Mechanism of Tolerance of Pythium Species to Ethazol

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ABSTRACT

Pythium sylvaticum, P. vexans, P. ultimum, and *P. debaryanum* became tolerant to fungistatic activity of ethazol after a time proportional to the initial concentration. Mycelial extracts from nontreated mycelium or menadione (2-methyl-1,4-naphthoquinone) partially relieved ethazol inhibition of growth and respiration. The occurrence of an antimycin A-insensitive pathway in the presence of menadione was demonstrated in *P. debaryanum.* The compounds present in extracts of nontreated mycelium that reversed ethazol inhibition were purified by gel filtration, partitioning in petroleum ether, and ion exchange fractionation. The two active fractions developed as ultraviolet-fluorescent spots on thin-layer chromatograms. Based on ultraviolet absorption spectra and color reactions with neotetrazolium chloride, the compounds that reversed ethazol inhibition were tentatively identified as ubiquinone derivatives. These purified ubiquinones reversed ethazol toxicity in bioassays with *P. ultimum.* The tolerance by *Pythium* spp. probably depends on increased use of an alternate pathway of electron transport mediated by ubiquinone. This circumvents electron transfer through the ethazol-sensitive site in the terminal respiratory chain.

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Additional key words: antimycin A-tolerant respiration, fungicide, polarographic measurement.

Ethazol [5-ethoxy-3-(trichloromethyl)-1,2,4-thiadiazole] inhibits respiration in *Pythium* spp. by blocking electron transfer between cytochromes b and c in the terminal respiratory chain (10). With increased use of the chemical, mutations may occur giving rise to ethazol-resistant strains. Sublethal concentrations of the fungicide may also bring about the increased formation of a substance or substances which confer tolerance. This research elucidated the components in the mycelial extracts from *Pythium vexans, P. sylvaticum, P. ultimum,* and *P. debaryanum* that reverse ethazol inhibition, and the mechanism involved in tolerance. A preliminary report has been published (9).

MATERIALS AND METHODS.—*Isolates and growth conditions.—Pythium ultimum* Trow (67-1), *P. sylvaticum* Campbell & Hendrix (P-22), *P. debaryanum* Hesse (P-6), and *P. vexans* de Bary (P-18) were selected from 11 species of *Pythium* and *Phytophthora* because of their faster growth and higher tolerance to ethazol. To assay toxicity, the isolates were grown in 100 ml of medium containing glucose, yeast extract, peptone, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, and distilled water (15) in 250-ml Erlenmeyer flasks. The cultures were incubated at 26 C for 3 days on a reciprocal shaker. Millipore-filtered ethazol was then added to give the desired final concentrations, and the cultures were shaken for an additional 18 hours. Treated mycelium (10-12 mg dry weight) was rinsed with sterile deionized water, blotted dry and inoculated on potato dextrose agar (PDA) in petri plates. Mycelial growth was measured over a 120-hour period following inoculation. Mycelium from 3- to 4-day-old potato dextrose (PD) broth shake cultures were used in respiratory measurements. Mycelial extracts and other test solutions used in bioassays were sterilized through 0.22-μM Millipore filters in autoclaved syringes. Plate cultures were incubated at room temperature (27 $\pm$ 1 C). Mycelial diameters were measured at various intervals.

Preparation of mycelial extracts.—Mycelium of each isolate was grown for 5 days in PD broth shake cultures, removed by filtration, rinsed thoroughly with deionized water, and suspended in 95% ethanol. Suspensions were homogenized in a Sorvall Omnimixer and filtered. The filtrate was concentrated under reduced pressure in a rotary vacuum evaporator. The extracts were clarified by mixing with Celite and filtering.

Partial purification and identification of the active compounds in mycelial extracts.—The components of the mycelial extracts capable of reversing ethazol inhibition (hereafter referred to as active components) were initially purified by gel filtration (Sephadex G-25, fine) in a 30 $\times$ 1.5-cm column. The bed was stabilized by washing with 0.1 M sodium phosphate buffer, pH 7, for 6 hours. The concentrated extracts were mixed with small amounts of molecular weight markers (blue dextran, raffinose, maltose, and glucose). The samples were introduced on top of the bed and eluted with the same buffer. The flow rate was regulated with a Buchler Polystaltic pump. After the void volume had passed through the column, forty 0.75-ml fractions were collected. The samples were

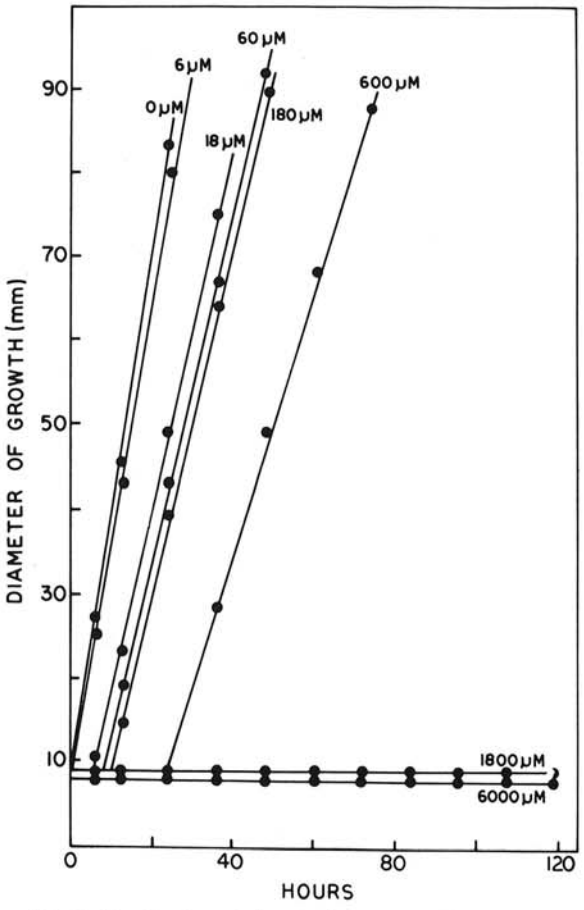

Fig. 1. Fungistatic and fungitoxic concentrations of ethazol to *Pythium sylvaticum*. The isolate was grown in shake culture at 26 C for 3 days. Millipore-filtered ethazol was then added to give the desired final concentrations and the cultures were incubated for an additional 18 hours. Then mycelium was rinsed with sterile deionized water, blotted dry and seeded (10-12 mg dry weight) on PDA. Mycelial growth was measured at various intervals.

assayed with the anthrone reagent for the sugar markers and colorimetrically for blue dextran. The active fractions determined by bioassay, and later by absorption measurements at 275 nm, were concentrated to dryness. The residues were taken up in petroleum ether, concentrated under reduced pressure in a rotary evaporator, and dried in a stream of N_2 gas. The latter steps were repeated twice. Aqueous solutions of the final residues were mixed with Dowex 50 [H^+ form, 74-38 μm particle size (200-400 mesh)], filtered, and then mixed with Dowex 1 [formate form, 293-149 particle size (50-100 mesh)]. The active compounds were eluted from the anion exchange resin with 1 mM pH 6 potassium phosphate buffer.

The final preparations were tested for amino acids, organic acids, and sugars by thin-layer chromatography on Eastman cellulose chromatograms. Solvent systems used were: (i) *n*-butanol:acetic acid:water (64:10:57, v/v) for amino acids, (ii) ethanol:ammonium hydroxide (99:1, v/v) for other organic acids, and (iii) *n*-butanol:ethanol:water (40:10:50, v/v) for sugars. Chemicals used for detection were: (i) ninhydrin (1% in absolute ethanol with 0.1% 8-hydroxyquinoline) for amino acids, (ii) bromphenol blue (23) for organic acids, and (iii) sodium hydroxide-silver nitrate (6) for sugars.

Chromatograms were developed on Eastman and Polygram cellulose with fluorescent indicator, developed in 1-propanol:water solution (4:1, v/v), and dried with a stream of N_2 gas, were used to detect the ultraviolet (UV)-absorbing and -fluorescing components. Some developed chromatograms were tested for quinones with neotetrazolium chloride (19). In others, the UV-fluorescing spots were scraped from the plates, eluted with sterile deionized water, and bioassayed for reversal of ethazol inhibition.

Ultraviolet spectrophotometry.—Absorption spectra were determined with a Beckman DB-Grating Spectrophotometer. The UV-absorption spectra of 10 μM ethazol were taken individually, and in mixture. Spectra of the purified active fractions hydrolyzed with 100 mM HCl for 2 hours at 75 C, and subsequently reduced with $NaBH_4$, also were measured. In all cases, petroleum ether was used as the solvent.

RESULTS.—*Fungistatic and fungitoxic concentrations of ethazol.*—Growth of *Pythium ultimum, P. sylvaticum, P. debaryanum,* and *P. vexans* was inhibited by 6 to 6,000 μM ethazol. In the liquid medium (15), concentrations <1,800 μM were fungistatic, while those >1,800 μM or higher were fungitoxic (Fig. 1). *Pythium* species removed from ethazol concentrations used in practice (600 μM or less) (18) eventually overcame the effects of the fungicide. The time required to reverse the inhibition was proportional to the initial ethazol concentration. After 24 hours on

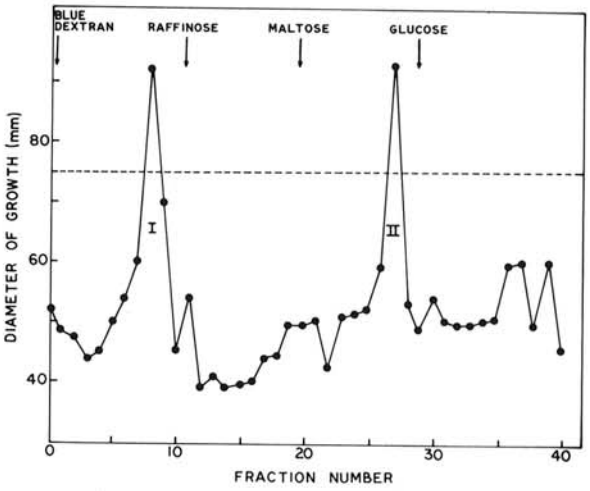

Fig. 2. Bioassay for reversal of ethazol inhibition by fractions from gel-filtered mycelial extracts with *Pythium debaryanum*. The concentrated extracts (3 ml from 1.5 gm mycelial dry weight) were eluted from Sephadex G-25 with 0.1 M phosphate buffer, pH 7. The elution points of the molecular weight markers (blue dextran, raffinose, maltose and glucose) are indicated on the graph. The samples were mixed with 4.5 μM ethazol in PDA and bioassayed by placing 10-12 mg of *P. debaryanum* mycelium in the center of each plate. The horizontal dashed line in the graph represents growth of the 4.5 μM ethazol control.

PDA, *P. sylvaticum* overcame the fungistatic effect of 600 μM ethazol. Once the inhibition was overcome, growth rates were linear, and were close to those of the control. Growth of the organisms did not resume after treatment with 1,800 μM to 6,000 μM ethazol 120 hours after inoculation.

Potato-dextrose agar cultures of *P. vexans, P. sylvaticum,* and *P. ultimum* locally treated with a solid formulation of ethazol near the mycelial tips eventually grew over the fungicide.

Ethazol inhibition reversed by mycelial extracts.—To determine whether a soluble fungal metabolite was involved in the ability of *Pythium* species to overcome ethazol inhibition, mycelial extracts were bioassayed. Mycelial extracts [2 ml from 700 mg of mycelium (dry weight basis)] incorporated into 22-ml of medium containing 4.5-μM ethazol prevented the fungicide-induced inhibition of growth. Radial growth of *P. debaryanum* was inhibited by 65% on PDA containing 4.5-μM ethazol. Assay of mycelial extracts by gel filtration revealed that two fractions were involved in reversing 4.5-μM ethazol inhibition of *P. debaryanum* (Fig. 2). The first active component (peak I) was eluted before raffinose, while the second one (peak II) was eluted before glucose. The extracts alone partially inhibited growth of the control. The active compounds were not detected in culture filtrates. The filtrates enhanced the toxic action of the fungicide. Addition of the filtrates to PDA also decreased the rate of mycelial growth in the control.

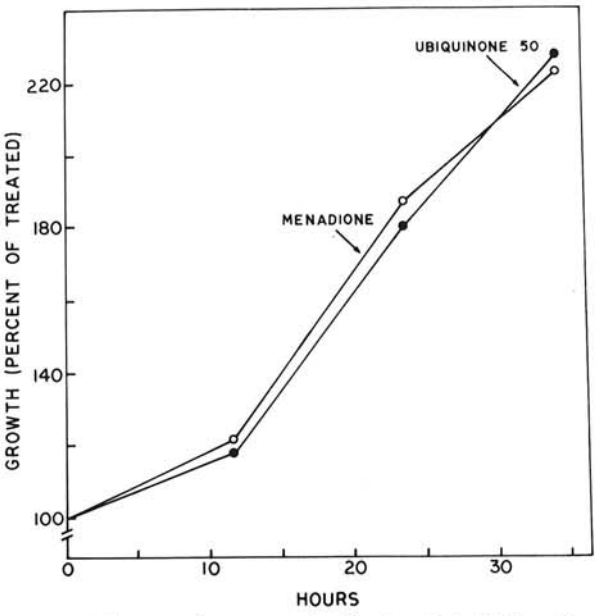

Fig. 4. Bioassay for reversal of ethazol inhibition by ubiquinone-50 and menadione with *Pythium ultimum.* Menadione (1.4 mM) and ubiquinone-50 (96.5 μM) were mixed with 4.5 μM ethazol in PDA and bioassayed by placing 10-12 mg of *Pythium ultimum* mycelium in the center of each plate. Plate cultures were incubated at room temperature (27±1 C).

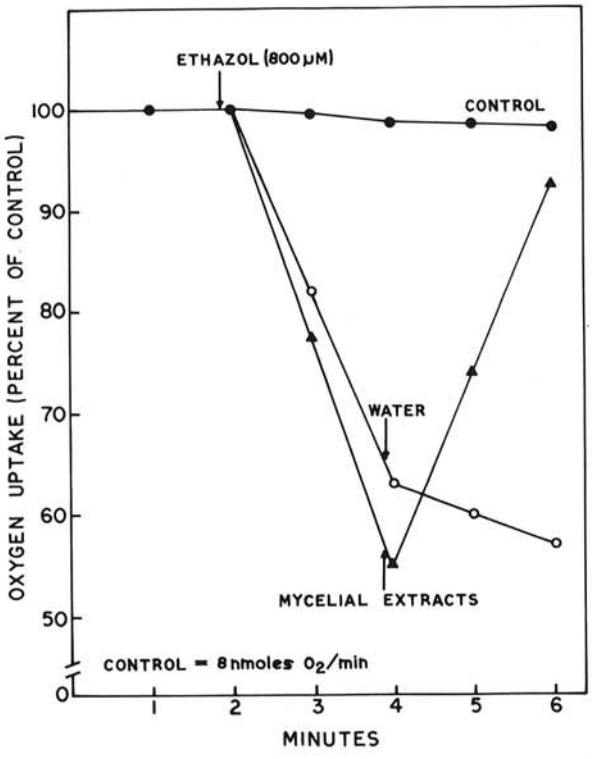

Fig. 3. Ethazol inhibition of respiration (oxygen uptake) of *Pythium vexans* reversed by its mycelial extracts. Oxygen uptake by mycelial fragments of 3-day-old shake cultures of *P. vexans* was measured polarographically with a recording oxygen electrode at 27 C. The assay solution consisted of 50 mM potassium phosphate buffer, pH 7.4, plus 50 mM potassium chloride. The vertical arrows indicate the time when ethazol water and mycelial extracts were added. Glucose (100 mM) was used as the substrate.

Respiration by mycelial fragments was measured polarographically with a recording oxygen electrode (7) using the procedures and conditions described earlier (10). Ethazol (800 μM) decreased glucose (100 mM) oxidation in intact cells of *P. vexans* by about 40% after 2 minutes of treatment. However, the introduction of concentrated mycelial extracts [400 μliters from 250 mg of mycelium (dry weight basis)] from the same fungus reversed the rate of oxygen uptake by 38% within 2 minutes (Fig. 3).

Bioassay of compounds for reversal of ethazol inhibition.—A number of commercially available low molecular weight metabolites were tested for their effect on ethazol inhibition. Many vitamins, amino acids, and thiols were tried, but none reversed ethazol toxicity on PDA cultures. Nicotinic acid, biotin, calcium pantothenate, choline, pyridoxine, folic acid, ascorbic acid, tocopherol acetate, glutamic acid, aspartic acid, methionine, dithiothreitol, mercaptoethanol, cysteine, and reduced glutathione slightly reversed ethazol inhibition. Thiamine and its phosphorylated derivatives, lecithin, adenine, and niacinamide did not reverse ethazol toxicity. The concentrations used (1 mM to 3mM) for these chemicals did not significantly alter growth rates when mixed with the control.

Two quinone compounds, menadione (1.4 mM), and ubiquinone-50 (96.5 μM) effectively reversed ethazol (3 μM) inhibition of *Pythium* spp. After 36 hours of

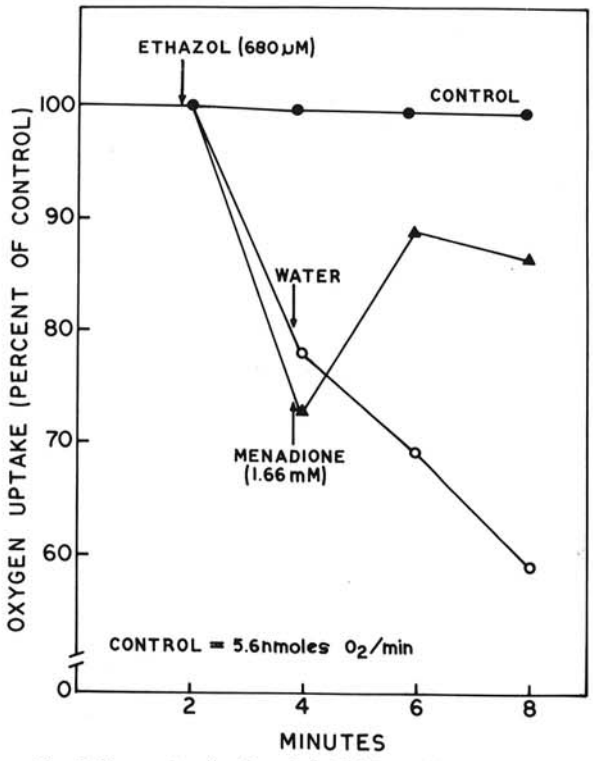

Fig. 5. Reversal of ethazol inhibition of respiration by menadione in *Pythium vexans*. Oxygen uptake by mycelial fragments from 4-day-old shake cultures of *P. vexans* was measured polarographically with a recording oxygen electrode at 27 C. The arrows indicate the time when ethazol, water, and menadione were added.

incubation, the synthetic vitamin K and coenzyme Q reversed inhibition of *P. ultimum* by 125 and 130%, respectively (Fig. 4). Both of these compounds decreased growth rates when added to the control.

The results reported earlier (10) suggested that the primary site of ethazol action is in the terminal respiratory chain. Since menadione and ubiquinone-50 can oxidize both the succinate- and NADH-driven electron transport chain (22), and also have properties similar to the active components in the extracts, they were investigated further. In *P. vexans,* 680 μM ethazol inhibited respiration by about 25% in 2 minutes. Addition of 1.66 mM menadione increased the rate of oxygen uptake by 17% 2 minutes later (Fig. 5). Ubiquinone-50 did not effectively release ethazol inhibition of respiration within 6 minutes. The nontreated mycelium respired at a constant rate. Dilution of treated mycelium with water continuously decreased the rate of oxygen uptake. *Pythium sylvaticum, P. ultimum,* and *P. debaryanum* responded similarly to the treatments.

TABLE I. Reversal of ethazol inhibition of growth of *Pythium ultimum* in agar by the two purified fractions isolated from mycelium[a]

Treatment	Mycelium diameter (mm)	
	24 hr	48 hour
Control	36.0	92.0
4 μM ethazol	9.1	9.2
I[b] + 4 μM ethazol	39.5	92.0
II[c] + 4 μM ethazol	36.5	92.0

[a]Compounds added to PDA (100 μg) and mycelial growth measured after 24 and 48 hours.
[b]First peak fraction.
[c]Second peak fraction.

Tests for purity and bioassay of the active fractions from mycelial extracts.—After gel filtration, partitioning in petroleum ether, and ion-exchange fractionation, the active compounds were tested for purity by thin-layer chromatography. Color reactions did not reveal the presence of amino acids, organic acids, and sugars. The compounds developed as single ultraviolet (UV) fluorescent spots on Eastman cellulose chromatograms. These results were confirmed on Polygram cellulose chromatograms with fluorescent indicator. The purified active compounds completely reversed 4 μM ethazol inhibition of *P. ultimum* in PDA (Table 1). Both the first and second peak fractions when mixed with ethazol resulted in slightly greater than or equal growth rates as the control.

Identity of the active compounds.—The purified compounds that reverse ethazol inhibition were tentatively identified based on their UV absorption spectra and color reactions on thin-layer chromatograms. Both the oxidized first and second active fractions had sharp peaks at 275 nm (Fig. 6). Upon reduction with sodium borohydride, the extinction at 275 nm decreased. This was accompanied by a gradual broadening and shift of the peak towards 290 nm. The developed spots on thin-

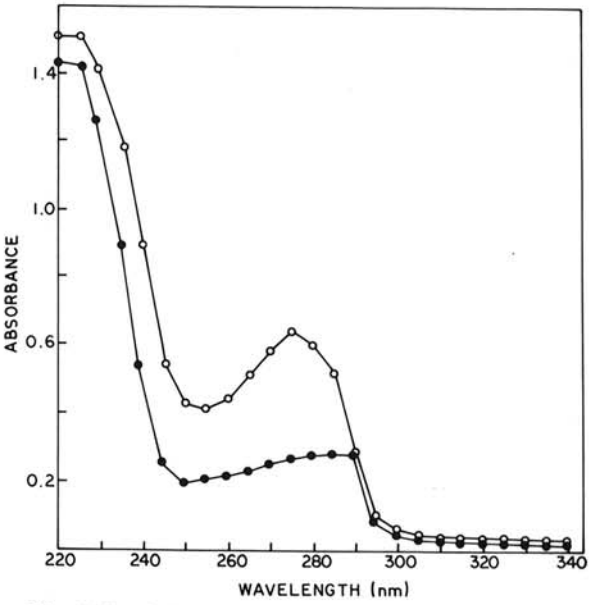

Fig. 6. Ultraviolet absorption spectra of the active fraction (peak II, Fig. 2) in petroleum ether. Spectra were determined with a Beckman DB-Grating Spectrophotometer. Spectra of the peak I (Fig. 2) active fraction were similar. Legend: o——o, spectrum of the peak II fraction purified through the thin-layer chromatography step; ▲——▲, spectrum of the peak II fraction hydrolyzed with 100 mM HCl for 2 hours at 75 C and subsequently reduced with $NaBH_4$.

layer plates also gave positive color reactions for quinones with neotetrazolium chloride. The above properties are consistent with those known for ubiquinones (3, 4, 13, 20, 27). Compounds which decrease in absorbancy in the 275-nm region when treated with borohydride have been shown by mass spectrographic analysis (3, 5), Craven's test (27), Dam-Karrer assay (27), and Irrevere-Sullivan reaction (27) to be almost entirely ubiquinones.

Comparative effect of menadione on respiratory inhibition by ethazol and antimycin.—The influence of menadione on ethazol and antimycin-induced respiratory inhibition was determined polarographically (7, 10). Menadione-mediated transfer of electrons, which is insensitive to antimycin A (22), was detected in mycelial fragments of *P. debaryanum*. Its approximate site in the terminal respiratory chain was verified with sodium azide. With succinate (100 mM) as substrate, 3.75 mM antimycin A and 13.3 mM sodium azide decreased oxygen uptake by 43 and 42%, respectively. Menadione (1.66 mM) reversed the antimycin-inhibited respiration by 17% after 4 minutes. This observation is analogous to the menadione release of ethazol-inhibited respiration in *P. vexans* (Fig. 5). Menadione did not effectively release the sodium azide inhibition.

Chemical reactivity of menadione and ethazol.—Since menadione considerably reduced toxicity, the possibility of a direct chemical combination resulting in ethazol inactivation was therefore considered. The UV absorption spectra of 10 μM menadione, 50 μM ethazol, and a mixture of the two were examined. When menadione and ethazol were mixed together, spectral shifts were not detected.

DISCUSSION.—Ethazol acts primarily as a fungistatic agent against *Pythium* spp. Relatively high concentrations (1.8 mM or higher) are needed to cause permanent toxicity. This result suggested that the fungus was either able to detoxify the fungicide, or was able to relieve or circumvent a blocked metabolic step. The finding that even low levels of the fungicide (6 μM) inhibited growth in shake cultures for extended periods of time argued against an active detoxification of the fungicide. This prompted the search for possible compounds formed by the fungi which could reverse the inhibition.

The compounds that prevented the inhibition were isolated from nontreated mycelial fragments. The active components of the extracts are therefore preformed, and probably play role(s) in normal metabolism. However, ethazol might have induced their accumulation in treated *Pythium* spp.

The procedures used in the purification of the extracts eliminated the possibility that the active components were proteins, amino acids, sugars and organic acids. The active compounds had low molecular weights (approximately 200 and 800) (Fig. 2). Their solubility in petroleum ether indicated a hydrophobic structure. Ion exchange fractionation revealed that they are negatively charged. Thin-layer chromatography showed the purified biologically active preparations (Table 1) to be essentially free of major contaminants. Both the UV absorption spectra (Fig. 6) and color reaction were consistent with the identification of the active compounds as ubiquinone derivatives. High-resolution mass spectrometry would be necessary for determining their exact structure. This method was used in identifying ubiquinones in bacteria (5). Quinones and quinonoid products in fungal cells have been extensively reviewed (1, 14, 25). It would be interesting to determine the precise identity of the compounds that reduce ethazol inhibition.

In the bioassay of several vitamins, amino acids, thiols, and coenzymes for their effects on ethazol inhibition, only menadione and ubiquinone-50 (Fig. 4) effectively released ethazol inhibition. These quinone compounds play a role in respiration, and therefore indicated that relief of inhibition is linked with the terminal respiratory chain. This would be consistent with our finding that ethazol acts primarily by inhibiting respiration (10).

The immediate release of ethazol-inhibited respiration in mycelial fragments by mycelial extracts (Fig. 3) is similar to that due to menadione (Fig. 5). This suggested an analogy in function of the active components in the extracts with menadione and ubiquinone. The pattern of respiratory inhibition by ethazol closely resembles that due to antimycin A, inhibition of which also is released by menadione, and indicates the near identity of the ethazol and antimycin A-sensitive sites. In the presence of menadione reductase, menadione mediates transfer of electrons through an antimycin A-insensitive pathway, and its occurrence was demonstrated in *P. debaryanum*. Since neither the extracts nor menadione reversed sodium azide inhibition, the bypass occurs before the cytochrome oxidases. The ubiquinone-mediated bypass resembles that mediated by menadione (22), the TMPD shunt (11, 12), and the antimycin A-resistant respiration of *Ustilago maydis* (8, 21), *Neurospora sitophila* (21), *Rhodotorula glutinis* (16, 17), *Saprolegnia* sp. (26), and skunk cabbage mitochondria (24).

The shunt mediated by menadione, ubiquinone, and mycelial extracts over the cytochrome b-c site probably plays a minor role in electron transport, since toxicconcentrations of ethazol caused an instantaneous decrease in oxygen uptake. It is likely that endogenous levels of ubiquinone in mycelium are too low to act as a shunt pathway. The inhibition was not relieved immediately unless menadione or the mycelial extracts were added to the treated mycelium.

The absence of spectral shits when menadione and ethazol were mixed together eliminated the possibility of direct fungicide inactivation by menadione. Hence the reversal of ethazol inhibition probably is due to a metabolic role of menadione. The possibility, however, of menadione competitively inhibiting ethazol at a common binding site in the electron-transport chain was not eliminated.

Unlike menadione, ubiquinone-50 could not immediately reverse ethazol inhibition of respiration in mycelial fragments and isolated mitochondria. This contrasts to the ability of this compound to effectively reduce toxicity in culture (Fig. 4). There may be a permeability problem in the cytoplasmic and mitochondrial membranes with ubiquinone-50 so that immediate effects are not detected. More likely, is the possibility that ubiquinone-50 must be metabolically modified before it can function in *Pythium* spp. as an electron transport bypass. If this were the case, enzymic conversion, possibly by adaptive enzymes, is a prerequisite to activity, and thus again no immediate

effects would be detectable.

The ability of ubiquinones to act in an electron bypass capacity has been demonstrated by others (2, 20). Ubiquinones can account for the possible mechanism of tolerance against the fungicide. Since ethazol reduced growth and respiration in *Pythium* spp., the compounds that mediate electron transfer through a bypass of the vulnerable site between cytochromes b-c should reverse the inhibition.

Regarding the significance to fungicide action, low ethazol concentration will likely prevent establishment of the damping-off pathogens by inhibiting spore germination and germ tube elongation. However, at low concentrations, the fungicide is unlikely to completely inhibit the actively growing mycelia.

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