The Mode of Action of Alternaric Acid on *Myrothecium verrucaria*

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ABSTRACT

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Alternaric acid inhibited the endogenous respiration of *Myrothecium verrucaria* but maximum effects of the toxin were reached after 5–6 min. The toxin reduced the production of $^{14}CO_2$ by 80–95% during aerobic respiration when (1-^{14}C)-acetate, (2-^{14}C)- acetate or (U-^{14}C)-glucose were the substrates. Alternaric acid totally inhibited the incorporation of ^{14}C from (U-^{14}C)-glucose into the cell wall, protein, nucleic acid, lipid, and trichloroacetic acid-extractable cell fractions of the fungus. The toxin did not cause leakage of electrolytes from the mycelium of *M. verrucaria*. Oxidation of exogenous substrates by isolated mitochondria, and oxidative phosphorylation, were not affected by the antibiotic. Alternaric acid had no noticeable effect on the activity of any enzyme of the Embden-Meyerhof-Parnas pathway, the hexose monophosphate pathway, or the tricarboxylic acid cycle. Alternaric acid reduced the uptake of several common metabolites by the mycelium, although not all to the same extent. The inhibition of uptake occurred within the first 2 min. Thus, the mode of action of alternaric acid appears to be the interference of uptake of metabolites by the mycelium.

Alternaria solani, the causal agent of early blight of tomato and potato, produces a toxic metabolite, alternaric acid, in culture. Brian et al (4) and Pound and Stahmann (13) demonstrated that treatment of tomato plants with alternaric acid caused lesions similar to those produced by the pathogen. The production of alternaric acid in culture varied with the isolate of pathogen, and there was no correlation between pathogenicity and the production of the toxin (4). Evidence for the production of the toxin in the host plant during pathogenesis always was indirect and alternaric acid has not been demonstrated in the infected host.

Alternaric acid is selectively antifungal, inhibiting the growth of some species of fungi at 1 μg/ml or less, but not affecting other fungal species even at 100 μg/ml (3). Alternaric acid is not bactericidal (3), but is phytotoxic to many and diverse types of higher plants (2). There is no information on the mechanism of action of alternaric acid on fungi or higher plants.

The ultimate aim of our research was to determine the mode of action of alternaric acid on higher plants but the action of the toxin on *Myrothecium verrucaria* was first studied as a model for further investigations on higher plants. This paper describes the effects of alternaric acid on the growth and physiological processes of the mycelium of *M. verrucaria*.

MATERIALS AND METHODS

Fungus cultures. An alternaric acid producing strain of *Alternaria solani* (Ell. and G. Martin) L. R. Jones and Grant was obtained from the American Type Culture Collection, ATCC 11078. The liquid medium for the production of alternaric acid consisted of: sucrose, 100 g; KNO_3, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 1.0 g; minor element concentrate, 1.0 ml; and distilled water to 1 L. The minor element concentrate consisted of: $FeSO_4 \cdot 7H_2O$, 100 mg; $MnSO_4 \cdot H_2O$, 10 mg; $K_2MoO_4 \cdot 5H_2O$, 10 mg; and distilled water to 1L. The reaction of the medium was adjusted to pH 5.5–6.5 before autoclaving. Inoculum for the liquid cultures consisted of disks cut with a 5-mm diameter cork borer from the edge of 10-day-old petri dish colonies of *A. solani*. Four disks were placed in each 500-ml Erlenmeyer flask containing 150 ml of the liquid medium. The cultures were grown at 26 C for 15 days on a reciprocal shaker at 96 strokes per minute. The alternaric acid produced by the cultures was extracted, purified, and characterized by the methods of Grove (9). The compound thus obtained met the criteria of melting point, UV absorption spectra, extinction coefficients, and biological activity for pure alternaric acid, and was used throughout this study.

Myrothecium verrucaria (Alb. and Schw.) Ditm. ex Fries was obtained from the American Type Culture Collection, ATCC 9095, and was maintained on PDA. Submerged liquid cultures were grown in Difco Czapek Dox Broth (CDB) modified by adjusting the pH to 3.6 with 1 N HCl prior to autoclaving. Portions (100 ml) of CDB in 500-ml Erlenmeyer flasks were each seeded with 3 ml of a suspension of 4-day-old mycelium which had been macerated in a Waring Blendor. The cultures also were grown at 26 C on a reciprocal shaker for the periods required in the different experiments.

Growth measurements. All fungal growth measurements were made on cultures grown in 50 ml of CDB in 250-ml Erlenmeyer flasks originally seeded with 1 ml of homogenized mycelium (2 mg dry weight per milliliter). The average dry weight of the mycelium was determined in triplicate and each experiment was repeated at least twice.

The dry weights of cultures were measured at 12-hr intervals for 6 days. At 4 days, the cultures were in late log phase and were used for subsequent experiments unless otherwise indicated. The toxic effect of ethanol, the solvent for alternaric acid, was determined at concentrations of 0.05 to 5% in the medium. Toxic action resulted in reduction of growth after 4 days and was determined by measuring the dry weight of mycelium.

The effects of alternaric acid on the growth of the fungus also was determined on 4-day-old mycelium by comparing the dry weight of cultures containing 0.5% ethanol to the cultures grown in the presence of the antibiotic. Alternaric acid in ethanol solution was added to triplicate flasks containing 50 ml of CDB at pH 3.5 to give concentrations of 0.05 to 2.0 μg/ml and 0.5% (v/v), respectively.

The effects of the pH of the biological activity of alternaric acid were measured in triplicate flasks containing 1 μg/ml alternaric acid in 50 ml of CDB medium for each pH level. The control flasks for each pH contained 0.5% ethanol. The effects of pH on the antibiotic were determined at initial pH levels ranging from 2.5 to 7.0, after 4 days of growth.

Effects of alternaric acid on respiration. Three-day-old liquid submerged cultures of *M. verrucaria* were harvested and suspended in 0.1 M sodium citrate, sodium phosphate buffer (CP buffer) pH 3.8 (6) (10 mg dry weight of mycelium per milliliter). Oxygen

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consumption was measured with a Biological Oxygen Monitor, Model 59 (Yellow Springs Instrument Co., Yellow Springs, OH 45387). All measurements were made at 30 C in 5 ml of media, consisting of 2 ml of the mycelial suspension and 3.0 ml of the CP buffer. The rates of oxygen consumption by the mycelium were first measured just prior to the addition of the inhibitors and at various times thereafter. Alternaric acid and antimycin A were added in 50 μl of ethanol to give the concentrations described in the results.

The method of Slater et al (15) was used to collect the $^{14}CO_2$ evolved during aerobic respiration from (U-^{14}C)-glucose, (1-^{14}C)-acetate, and (2-^{14}C)-acetate. Sodium (1-^{14}C)-acetate, (32 mCi/mmol) and sodium (2-^{14}C)-acetate (61 mCi/mmol) were purchased from ISC Isotope Specialists, Burbank, CA 91500, and (U-^{14}C)-glucose (240 mCi/mmol) from New England Nuclear, Boston, MA 02118. The $^{14}CO_2$ was collected in 125-ml Erlenmeyer flasks which contained 10 ml of mycelial suspension. The mycelial suspension (10 mg dry weight per milliliter) was made by placing freshly harvested 4-day-old mycelium in the CP buffer pH 3.5. At the start of the experiment, either 100 μl of ethanol (control flasks) or alternaric acid in 100 μl of ethanol were added to give the concentrations described in the results. The ^{14}C compound then was added and the flasks were sealed.

The flasks were placed on a reciprocal shake at room temperature for 60 min, then 2 ml of 10% trichloroacetic acid (TCA) were injected into the flasks. Shaking was continued for an additional 60 min to collect all the $^{14}CO_2$ in 0.5 ml of Hyamine® (New England Nuclear, Boston, MA 02118). Radioactivity was detected with a Packard Tri-Carb scintillation spectrometer, Model 3003 with 10 ml of Bray's scintillation cocktail (1) after adding 100 μl of the Hyamine containing the absorbed $^{14}CO_2$. Enzymes of the Embden-Meyerhof-Parnas pathway, hexose monophosphate pathway, and the tricarboxylic acid cycle were assayed by the methods described by Reilly and Gottlieb (14).

Pyruvate dehydrogenase was assayed by the method of Land and Clark (11), and NADH Cytochrome C reductase by the method of Dowler, Shaw and Gottlieb (5). Mitochondrial oxidation and oxidative phosphorylation were determined by the methods described by Reilly and Gottlieb (14). Alternaric acid was added to the enzymes assay mixtures in 10 μl of ethanol to give a concentration of 8.3×10^{-5}M. Ethanol alone was used as a control for each assay.

Incorporation of ^{14}C into cells. Freshly harvested 3-day-old mycelia were suspended in 0.1 M CP buffer, pH 3.8, containing glucose at 5×10^{-4}M. The suspension was dispensed in 250-ml Erlenmeyer flasks (150 mg dry weight of mycelium per 25 ml). The control flasks contained 0.25 ml of ethanol, but no antibiotic in the suspension. Alternaric acid was added in 0.25 ml ethanol to give the concentrations described in the results. Cycloheximide and sodium azide were added as aqueous solutions. Each treatment was made in triplicate.

These cell suspensions were placed on a reciprocal shaker for 15 min, then 2.5 μCi of (U-^{14}C)-glucose (specific activity 240 mCi/mM), were added. After 60 min, the mycelia were collected on pre-weighed filter paper, washed three times with distilled water, and dried to constant weight at 70 C in a vacuum oven. The dried cells were ground and 50 mg were used for the cell fractionation procedure of Gottlieb and Van Etten (8).

The radioactivity of each fraction was determined on 0.05 ml portions of the fraction in 10 ml of Bray's cocktail (1).

Leakage of cell constituents. Comparisons of the effect of the antibiotic filipin (The Upjohn Co., Kalamazoo, MI 49001) with that of alternaric acid were made in triplicate flasks containing 25 ml of a suspension of 4-day-old cells (9 mg dry weight mycelium per ml) in 0.1 M glucose in double distilled water. After adjustment of the pH to 3.5 with 0.2 N HCl, the conductivity was 40–60 μmho. Additions to the flasks were: 25 μl ethanol; 25 μl ethanol containing 500 μg filipin, and 25 μl ethanol containing 500 μg alternaric acid. A Copenhagen Radiometer Conductivity Meter, The London Co., Westlake, OH 44145 with a type CDC 114 conductivity cell was used and measurements were made at various incubation times from 0 to 120 min. After the last measurement, the entire suspension was filtered and the filtrate was analyzed for ninhydrin-positive materials (12) with leucine as the amino acid standard. Duplicate 1-ml portions of the treatments also were assayed for leakage by determining the amounts of 260 nm absorbing materials with a Beckman DU spectrophotometer.

Uptake of metabolites. To determine the uptake of metabolites, 2-day-old cultures were harvested and suspended in 0.15 M CP buffer, pH 3.75 in amounts equivalent to 10 mg dry wt mycelium per milliliter. The experiments were carried out in 10 × 75 mm test tubes to which were added 10 μl of ethanol, or alternaric acid in ethanol. Another series contained antimycin in ethanol and 0.15 M CP buffer, pH 3.75. These preparations had a final volume of 0.5 ml. The various concentrations of inhibitors and ^{14}C-labeled substrates are described in the results section. The experiments were started by adding 1.0 ml of the mycelial suspension to the tubes. At the desired time, the contents of the tubes were removed by aspiration with a U-shaped tube attached to a Millipore filter apparatus. The mycelia were collected on 2 × 2-cm squares of Miracloth (Chicopee Mills, Inc., Milltown, NJ 08850) then washed with a total of 15 ml of a 0.1 M unlabeled solution of the substrate being studied. The Miracloth squares containing the mycelia were placed into scintillation vials, 10 ml of Bray's cocktail (1) was added to each vial, and radioactivity was determined as described previously.

RESULTS

Effects of alternaric acid on growth. Ethanol, the solvent for alternaric acid, was relatively innocuous to the fungus. Ethanol at 0.05 to 0.5% did not inhibit fungal growth, but in higher concentrations growth was progressively reduced and was totally inhibited at 5%. Five tenths percent ethanol therefore was used in all experiments that required the addition of alternaric acid.

When alternaric acid was added to CDB medium at pH 3.5, it greatly reduced the growth of the fungus. Above 0.05 μg/ml there was an increasing effect, with total inhibition at 0.75 μg/ml (1.8×10^{-6}M) (Fig. 1). At pH 3.5, the LD_{50} of the toxin was 0.25 μg/ml (6.1×10^{-7}M). Alternaric acid was active at and below pH 4.0, but above this pH, its growth inhibitory activity decreased (Fig. 2). At pH 4.0, inhibition of growth was 100% with 1μg/ml (2.4×10^{-6}M), but at pH 4.5 it was only 64%. Above pH 5.0, alternaric acid did not inhibit the growth of *M. verrucaria* (Fig. 2).

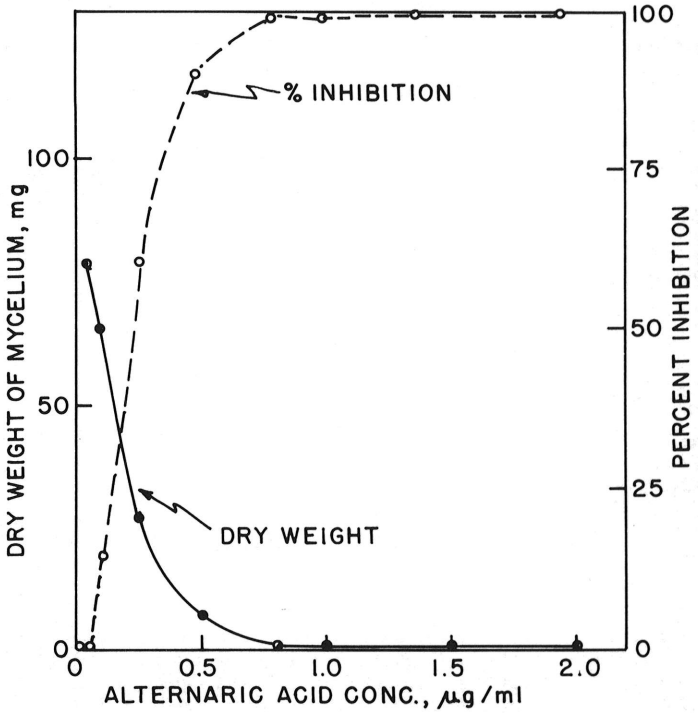

Fig. 1. Inhibition of growth of the mycelium of *Myrothecium verrucaria* by increasing concentrations of alternaric acid. The data are expressed as dry weight of mycelium per flask and as the percent inhibition of growth. The dry weights were determined from 4-day-old cultures grown on a reciprocal shaker, 96 spm, at 26 C.

Effects of alternaric acid on respiration. Endogenous consumption of oxygen by *M. verrucaria* decreased as the period of exposure to alternaric acid (2.4×10^{-4} M) increased (Table 1). The maximum reduction of endogenous oxygen consumption was 83% and occurred 5 min after the antibiotic was added. Antimycin A (1×10^{-5}M), a specific inhibitor of mitochondrial oxygen consumption, reduced it about 85% within the first min but no further inhibition occurred within the next 5 min. Ethanol at 1% (v/v) had no observable effect over a 6-min period. The maximum inhibition obtainable was 83% at 8.3×10^{-5}M alternaric acid with no further increase even at 1.02×10^{-4}M.

The inhibitory effect of the alternaric acid on respiration also was observed in the marked reduction of the production of carbon dioxide by the fungus. Alternaric acid at 2.4×10^{-4}M, reduced the aerobic production of $^{14}CO_2$ from uniformly labeled glucose by 83%; from (1-^{14}C)-acetate, 89%; and (2-^{14}C)-acetate, 95% (Table 2). In cell-free preparations there was no obvious interference by alternaric acid (8.3×10^{-5}M) with the activity of the individual enzymes of the Embden-Meyerhof-Parnas pathway, the hexose monophosphate pathway, or the tricarboxylic acid cycle. Also not affected were pyruvate dehydrogenase and NADH cytochrome C reductase. Isolated mitochondria oxidized succinate and NADH, but not pyruvate. Alternaric acid (1.9×10^{-4}M) did not inhibit this oxidation or oxidative phosphorylation. Antimycin (1.8×10^{-6}M), however, totally inhibited both NADH oxidation and oxidative phosphorylation by the mitochondria (authors' data, *not presented).*

Effects of alternaric acid on synthetic activities in *Myrothecium verrucaria.* With increasing concentrations of alternaric acid, the inhibition of the incorporation of labeled carbon from (U-^{14}C) glucose into various cell fractions also increased (Fig. 3). At the lowest concentration, 2.0×10^{-5}M, there was stimulation of the incorporation but at higher concentrations there was partial inhibition of incorporation until at 1.2×10^{-4}M, there was no incorporation of the carbon label into cell protein, nucleic acid, lipid, or cell wall fractions. In similar experiments, cycloheximide (6.5×10^{-5}M), a known inhibitor of protein synthesis, reduced the incorporation of label into the protein fraction of the cell. Sodium azide (3×10^{-3}M), an inhibitor of respiration, also inhibited the incorporation of ^{14}C except that the inhibition of incorporation was between 90 and 100% in all fractions. (Table 3). The effect of the alternaric acid on protein synthesis also was studied in a wheat germ cell-free system containing a mixture of ^{14}C-amino acids and a hemoglobin RNA message. The antibiotic did not prevent the incorporation of the amino acids into peptides, indicating that the innate ability of the cell to synthesize protein was not diminished (R. P. Ricciardi, *unpublished).*

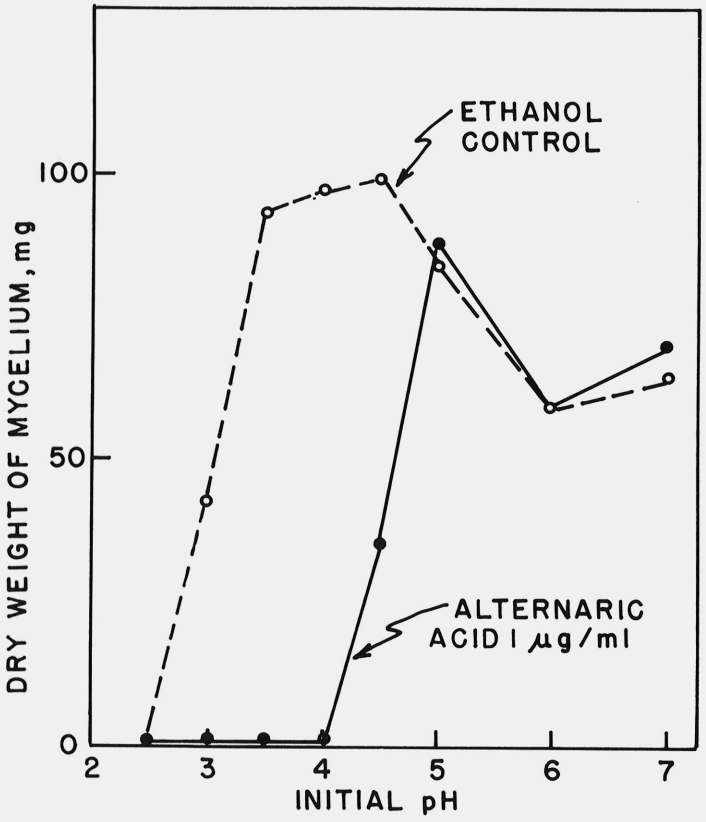

Fig. 2. The effects of pH on alternaric acid inhibition of the growth of *Myrothecium verrucaria.* The data are expressed as dry weight of mycelium per flask. Initially, the 250-ml Erlenmeyer flasks contained 50 ml of CDB at the indicated pH values. Control flasks contained 0.5% ethanol (v/v), whereas the alternaric acid treated flasks contained 1 µg/ml of the toxin and 0.5% ethanol (v/v). One milliliter of mycelial suspension (2 mg dry weight of mycelium per milliliter) was added to each flask. The dry weight of the mycelium in each flask was determined after 4-days of growth on a reciprocal shaker, 96 spm, at 26 C.

TABLE 1. Effect of alternaric acid and Antimycin A on oxygen consumption by *Myrothecium verrucaria*

Time after antibiotic addition (min)	2.4×10^{-4}M alternaric acid		1×10^{-5}M Antimycin A	
	Oxygen consumption[a] (µl/min/vial)	Inhibition (%)	Oxygen consumption[a] (µl/min/vial)	Inhibition (%)
0	2.86	...	2.86	...
1	2.52	12	0.43	85
2	1.43	50	0.43	85
3	0.84	71	0.43	85
4	0.67	77	0.43	85
5	0.50	83	0.43	85
6	0.50	83	0.43	85

[a]Oxygen consumption was measured in a total of 5 ml, consisting of 3 ml of 0.1 M sodium citrate sodium phosphate buffer (pH 3.8), and 2 ml of freshly harvested mycelium suspended in the same buffer (10 mg dry weight of mycelium per milliliter). Alternaric acid and Antimycin A were added in 25 µl of enthanol to give final concentrations as indicated above.

TABLE 2. The effects of alternaric acid and antimycin A on the production of $^{14}CO_2$ by *Myrothecium verrucaria* during aerobic respiration of various substrates

Amount of substrate and antibiotic[a]	(µmoles $^{14}CO_2$/min/mg dry wt mycelium)	Inhibition (%)
(U-^{14}C)-Glucose (0.012 µmoles)		
Control	2.59×10^{-6}	0
Alternaric acid (2.4×10^{-4}M)	5.11×10^{-7}	83
Antimycin A (9×10^{-5}M)	2.11×10^{-6}	28
Sodium (1-^{14}C)-acetate (0.205 µmoles)		
Control	4.28×10^{-7}	0
Alternaric acid (6.0×10^{-5}M	3.42×10^{-7}	20
Alternaric acid (1.2×10^{-4}M)	1.23×10^{-7}	71
Alternaric acid (2.4×10^{-4}M)	4.78×10^{-8}	89
Alternaric acid (3.6×10^{-4}M)	2.59×10^{-8}	94
Antimycin A (9×10^{-5}M)	2.96×10^{-7}	31
Sodium (2-^{14}C)-acetate (0.205 µmoles)		
Control	1.40×10^{-6}	0
Alternaric acid (1.2×10^{-4}M)	4.92×10^{-7}	65
Alternaric acid (2.4×10^{-4}M)	7.56×10^{-8}	95
Antimycin A (9×10^{-5}M)	6.34×10^{-7}	55

[a]Control rates were determined in the presence of 100 µl of ethanol, the solvent for alternaric acid and antimycin A. Alternaric acid and antimycin A were added in 100 µl of ethanol to give final concentrations indicated.

Effects of alternaric acid on permeability. Alternaric acid, at 100 μg/ml (2.4×10^{-4}M), caused a small but linear increase in leakage of electrolytes from the mycelium of *M. verrucaria* as shown by conductivity measurements during a 2-hr period (Fig. 4). Filipin, a known inducer of leakage, caused a much more rapid loss of electrolytes in the first hour but only a slight additional loss between the first and second hour. Ethanol, the solvent for alternaric acid and filipin, reduced conductivity over the 2-hr period. The pH of the medium remained constant at 4.0 during the 2-hr period.

After 2 hr, the concentrations of ninhydrin positive materials, calculated with leucine as the standard, were 0.26, 0.52, and 0.82 mM in the ethanol, alternaric acid and filipin treated media, respectively. No 260-nm-absorbing compounds were detected in the medium treated with ethanol. The alternaric acid treated medium had an $A_{260\ nm}$ of 0.02 and the filipin treated medium had an $A_{260\ nm}$ of 0.20.

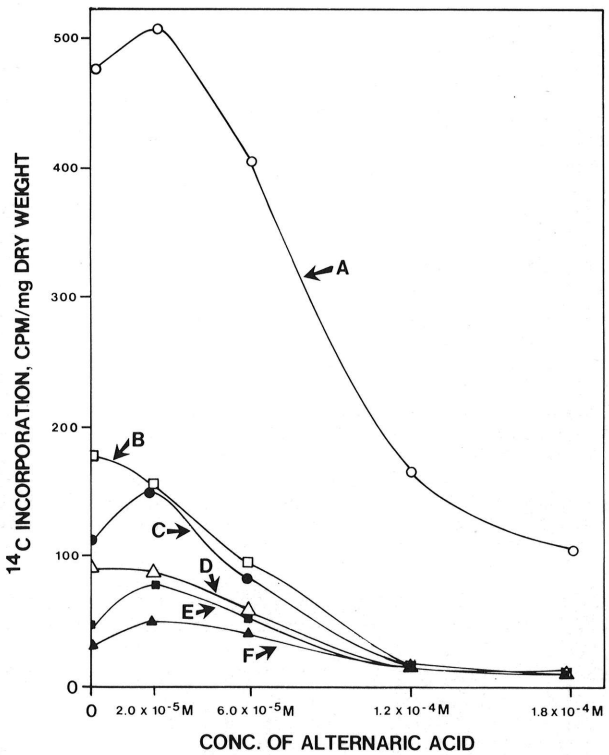

Fig. 3. The effects of increasing concentrations of alternaric acid on the incorporation of ^{14}C from (U-^{14}C)-glucose into cell fractions of the mycelium of *Myrothecium verrucaria*. A, cell pool; B, nucleic acids; C, cell wall; D, protein; E, lipids; F, alcohol soluble protein.

Alternaric acid at 1.6×10^{-4}M reduced the uptake of glucose, phenylalanine, and acetate, 80%; 2-deoxyglucose and leucine, 74%; uracil, 47% and pyruvate, 22% (Table 4). Inhibition of six out of the seven compounds began with the first 2 min; only uracil required a slightly longer time. The inhibition in uptake of glucose, which was typical of all the compounds is seen in Fig. 5.

Antimycin A, a respiratory inhibitor, reduced the uptake of phenylalanine, 2-deoxyglucose, uracil, acetate, and pyruvate between 2 and 14% over a 4-min period, whereas alternaric acid reduced their uptake between 32 and 68% (Table 5).

DISCUSSION

The inhibition of growth of *M. verrucaria* occurred at 1.8×10^{-6}M alternaric acid but the specific metabolic functions were inhibited between 8.3×10^{-5} and 1.2×10^{-4}M. However, the growth experiments were conducted over a 4-day period whereas the metabolic studies measured effects over much shorter time spans. If growth studies had been conducted over a shorter time period, most likely the concentration of alternaric acid required to inhibit would have been higher. In addition to its effect on growth, alternaric acid inhibited endogenous aerobic respiration at concentrations only slightly greater than that for growth inhibition of *M. verrucaria*. Despite the decrease in respiration, the antibiotic did not decrease the individual activity of the various enzymes of the Embden-Meyerhof-Parnas pathway, the hexose monophosphate shunt, and the tricarboxylic acid pathway. In this, the action of alternaric acid is not unique, for such inhibitions have not been shown for other antibiotics (7).

Interference with aerobic mitochondrial respiration is not an uncommon effect of antibiotics. Alternaric acid failed to do this even though it inhibited the oxygen consumption of whole cells. On whole cells and at similar concentrations of the two antibiotics, antimycin A produced maximal inhibition of respiration in one fifth the time that was needed for alternaric acid. Thus, their mechanisms of action probably differ. Aerobic respiration of the mycelium of *M. verrucaria* is similar to that of other fungi in that only part of this respiration (about 85%) is sensitive to antimycin and sodium azide.

The studies on the incorporation of label from (U-^{14}C)-glucose showed that alternaric acid reduced ^{14}C incorporation into protein, nucleic acid, lipid, and cell wall functions. However, the inhibition of incorporation was about the same for all so that synthesis in these functions could not be considered specific points of primary attack by the antibiotic.

A comparison of the action of cycloheximide, an inhibitor of protein synthesis, with alternaric acid is enlightening. Cycloheximide had very little effect on syntheses other than that of protein whereas alternaric acid again inhibited incorporation of ^{14}C into all

TABLE 3. The effects of alternaric acid, cycloheximide, and sodium azide on the incorporation of ^{14}C from (U-^{14}C)-glucose into cell fractions of *Myrothecium verrucaria*

	EXPERIMENT 1					EXPERIMENT 2				
	Control (0.5% ethanol)	Alternaric acid (2.4 $\times 10^{-4}$M)		Cycloheximide (6.5×10^{-5}M)		Control (0.5% ethanol)	Alternaric acid (2.4×10^{-4}M)		Sodium azide (3×10^{-3}M)	
Cell fraction	CPM[a] (no.)	CPM[a] (no.)	Inhib. (%)	CPM[a] (no.)	Inhib. (%)	CPM[a] (no.)	CPM[a] (no.)	Inhib. (%)	CPM[a] (no.)	Inhib. (%)
Cell pool	2,015	66	97	1,936	4	6,592	340	95	320	95
Nucleic acid	780	0	100	240	69	1,932	56	97	42	98
Protein	362	0	100	75	80	1,392	0	100	0	100
Lipid	104	0	100	111	0	917	0	100	0	100
Cell wall	346	18	95	142	59	436	0	100	0	100
Alcohol sol. protein	69	0	100	14	80	163	0	100	0	100
Total	3,676	84	99	2,518	31	11,432	396	96	362	96

[a]CPM is expressed as counts per min per mg dry weight of mycelium, and is the mean of three replicates per experiment. Experiment 1: The equivalent of 150 mg dry weight of mycelium was suspended in 50 ml of 0.1 M sodium citrate sodium phosphate buffer, pH 3.8 containing glucose at 5×10^{-4}M. Experiment 2: The volume of the medium was reduced to 25 ml but still contained the equivalent of 150 mg dry weight of mycelium.

fractions to the same degree. Furthermore, cycloheximide inhibited peptide synthesis in a cell free system whereas alternaric acid did not inhibit this system. Sodium azide, which inhibits mitochondrial respiration, resembled alternaric acid in that it inhibited all macromolecular syntheses.

One common regulatory mechanism for all the syntheses studies might be energy formation which is needed for all such reactions. This mechanism could be ruled out, however, because there was no measurable inhibition of oxidative phosphorylation by alternaric acid.

Another mechanism that plays a role in cellular activities is that involving the movement of electrolytes and nutrients in and out of the cell or its organelles. A number of antibiotics such as the polyene, nystatin, react with the sterols of the cell membrane changing its permeability and allowing the leakage of metabolites from the cell with the resulting inhibition of growth, oxygen consumption, and other cell functions (10). Alternaric acid in contrast, caused no such leakage of electrolytes or ninhydrin-positive and 260-nm-absorbing materials, thereby ruling it out as the primary mechanism of action.

Another membrane function that might control other metabolic

TABLE 4. The effects of alternaric acid on the rate of uptake of various compounds by the mycelium of *Myrothecium verrucaria*

Compound	Rate of uptake (nmoles/min/tube)		Inhibition (%)
	Control	Alternaric acid	
(U-^{14}C)-Glucose	0.830	0.160	81
(U-^{14}C)-Phenylalanine	0.345	0.066	80
Sodium (1-^{14}C)-acetate	6.390	1.280	80
(1-^{14}C)-2-Deoxyglucose	0.042	0.011	74
(U-^{14}C)-Leucine	1.860	0.573	69
(2-^{14}C)-Uracil	0.180	0.095	47
Sodium (U-^{14}C)-pyruvate	1.010	0.788	22

[a]The uptake was determined by adding 1 μmole of the compound being studied to a total volume of 1.5 ml of cell suspension. The control contained 10 μl of ethanol. Alternaric acid was added in 10 μl of ethanol to give a final concentration of 1.6×10^{-4}M.

TABLE 5. The effects of alternaric acid and antimycin A on the uptake of metabolites by the mycelium of *Myrothecium verrucaria* during a 4-min exposure

Metabolite and treatment	Uptake (nmoles/4 min/tube)	Inhibition (%)
(U-^{14}C)-Phenylalanine		
Control	0.71	...
Alternaric acid	0.48	32
Antimycin A	0.61	14
(1-^{14}C)-2-Deoxyglucose		
Control	45.20	...
Alternaric acid	15.60	65
Antimycin A	44.20	2
(2-^{14}C)-Uracil		
Control	0.36	...
Alternaric acid	0.19	47
Antimycin A	0.37	0
Sodium (1-^{14}C)-acetate		
Control	29.60	...
Alternaric acid	4.10	86
Antimycin A	27.60	7
Sodium (U-^{14}C)-pyruvate		
Control	0.60	...
Alternaric acid	0.27	55
Antimycin A	0.58	3

[a]The amounts of metabolites added were: phenylalanine, 0.001 μmole; 2-deoxyglucose, 5 μmoles; uracil, 0.009 μmoles; sodium acetate, 0.25 μmoles; and, sodium pyruvate, 0.14 μmoles. The concentration of antimycin A was 1.0×10^{-5}M, and alternaric acid 1.6×10^{-4}M. The control contained 10 μl of ethanol, the solvent for alternaric acid and antimycin A.

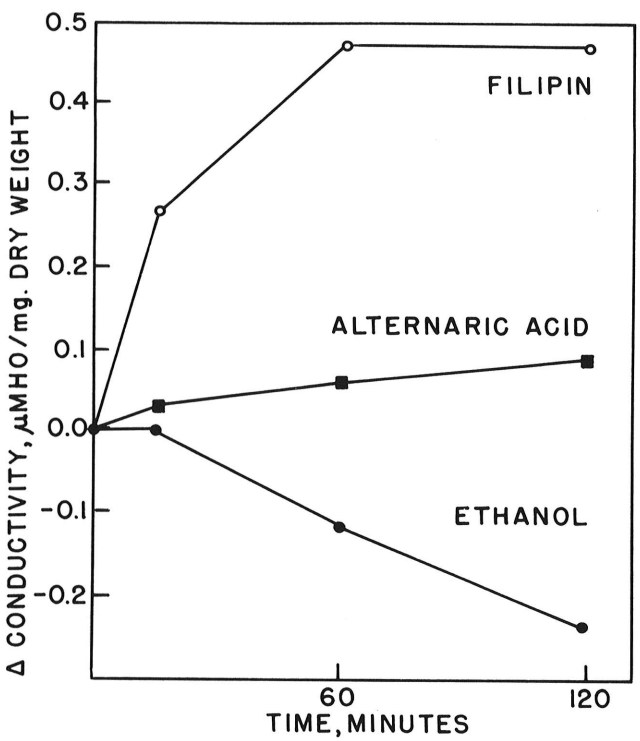

Fig. 4. The effects of 100 μg/ml alternaric acid, 25 μl ethanol and 100 μg/ml filipin on the leakage of electrolytes from the mycelium of *Myrothecium verrucaria*. Alternaric acid and filipin were added in 25 μl of ethanol. The data are expressed as the change in conductivity per mg dry weight to time. The initial conductivity of the cultures was between 40 to 60 μmho.

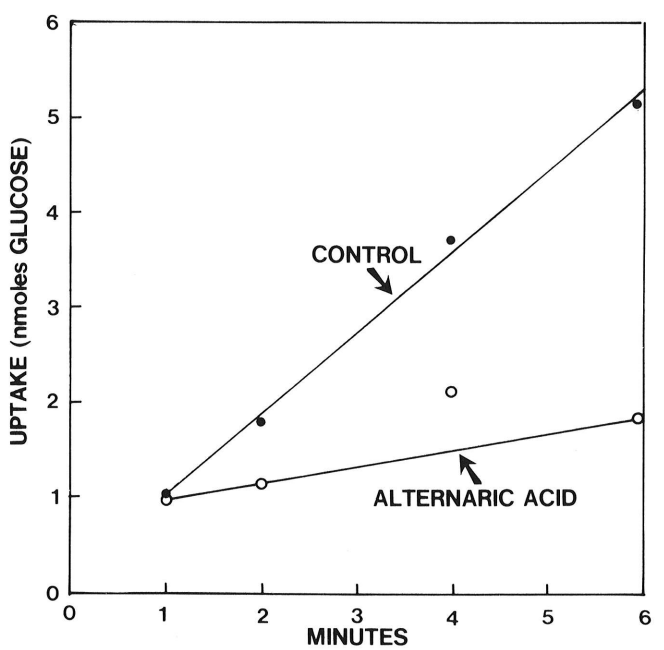

Fig. 5. The relationship between time and the effects of alternaric acid on the uptake of (U-^{14}C)-glucose by the mycelium of *Myrothecium verrucaria*. The control contained 10 μl of ethanol, alternaric acid (1.6×10^{-4}M, final concentration) was added in 10 μl of ethanol. Uptake was measured by adding 1 μmole of (U-^{14}C)-glucose to a total volume of 1.5 ml of mycelial suspension (10 mg dry weight per milliliter).

pathways is the uptake of nutrients by the cell. In fungi, this is usually an energy requiring process. A reduction in the uptake of glucose, for example, would result in a decrease both in oxygen consumption and the synthesis of the various macromolecules. Studies reported here revealed a decrease in the uptake of glucose, amino acids, uracil, acetate, and pyruvate by *M. verrucaria* within less than 2 min. Inhibition of oxygen consumption, on the other hand, required 4–5 min.

We propose that the primary site of action of alternaric acid on *M. verrucaria* is the inhibition of the mechanisms responsible for the uptake of nutrients by the fungus and the passage of metabolites through the organelle membranes. All other inhibitory effects of the antibiotic, such as on respiration and cell syntheses, result from and can be explained by the reduction in membrane permeability. If the movement of metabolites through the mitochondrial membranes were inhibited, respiration would inevitably decrease.

The results obtained with *M. verrucaria* furnish a model for future studies on the toxic effects of alternaric acid in the diseases caused by *Alternaria solani*.

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