungus. The peculiarities of the electron transport system between different organisms, and the ability to synthesize and accumulate compounds that can help circumvent the ethazol-sensitive site, may also account for the selective action of the fungicide (10).

The similarity in target of inhibition between ethazol and antimycin A was confirmed in mitochondria from *P. ultimum* (Fig. 5), and beef heart. The slight increase in respiration of KCN-treated *P. debaryanum* mycelium upon addition of TMPD could be due to incomplete action of the inhibitor. In *P. ultimum* mitochondria, TMPD did not release KCN inhibition of oxygen uptake. The pattern of response to KCN is, therefore, different from those treated with antimycin A and ethazol. This suggested that TMPD bypass occurs before cytochromes a, a_3 and confirms earlier observations (14, 17, 18, 26). The TMPD-induced respiration of the antimycin A-blocked system is inhibited by KCN. Since ethazol inhibition was reversed by cysteine and ascorbic acid (Fig. 4), this further supports the finding that the site acted upon by ethazol is before cytochrome c. Other antifungal chemicals reported to act in the electron transport system include carboxin (7, 22, 34, 36), pyrrolnitrin (23, 33, 37, 38), and diazoben (31). None of these acts specifically on the site inhibited by ethazol.

Inhibitor studies (Fig. 2, 3, 4, and 5, Table 1) suggested that the main system for terminal oxidation of substrates in *Pythium* spp. is through the typical flavoprotein and electron transport system. These results, and the observation that NADH oxidase system seem to play a major role in *Pythium* spp. respiration (Fig. 5), support earlier reports (5, 32).

The inhibition of the electron transport system by ethazol would consequently prevent chemical energy formation and storage. Energy is required for synthesis of protein, nucleic acid, and other cell components. Therefore, inhibition of energy production will result in reduced levels of biosynthesis, and thus to an inhibition of growth. In contrast, inhibition of biosynthesis or cell division may have only little or no detectable effect on respiration within the normal period of observation (19, 27).

LITERATURE CITED

1. AL-BEDAWI, A. S., and J. B. SINCLAIR. 1969. Evidence of systemic activity by Terrazole against Rhizoctonia solani in cotton seedlings. Phytopathology 59:68-70.
2. CANTAROW, A., and B. SCHEPARTZ. 1967. Biochemistry. 4th ed. W. B. Saunders Co., Philadelphia. 898 p.
3. DEKKER, J. 1971. Problems of selectivity in the field of systemic fungicides. Acta Phytopathol. Acad. Sci. Hung. 6:329-337.
4. DEMEL, R. A., and L. L. M. VAN VEENEN. 1965. Penetration of lipid monolayers by polyene antibiotics. Correlation with selective toxicity and mode of action. J. Biol. Chem. 240:2749-2753.
5. DOWLER, W. M., P. D. SHAW, and D. GOTTLIEB. 1963. Terminal oxidation in cell-free extracts of fungi. J. Bact. 86:9-17.
6. ESTABROOK, R. W. 1967. Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. Pages 41-48 *in* R. W. Estabrook and M. E. Pullman, eds. Methods in enzymology. Vol. 10. Oxidation and phosphorylation. Academic Press, New York. 818 p.
7. GEORGOPOULOS, S. G., E. ALEXANDRI, and M. CHRYSAYI. 1972. Genetic evidence for the action of oxathiin and thiazole derivatives on the succinic dehydrogenase system of Ustilago maydis mitochondria. J. Bact. 110:809-817.
8. GOTTLIEB, D., and P. D. SHAW. 1970. Mechanism of action of antifungal antibiotics. Annu. Rev. Phytopathol. 8:371-402.
9. GREEN, D. E. 1966. The mitochondrial electron transfer system. Pages 309-326 *in* M. Florkin and E. H. Stotz, eds. Comprehensive biochemistry. Vol. 14. Biological oxidations. Elsevier Publishing Co., Amsterdam, The Netherlands. 514 p.
10. HALOS, P. M. 1975. Biochemical bases of sensitivity and

resistance by Pythium species to ethazol. Ph.D. Thesis, University of California, Berkeley. 111 p.
11. HARTREE, E. F. 1972. Determination of protein: A modification of the Lowry method that gives a linear photometric response. Anal. Biochem. 48:422-427.
12. HUIJING, F., and E. C. SLATER. 1961. The use of oligomycin as an inhibitor of oxidative phosphorylation. J. Biochem. Tokyo 49:493-501.
13. IKUMA, H., and W. D. BONNER, JR. 1967. Properties of higher plant mitochondria. I. Isolation and some characteristics of tightly coupled mitochondria from dark-grown mung bean hypocotyls. Plant Physiol. 42:67-75.
14. JACOBS, E. E. 1960. Phosphorylation coupled to electron transport initiated by substituted phenylenediamines. Biochem. Biophys. Res. Commun. 3:536-539.
15. LARDY, H. A., D. JOHNSON, and W. C. MC MURRAY. 1958. Antibiotics as tools for metabolic studies. I. A survey of toxic antibiotics in respiratory, phosphorylative and glycolytic systems. Arch. Biochem. Biophys. 78:587-597.
16. LARDY, H., and W. C. MC MURRAY. 1959. The mode of action of oligomycin. Fed. Proc. 18:269.
17. LEE, C. P., K. NORDENBRAND, and L. ERNSTER. 1965. Electron transport and oxidative phosphorylation in the cytochrome b-c region of the respiratory chain as studied with the tetramethyl phenylene diamine shunt over the antimycin A-sensitive site. Proc. Intern. Symp. Oxidases Related Redox Systems. John Wiley & Sons, New York. 960 p.
18. LEE, C. P., G. L. SOTTOCASA, and L. ERNSTER. 1967. Use of artificial electron acceptors for abbreviated phosphorylating electron transport: flavin-cytochrome c. Pages 33-37 *in* R. W. Estabrook and M. E. Pullman, eds. Methods in enzymology. Vol. 10. Oxidation and phosphorylation. Academic Press, New York. 818 p.
19. LUKENS, R. J. 1971. Chemistry of fungicidal action. Springer-Verlag, New York. 136 p.
20. MC CAIN, A. H. 1971. Common names of fungicides. Calif. Plant Pathol. No. 5:2-3.
21. MATHRE, D. 1968. Uptake and binding of oxathiin systemic fungicides by resistant and sensitive fungi. Phytopathology 58:1464-1469.
22. MATHRE, D. E. 1971. Mode of action of oxathiin systemic fungicides. III. Effect on mitochondrial activities. Pestic. Biochem. Physiol. 1:216-224.
23. NOSE, M., and K. ARIMA. 1969. On the mode of action of a new antifungal antibiotic pyrrolnitrin. J. Antibiot. Tokyo 22:135-141.
24. RICH, S., and J. G. HORSFALL. 1954. Relation of polyphenol oxidases to fungitoxicity. Proc. Nat. Acad. Sci., USA 40:139-145.
25. RIESKE, J. E. 1967. Antimycin A. Pages 542-584 *in* D. Gottlieb and P. Shaw, eds. Antibiotics. I. Mechanisms of action. Springer-Verlag, New York. 785 p.
26. SANADI, D. R., and E. E. JACOBS. 1967. Assay of oxidative phosphorylation at the cytochrome oxidase region (Site III). Pages 38-41 *in* R. W. Estabrook and M. E. Pullman, eds. Methods in enzymology. Vol. 10. Oxidation and phosphorylation. Academic Press, New York. 818 p.
27. SIJPESTEIJN, A. K. 1970. Biochemical modes of action of agricultural fungicides. World Rev. Pest Control 9:85-93.
28. SLATER, E. C. 1967. Application of inhibitors and uncouplers for a study of oxidative phosphorylation. Pages 48-57 *in* R. W. Estabrook and M. E. Pullman, eds. Methods in enzymology. Vol. 10. Oxidation and phosphorylation. Academic Press, New York. 818 p.
29. SMITH, A. L. 1967. Preparation, properties, and conditions for assay of mitochondria: slaughterhouse material, small-scale. Pages 81-86 *in* R. W. Estabrook and M. E. Pullman, eds. Methods in enzymology. Vol. 10. Oxidation and phosphorylation. Academic Press, New York. 818 p.
30. SOTTOCASA, G. L., B. KUY LENSTIERNA, L. ERNSTER, and A. BERGSTRAND. 1967. An electron transport system associated with the outer membrane of liver mitochondria. J. Cell. Biol. 32:415-438.
31. TOLMSOFF, W. J. 1962. Biochemical basis for biological specificity of Dexon (*p*-dimethyl amino-benzenediazo-sodium sulfonate) as a fungistat. Phytopathology 52:755 (Abstr.).
32. TOLMSOFF, W. J. 1962. Respiratory activities of cell-free particles from Pythium ultimum, Rhizoctonia solani and sugar beet seedlings. Phytopathology 52:755 (Abstr.).
33. TRIPATHI, R. K., and D. GOTTLIEB. 1969. Mechanism of action of the antifungal antibiotic pyrrolnitrin. J. Bact. 100:310-318.
34. ULRICH, J. T., and D. E. MATHRE. 1972. Mode of action of oxathiin systemic fungicides. V. Effect on electron transport system of Ustilago maydis and Saccharomyces cerevisiae. J. Bact. 110:628-632.
35. WESCOTT, E. W., and H. D. SISLER. 1964. Uptake of cycloheximide by a sensitive and a resistant yeast. Phytopathology 54:1261-1264.
36. WHITE, G. A. 1971. A potent effect of 1,4-oxathiin systemic fungicides on succinate oxidation by a particular preparation from Ustilago maydis. Biochem. Biophys. Res. Commun. 44:1212-1219.
37. WONG, D. T., and J. M. AIRALL. 1970. The mode of action of antifungal agents: effects of pyrrolnitrin on mitochondrial electron transport. J. Antibiot. 23:55-62.
38. WONG, D. T., J. HORNG, and R. S. GORDEE. 1971. Respiratory chain of a pathogenic fungus, Microsporium roseum: effect of the anti-fungal agent pyrrolnitrin. J. Bact. 106:168-173.