Effects of carboxin, an oxathiin systemic fungicide, on cell permeability and DNA dependent RNA synthesis

by Owen Daniel Shively

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Botany
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Abstract:
Mycelial discs of Rhizoctonia solani were treated with the systemic fungicide carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) for periods of 0 to 10 hours. The fungus was then removed by filtration and the filtrates analyzed for the presence of various intracellular materials to determine if carboxin alters fungal plasma membranes. Electrical conductance was used to monitor leakage of ionic materials. Carboxin "treatment consistently resulted in greater electrical filtrate conductance. Potassium ions were lost from cells after carboxin treatment but their presence was not sufficient to explain total conductivity increases. Organic acids and phosphate containing materials were also released after carboxin treatment and both could be responsible for increases in conductance. Phosphate losses were sufficient to retard further growth of the organism after fungicide removal and placement on a medium without phosphate. Starvation of the mycelial discs, in deionized water, markedly reduced respiratory metabolism. Carboxin treatment, after starvation, did not result in cell leakage indicating that membrane damage did not occur. The leakage of organic acids is difficult to explain unless membrane damage exists as weak acids do not readily penetrate plasma membranes. However, a probable source of such acids exists, in that carboxin treatment has previously been reported to inhibit respiratory metabolism with a build up of intermediate tricarboxylic acids. Oxidative phosphorylation is undoubtedly reduced with respiratory inhibition and this could explain the presence of phosphate materials in filtrates.

The leakage phenomenon, as observed with carboxin treatment, was similar, although slower and of less magnitude, to leakages induced in yeast by materials known to alter membrane structure. Carboxin does not appear to inhibit DNA dependent RNA polymerase in a cell-free system from soybeans. Attempts to isolate such a polymerase system from a fungus were unsuccessful and therefore definite conclusions were not established.

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Date March 3, 1971

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ABSTRACT

Mycelial discs of Rhizoctonia solani were treated with the systemic fungicide carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) for periods of 0 to 10 hours. The fungus was then removed by filtration and the filtrates analyzed for the presence of various intracellular materials to determine if carboxin alters fungal plasma membranes. Electrical conductance was used to monitor leakage of ionic materials. Carboxin treatment consistently resulted in greater electrical filtrate conductance. Potassium ions were lost from cells after carboxin treatment but their presence was not sufficient to explain total conductivity increases. Organic acids and phosphate containing materials were also released after carboxin treatment and both could be responsible for increases in conductance. Phosphate losses were sufficient to retard further growth of the organism after fungicide removal and placement on a medium without phosphate. Starvation of the mycelial discs, in deionized water, markedly reduced respiratory metabolism. Carboxin treatment, after starvation, did not result in cell leakage indicating that membrane damage did not occur. The leakage of organic acids is difficult to explain unless membrane damage exists as weak acids do not readily penetrate plasma membranes. However, a probable source of such acids exists, in that carboxin treatment has previously been reported to inhibit respiratory metabolism with a build up of intermediate tricarboxylic acids. Oxidative phosphorylation is undoubtedly reduced with respiratory inhibition and this could explain the presence of phosphate materials in filtrates. The leakage phenomenon, as observed with carboxin treatment, was similar, although slower and of less magnitude, to leakages induced in yeast by materials known to alter membrane structure. Carboxin does not appear to inhibit DNA dependent RNA polymerase in a cell-free system from soybeans. Attempts to isolate such a polymerase system from a fungus were unsuccessful and therefore definite conclusions were not established.

INTRODUCTION

Pesticides have been utilized for many years in the control of plant pathogens. Fungal and bacterial pathogens have been successfully controlled by surface treatment of host plants. However, protection is generally short term and continual application is necessary to prevent re-establishment of the pathogen. With the advent of systemic pesticides a number of years ago, notably longer control of insects and weeds has been achieved.

Systemic fungicides, developed within the last few years, are few in number and remain in the experimental stage. One such group of chemicals, the oxathiins, having a great deal of potential as systemic fungicides was discovered by Von Schmeling and Kulka (48). Since this time, other workers have noted successful control of a number of plant pathogens using the oxathiins (4, 10, 17, 36, 47).

The metabolic fate of systemic materials is much better understood within animal and microbial tissue than it is within plant tissue. Very few chemical compounds possess the necessary physical properties required for uptake by plants either through roots or leaves. After entry, these chemicals are subject to the chemical processes of the plant. Questions then, that for the most part remain unanswered and which must be considered before a systemic fungicide can be successfully utilized, are as follows: what does the chemical do to the plant, what does the plant do to the chemical,

and what does the chemical do to the fungus? The research presented in this paper was an attempt to explain the effects of carboxin on a sensitive fungus.

The use of fungicides dates back to ancient times. Archilles is recorded as having used sulphur compounds as disinfectants. Sulfur remained as the dominant fungicide until 1882 and this period is referred to as the first or sulfur era of fungicides. In 1882, with the development of Bordeaux Mixture, copper containing fungicides came to the front and a new era was begun. This second or copper era was prevalent until the development of organic fungicides, which were derivatives of dithiocarbamic acid and were patented in 1934 by Tisdale and Williams. The first of these derivatives to become successful was thiram which was at first found noteworthy as an insecticide and later was found by many workers to be a successful fungal seed treatment and protective spray (28). Ferbam (2, 23) and Ziram (19, 49), the ferric and zinc salts of dithiocarbamic acid, were later found to provide even better protection against a variety of plant pathogens. These first dithiocarbamates were prepared from monoamines and were soon improved by using diamine dithiocarbamates such as nabam, zineb and maneb. The dithiocarbamate fungicides have undoubtedly been the most useful of the organic fungicides developed to date (28).

Since the development of the dithiocarbamates as organic protective fungicides, other organic materials have also been developed into protective fungicides. Among these materials are the quinones, imidazolines and carboximides. Numerous antibiotics have also been studied in this respect (16).

The concept of injecting plants, especially trees, in an attempt to control disease through systemic action is not entirely new. Leonardo da Vinci described the injection of arsenic into apple trees. However, his purpose was that of controlling his human enemies rather than typical pathogens (39). Probably the first real attempt at systemic injections was that of Bolley in 1905 in an effort to control plum pockets disease. However, no details are available (28). Later attempts by Rumbold (40) to control silver leaf of plum, and by Muller (28) to control insects, pests, and diseases met with little success and even less interest. However, with Howard's successful systemic treatment of bleeding canker in hard maple and the emergence of Dutch Elm disease, interest in systemics began to pick up (28). Systemic fungicides at present, although still few in number, are the subject of a good deal of research and the amount of literature pertaining to the subject is steadily increasing. The systemic fungicides of most importance today are the oxathiins (8, 9, 11, 26, 27, 37), and benzimidazoles (42). The oxathiin,

carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) is utilized at present, with restrictions for seed treatment of cereals while the benzimidazole benomyl is utilized commercially as a systemic protectant of roses and other ornamentals. Thiabendazole has been successful in controlling Cercospora leaf spot on sugar beets (43).

An enormous amount of research, with respect to mode of action, has been conducted on all groups of fungicides. However, the exact site or sites at which any fungicide intervenes and thus inhibits the myriad of biological systems are in most cases unknown. According to Owens and Hayes (35), the dithiocarbamates interact with fungal enzymes, particularly those enzymes with sulfhydryl groups. The diamine dithiocarbamates are also known to inhibit various dehydrogenases and sulfhydral enzymes, and may be effecting other enzymes as well. The quinones are known to react with proteins and therefore also may inhibit enzyme catalyzed reactions. Inhibition of the oxidation-reduction reactions of the electron transport systems as well as an uncoupling of oxidative phosphorylation have also been attributed to these materials (38). Brown and Sisler (6) have demonstrated an effect on selective permeability of fungal membranes after treatment with imidazolines, although this does not necessarily kill the cells. Kerridge (22) has suggested

an effect on nucleic acid and protein synthesis. The trichloromethylthiodicarboximides inhibit the respiration of acetate to citrate. Apparently enzyme activity is inhibited by an attack on protein or coenzyme thio-groups (34). No effect on permeability has been noticed (30). Somers and Pring (40) have concluded that the guanidines react with the cell membrane altering its permeability. They have also presented evidence that the structure of other intracellular membranes may be disrupted. In addition, glucose oxidation is inhibited indicating enzymatic effects and ribonucleic acid (RNA) synthesis is inhibited.

The antibiotics Blasticidin S. and Kasugamycin apparently interfere with the final assembly of amino acids into protein without altering nucleic acid synthesis (16). Cycloheximide also inhibits protein synthesis (24). The Polyaxins, a family of nine different but related compounds, retard cell wall synthesis (16). The Polyene antibiotics appear to act on cell membranes. When whole cells are treated, mitochondrial respiration within these cells is reduced. When mitochondria are isolated and then treated, respiratory interference is not noted (13). However, if mitochondria are isolated following whole cell treatment, respiration is impeded (41). This effect on respiration was attributed to permeability changes in the mitochondrial membrane with subsequent

loss of cofactors or an inactivation of dehydrogenases. Desertomycin and asochitine induce leakage of cell constituents in a variety of cells (18, 33). Permeability changes in this instance are thought to result from an association between antibiotic and membrane protein. Protein extracted from membranes treated with these antibiotics had different electrophoretic responses than the same protein which was not treated. Griseofulvin (21), phleomycin (12), and lomofungin (15) have similar modes of action. Griseofulvin and phleomycin are associated with synthesis of deoxyribonucleic acid (DNA) while phytoactin and lomofungin are associated with synthesis of RNA. The anthracyclines apparently couple with DNA, the effect being an inhibition of RNA synthesis. There is also evidence that the activity of DNA polymerase is reduced (16).

Information regarding the mode of action of systemic fungicides is far from comprehensive. However, a good deal of research has provided several hypotheses. The benzimadazoles of which there are three, may inhibit the synthesis of nucleic acids. Benomyl, according to Clemons and Sisler is very unstable and rapidly forms benzimidazole carbamic acid methyl ester (BCM). These two compounds appear to be equally fungitoxic but because benomyl is transformed so readily to BCM it may be that BCM is entirely responsible for toxicity. Thiabendazole is also fungistatic although somewhat less

active than benomyl. Since the structure of the benzimidazole fungicides is similar to that of purines, these fungicides may interfere with the synthesis of the purine nucleotides adenosine and guanosine triphosphate. This being the case, incorporation of the benzimadazole into a ribose phosphate would be prerequisite to the action of the benzimidazoles (42).

The mode of action of the systemic oxathiins, is as yet unresolved. Carboxin selectively inhibits one group of fungi, the Basidiomycetes. A close analogue, F-427 [5 6-dihydro-2-methyl-N-(2-biphenylyl)-1 4-oxathiin-3-carboxamide], has a broader spectrum of activity among fungi which suggests that the selective nature of carboxin may be a result of its inability to penetrate the cells of nonsensitive fungi (9, 11, 26). Mathre (27) has reported an inhibition of acetate metabolism in carboxin treated cells as well as an inhibition of incorporation of phenylalanine into protein and uracil into RNA. Ragsdale and Sisler (27) have substantiated these results. The general concensus seems to be that carboxin is acting to block the tricarboxylic acid cycle (TCA cycle). The inhibition of nucleic acid and protein synthesis could be a secondary effect because sufficient energy and substrates are not being produced.

The selective nature of carboxin together with its ability to inhibit the TCA cycle suggested that an investigation of the effects of carboxin on plasma membranes was necessary to more fully understand its mechanism of action. Membrane alterations in susceptible fungi could explain carboxin's selectivity. Furthermore, a disruption of mitochondrial membranes could adversely affect the operation of the TCA cycle. Because of the possibility that carboxin's primary site of action might not involve the TCA cycle and because of repeated indications that carboxin affects nucleic acid synthesis, the necessity of a study involving the effect of carboxin on nucleic acid synthesis in a cell-free system was apparent.

MATERIALS AND METHODS

I. Permeability Studies:

Preparation of culture filtrates. -- Rhizoctonia solani Kuhn, the imperfect form of the basidiomycete Thanatephorus cucumeris (Frank) Donk, was utilized throughout the course of this investigation. The pathogen was originally isolated from cotton seedlings and stock cultures were maintained on modified Eckert's media composed of the following in g/l: glucose, 18; yeast extract, 3; peptone, 5; K_2HPO_4, 1.68; $MgSO_4$, 0.5. Cultures were transferred aseptically by placing a 1.0 cm disc of the stock mycelial mat into each new plate. Cultures were incubated for a period of 5 days at 25°C prior to use. Mycelial discs (10-15 per plate) were then removed using a 1 cm cork borer and washed on a 9.0 cm asperated porcelain Buchner funnel with 7 liters of sterile-deionized water.

Each treatment contained 24 washed mycelial discs in either 10 ml of sterile deionized water or 10^{-4}M carboxin. Carboxin was supplied by Uniroyal Chemical, Division of Uniroyal, Inc., Bethany, Connecticut. Samples were shaken on a Burrell wrist action shaker for the period of time indicated for each experiment. The discs were then removed by filtration using Whatman No. 4 paper in a 7.0 cm asperated Buchner funnel, oven dried at 100°C for 24 hours, and weighed.

The filtrate was transferred to test tubes and refrigerated at $4^{o}C$ until tests were initiated, always on the same day.

Analysis of filtrates. -- Conductivity was measured directly in μ mohs conductance using a Lab-Line Lectro Mho-Meter Model MC-1, Marck IV, with a cell constant of 0.1. The dipping cell was modified by plugging the flow pores with cork stoppers thus allowing the cell to be inverted and the filtrate poured into the cell in sufficient amounts to cover the electrodes.

A Beckman Infrared Analyzer, Model IR315, was utilized in the analysis of total organic carbon. Injections of 10-20 μl of filtrate were made depending on carbon content. Filtrate aliquots for total carbon analysis were injected directly. For organic carbon analysis, the filtrate was acidified with 6N HCl and purged with nitrogen for 10 minutes to remove CO_2 prior to injection. Characterization of organic carbon into major carbon containing groups was accomplished using Dowex 50W-X8 and Dowex 1-X8 ion exchange resins in 1 x 4 cm columns. Dowex 1 columns were converted to OH^- form with enough 0.5N NaOH to cause a complete color change, then washed with deionized water until the pH was less than 9.0. Dowex 50 columns were charged with 30 ml of 6N HCl and then washed with deionized water until the pH was that of water. Sample filtrates (15 ml) were passed first through Dowex 50 and followed by

30 ml of deionized water. This fraction was then passed through Dowex 1 and followed by 30 ml of deionized water. The amino acid fraction was eluted from the Dowex 50 column using 30 ml of 6N NH_4OH followed by 30 ml of deionized water. The organic acid fraction was eluted from the Dowex 1 column using 30 ml of 6N formic acid followed by 30 ml of deionized water. The three fractions were air dried and resuspended in 15 ml of deionized water. Aliquots from each fraction (20 µl) were injected into the Beckman Infrared Carbon Analyzer to determine the carbon content of each fraction.

A Beckman Atomic Absorption Accessory Model 1300 was used in conjunction with a Model 2400 Beckman DU Spectrophotometer to determine the presence of sodium, potassium and calcium ions in the filtrates.

An adaptation of the Somogyi method for the determination of glucose was used in the analysis of filtrates for the presence of reducing sugars (31). Optical densities were read at 540 nm using a Beckman DU - 2 Spectrophotometer.

The potassium persulfate method (29), was used as an assay for total phosphate. A modification of this procedure to allow use of a 24 ml volume is as follows: 5 ml of filtrate were brought to a volume of 25 ml with deionized water; 4 ml of 5% potassium persulfate were added; the sample was autoclaved for 30 min and then cooled to

room temperature; 1 ml acid ammonium molybdate reagent was added with shaking; then 3 drops of the stannous chloride reagent were added followed by a 10 min period for color development. A Klett-Summerson Photo-electric Colorimeter, Model 800-3, was used to read samples at 690 nm.

Respiration. -- Gas exchange was measured using standard manometric techniques with the use of the Gilson Differential Respirometer (46). Each flask contained 3 ml of either deionized water or 10^{-4}M carboxin and 4 mycelial discs (about 17.8 mg dry weight). Carbon dioxide was trapped by the addition of 0.2 ml of 20% KOH and fluted filter paper to the center well, and temperature was held constant at 30°C.

Growth effects. -- Mycelial discs incubated with and without 10^{-4}M carboxin for 5 hours were removed from the suspending medium and washed with 2 liters of deionized water. The discs were then placed on synthetic media with and without phosphate as described below:

WITH PHOSPHATE [a]	g/l	WITHOUT PHOSPHATE [b]	g/l
Sucrose	15.0	Sucrose	15.0
K_2HOP_4	1.3	KCl	1.7
KH_2PO_4	1.0	$(NH_4)_2SO_4$	1.0
$(NH_4)_2SO_4$	1.0	$MgSO_4 \cdot 7H_2O$	0.5
$MgSO_4 \cdot 7H_2O$	0.5		

[a] final pH of media was 7.0
[b] pH was originally adjusted to 7.0 with 3N HCl
pH was checked daily and adjusted to pH 7.0 with 3N NaOH

After 24 and 36 hours incubation at 24°C, the diameter of each culture was measured and the diameter of the original disc (1.0 cm) subtracted to establish the amount of new growth.

With the exception of those studies involving mycelial growth on media with and without phosphate, all experimental trials were duplicated at least 3 times with at least 3 replications of each experiment. In the phosphate studies, each trial was duplicated 5 times with replication of the experiment.

II. Ribonucleic Acid Synthesis:

Chromatin isolation. -- Soybeans (Glycine max L. var. Wayne) were grown in vermiculite at 27°C in the dark for 4 days prior to spraying with 1000 µg/ml of 2,4-dichlorophenoxyacetic acid (2,4-D) (25 ml per 400 plants). Plants were sprayed with 2,4-D to enhance

RNA polymerase activity. Twelve hours after spraying, hypocotyl sections taken 1 cm below the cotyledonary hook to the first lateral root were excised (32). Sections were then chilled and surface sterilized with 1% NaCl; they were then ground for 1 minute in a Waring blendor with an equal weight of grinding media (0.05M, tris-HCl pH 8.0; and chromatin was suspended in 2 ml of the tris buffer so that 0.2 ml of the mixture would contain the correct concentration of each. Then 0.2 ml of this mixture, 0.1 ml chromatin suspension, 0.1 ml ^{14}C-UTP, and 0.1 ml of either carboxin or deionized water was added to a 6 inch test tube and incubated at 37^{o}C for 0 and 30 min. The reaction was stopped by the addition of 4 ml of ice-cold 10% trichloroacetic acid and placed in a salt-ice solution for 5 min. Precipitates were then transferred to membrane filters (Millipore, type HA, 0.45μ) and washed with 50 ml of 5% cold trichloroacetic acid. The filters were then removed, dried under an infrared lamp, and counted in a Nuclear Chicago, Model 6804 liquid scintillation counter. The scintillation solution used was as follows: 4 ml absolute methanol and 12 ml of scintillation fluid containing 4g of PPO (2 5-diphenyloxazole) and 100 mg of POPOP 1,4-bis 2-(5-phenyloxazolyl) in 1 liter of toluene. All counts were corrected for background and for quenching by use of the channels-ratio method and the incorporation of ^{14}C-UTP was converted to pica

moles/100 µg DNA template utilized. Sterility was maintained throughout the experiment.

Deoxyribonucleic acid determinations. -- Chromatin DNA concentrations were determined according to the method of Burton (7) as follows: to 0.8 ml aliquots of chromatin samples, similar to those used in RNA synthesis studies, was added 0.8 ml of deionized water and 1.6 ml of 1N $HClO_4$. This mixture was heated at 70°C for 70 min in hot water and then dialyzed for 12 hours to remove sucrose from the grinding media. Of this dialyzed material 2 ml were then added to 4 ml of diphenylamine reagent and allowed to sit at 30°C for 24 hours for color development. Optical density at 590 nm was then determined using the Beckman DU-2 Spectrophotometer (7).

RESULTS

Permeability studies. -- If carboxin is attacking cell membranes as a site of action, an expected result would be the leakage of cellular components. If materials are released with carboxin treatment, the possibility exists that some of these materials could be charged and therefore electrical conductance should be an assay for their presence. To test this hypothesis, 24 mycelial discs were placed in 10 ml of 10^{-4}M carboxin or distilled water and shaken for periods of 0 to 10 hours followed by filtration to remove the discs. After filtration, the conductivity of the filtrates was determined. Conductance was consistently higher in carboxin treated samples (Fig. 1). After 10 hours contact with carboxin, filtrate conductivity increased an average of 37 µmhos over the zero time values as compared with an increase of 8µmhos in control filtrates. Increases in conductance were also observed in filtrates from carboxin treated samples after an exposure period of only 1 hour (Fig. 1).

This conductivity data indicated that carboxin was inducing a leakage from cells of materials ionic in nature. A logical assumption, based on this data, would be that carboxin was altering the semipermeable nature of the fungal plasma membrane thus allowing a release of intracellular materials.

Reducing sugars were not found in filtrates from either treated or untreated samples. If carbohydrates present in the culture medium

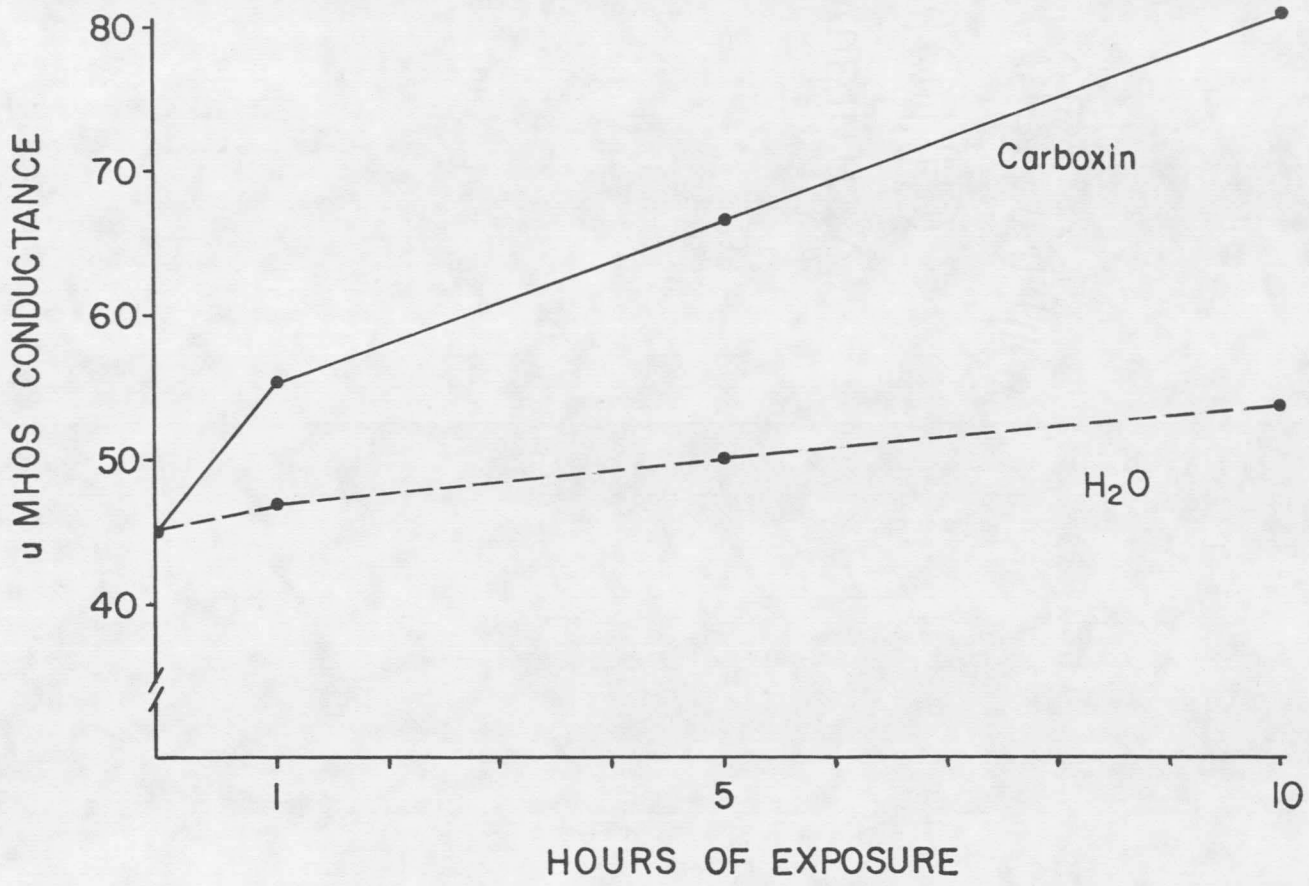

Figure 1. Conductance (μ mhos) of filtrates from samples containing 24 mycelial discs of Rhizoctonia solani exposed to 10^{-4}M carboxin or deionized water.

were not completely removed by washing of the mycelial discs, they were probably utilized as respiratory substrates. Endogenous sugars were undoubtedly consumed in the same manner.

The loss of inorganic ions from cells treated with carboxin was checked as a possible factor affecting conductivity. When sample filtrates were assayed for sodium and calcium ions, both were found; however, significant differences between treated and untreated systems were not observed (Table I and II). Potassium was found in significantly greater amounts in filtrates from carboxin treated samples (Table III). After a contact period of 10 hours, filtrates from treated samples contained an average of 6.65 mg potassium/ liter. Although this difference is statistically significant at the 5% level, it is not sufficient to explain an increase in conductance of 29 µmhos. Calculations show the above increase in potassium concentrations would contribute only 7% of the 29 µmhos conductivity increase.

Because increases in conductance could not be explained solely by the presence of inorganic ions, filtrates were assayed for the presence of organic materials. As can be observed in Figures 2 and 3, organic carbon concentrations were significantly higher in filtrates from treated samples.

Table I. Concentration of calcium ions in filtrates from samples containing mycelial discs of Rhizoctonia solani after 0 and 10 hours of exposure to 10^{-4}M carboxin or deionized water.

Exposure Time (Hrs)	Ca^{++} concentration (mg/l)	
	10^{-4}M Carboxin	Deionized Water
0	1.06 [a]	1.08
10	1.14	1.08

[a] Differences between values for treated and untreated samples are insignificant at the 5% level (t test).

Table II. Concentration of sodium ions in filtrates from samples containing mycelial discs of Rhizoctonia solani after exposure to 10^{-4}M carboxin or deionized water.

Exposure Time (Hrs)	Na^+ concentration (mg/1)	
	10^{-4}M Carboxin	Deionized Water
0	2.70 a/	3.22
1	2.66	2.81
5	2.68	2.65
10	2.76	2.76

a/ Differences between values for treated and untreated samples are insignificant at the 5% level (t test).

Table III. Concentration of potassium ions in filtrates from samples containing mycelial discs of *Rhizoctonia solani* after exposure to 10^{-4}M carboxin or deionized water.

Exposure Time (Hrs)	K^+ concentration (mg/l)	
	10^{-4}M Carboxin	Deionized Water
0	1.96 [a]	2.06
1	2.35	3.91
5	2.35	4.69
10	3.12	6.65

[a] Differences between values for 1, 5 and 10 hour treated and untreated samples are significant at the 1% level. Differences between 0 time values are nonsignificant at the 5% level (t test).

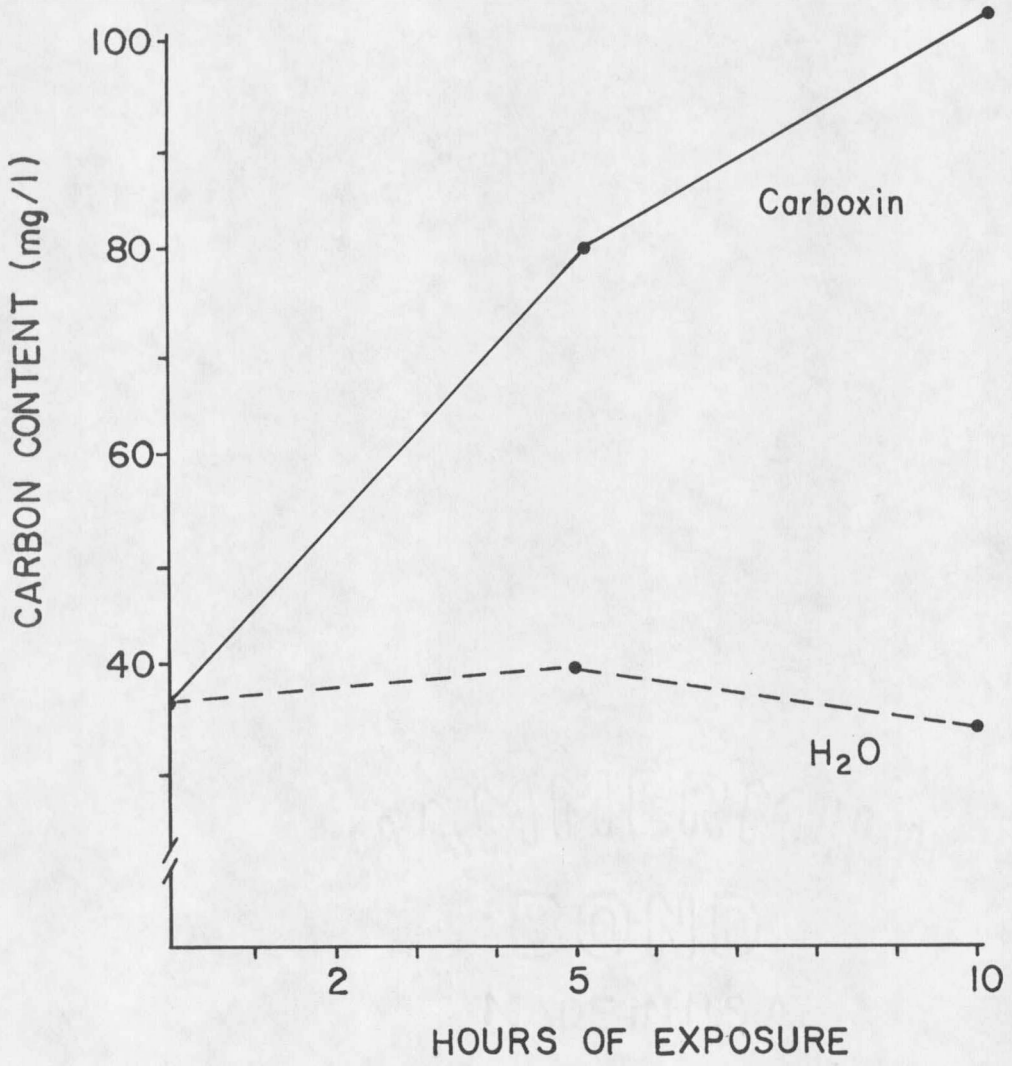

Figure 2. Effect of carboxin (10^{-4}M) on the release of organic carbon from mycelial discs of Rhizoctonia solani. Carbon content of samples was corrected for the amount of carbon in carboxin.

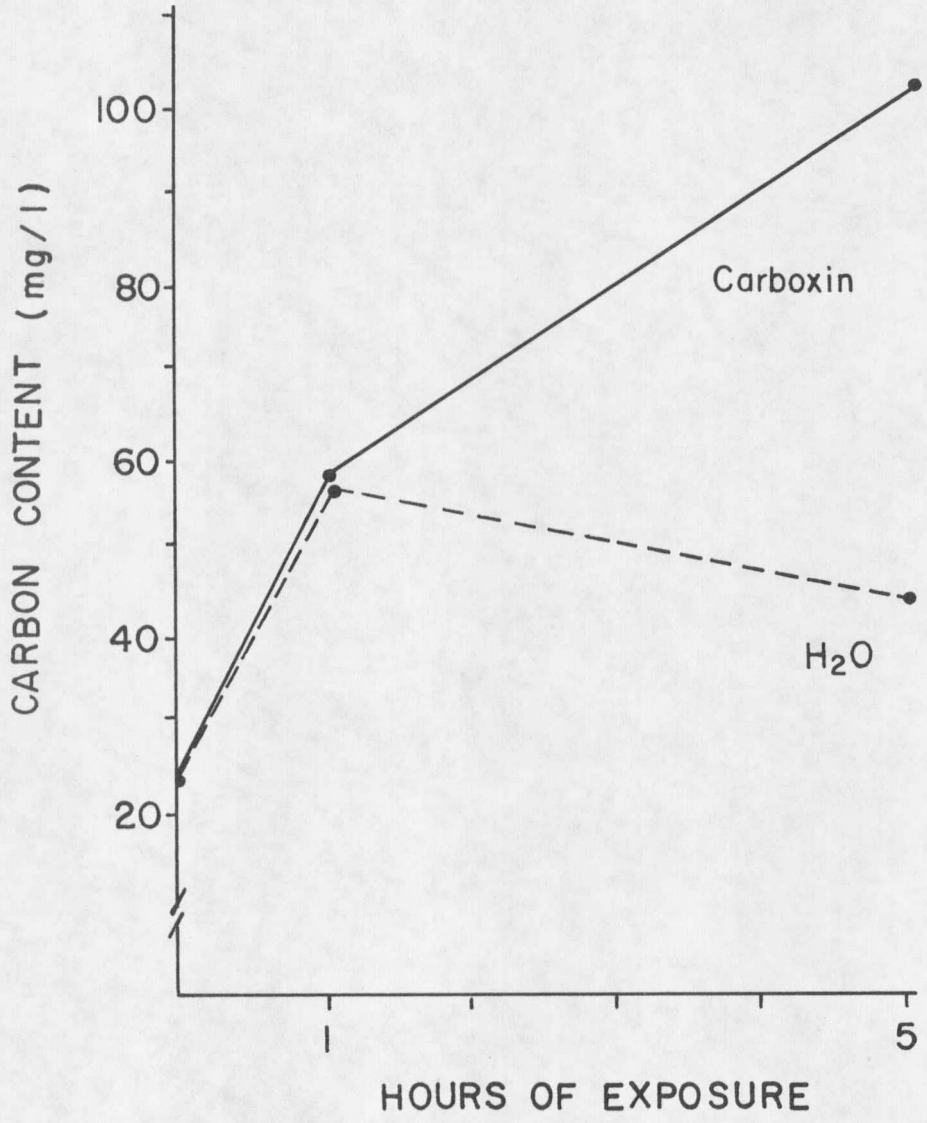

Figure 3. Effect of carboxin on the release of organic carbon from mycelial discs of Rhizoctonia solani. Carbon content of samples has been corrected for the amount of carbon in carboxin.

The presence of carbon compounds in filtrates from samples treated with carboxin did not in itself indicate that these materials were responsible for increases in conductivity. The data did, however, establish that an increase in organic compounds occurred with carboxin treatment and the possibility of these compounds being charged existed.

To determine the type of charge, if any, on the carbon compounds being liberated from carboxin treated cells, the filtrates from the samples were passed through columns of anion and cation exchange resins. The eluates from these columns plus the neutral fraction were then analyzed for the presence of organic carbon (Table IV). The major portion of the organic carbon was present in the anion fraction indicating that carboxin treatment resulted in the release of negatively charged organic materials. These materials undoubtedly affect conductance and could thus explain part of the conductivity increases in carboxin treated samples. The negative charge associated with these organic exudates suggests that they are organic acids.

Because of the phospholipid structure of plasma membranes and indications that carboxin may function to disrupt the structure of such membranes, filtrates were checked for the presence of phosphate. As seen in Figure 4, filtrates from carboxin treated samples

Table IV. Carbon content of the anion, cation, and neutral fractions of treated and nontreated 5 hour sample filtrates. Samples were treated with 10^{-4}M carboxin or deionized water. The carbon content of carboxin has been subtracted from carboxin treatment values.

	Fraction		
	Carbon content (mg/l)		
Treatment	Anion	Cation	Neutral
Carboxin	65.5	0	15
Water	14.5	0	16

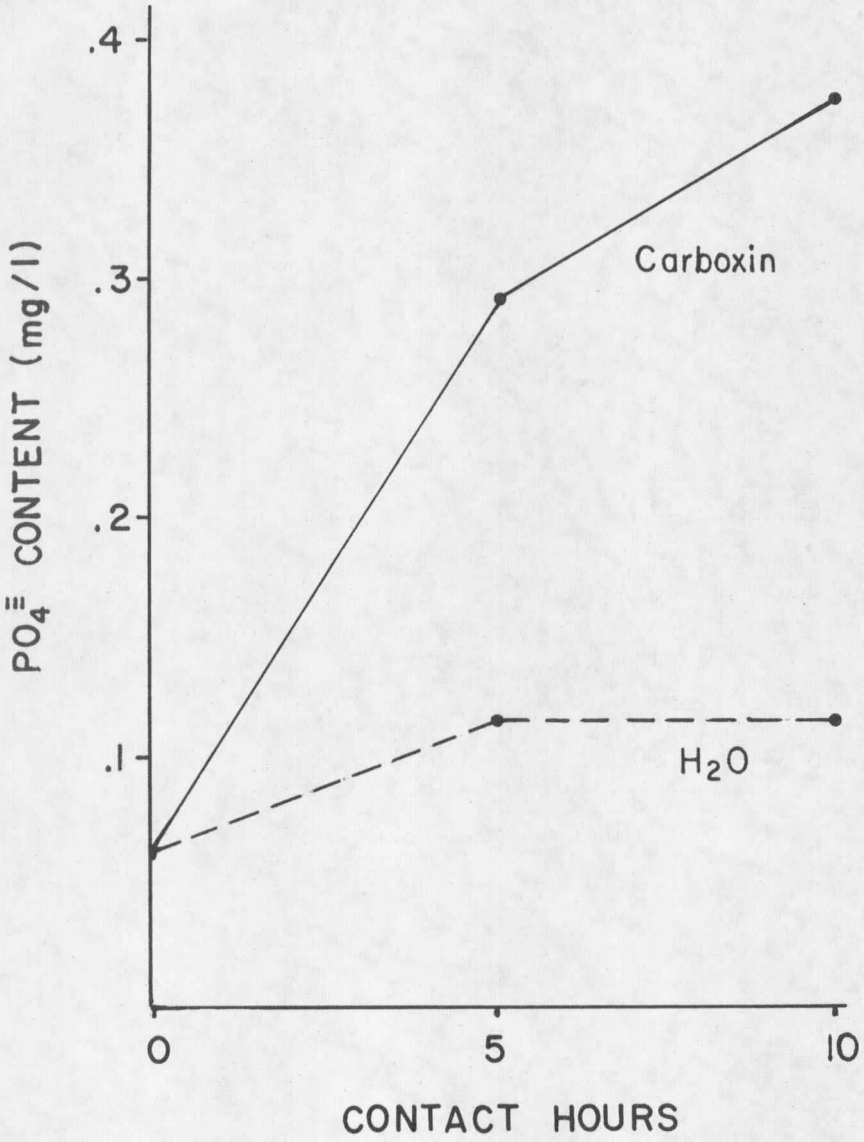

Figure 4. Effect of carboxin on release of phosphate from mycelial discs of Rhizoctonia solani.

contained greater amounts of phosphate than did filtrates from untreated samples.

To determine the effect of carboxin induced phosphate losses on growth, mycelial discs which had been subjected to 10^{-4}M carboxin or deionized water and then washed thoroughly were placed on a medium with and without phosphate ions. The data presented in Table V indicates that both treated and untreated mycelial discs resumed growth on the complete medium at the same rate. If carboxin treated discs were placed on media without phosphate, their growth was inhibited as compared to untreated discs.

Mathre (27) and Ragsdale and Sisler (37) have shown that metabolism of acetate is greatly inhibited in carboxin treated sensitive fungi. In addition, there appears to be a build up of succinate (27) in a manner similar to that described for malonate inhibition of the TCA cycle (3). If respiratory pathways are blocked in R. solani after carboxin treatment, with a concomitant build up of respiratory intermediate pools, the possibility exists that these materials could be exuded. To study this further, mycelial discs were starved by prolonged incubation in deionized water to reduce respiratory intermediate levels. Respiration rates were checked periodically at 30 minute intervals and fresh KOH added every 3 hours. With each KOH addition, the system was allowed to

Table V. Growth from mycelial discs of Rhizoctonia solani which had been treated for 5 hours with 10^{-4}M carboxin or deionized water, washed, and placed on a carboxin-free medium with and without phosphate.

	Complete Medium		Complete Minus $PO_4^{\equiv}$	
	Control	Carboxin	Control	Carboxin
Diameter of culture in cm after 36 hours of growth.	3.3	3.3	1.9	1.6
Diameter of culture in cm after 48 hours of growth.	6.7	6.7	3.5	2.7*

* Significantly different at the 5% level.

equilibrate for 30 minutes before readings were taken. As seen in Figure 5, after 24 hours the effect of starvation on O_2 uptake was evident. Respiration rates dropped considerably indicating a lack of endogenous respiratory substrates. With respiration proceeding under these conditions of starvation, the addition of carboxin should not result in a build up of intermediate pools and therefore losses from both control and treated cells should be similar and at a minimum. To test this hypothesis, mycelial discs were allowed to shake for 24 hours in deionized water and then placed in contact with carboxin or water for 6 additional hours after which the conductivity of sample filtrates was determined. As seen in Table VI, no significant differences in conductivity existed between treated and untreated systems.

RNA synthesis. -- Mathre (27) and Ragsdale and Sisler (37) have reported that RNA synthesis is inhibited in carboxin treated cells. However, it was not determined whether carboxin directly inhibits RNA synthesis or whether this effect is secondary resulting from a lack of high energy compounds being produced by the cell as a result of inhibition of the TCA cycle. To determine if carboxin could inhibit RNA polymerase directly, a DNA dependent RNA polymerase system from soybeans was used (32). The results of these tests

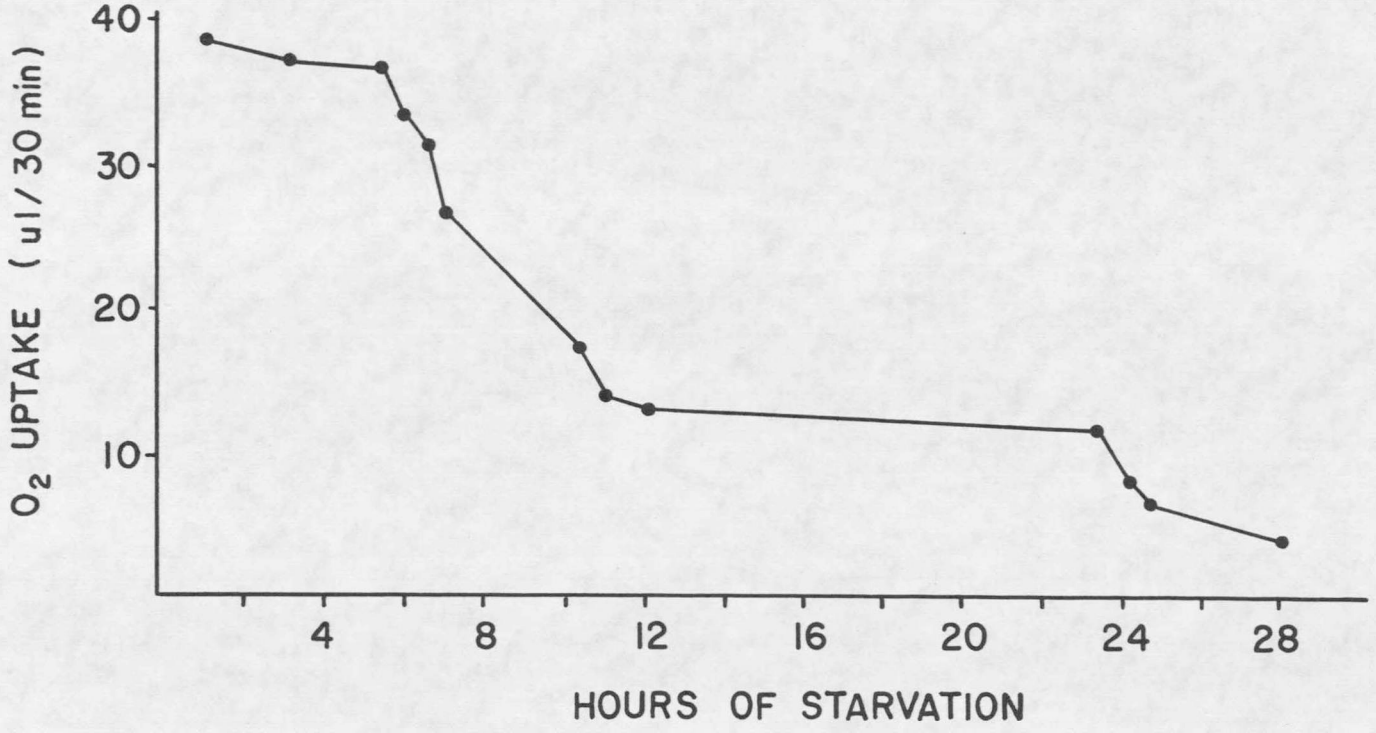

Figure 5. Effect of starvation on oxygen uptake by mycelial discs of Rhizoctonia solani in deionized water. Approximately 17.8 mg dry weight of R. solani flask.

Table VI. Conductance (μ mhos) of filtrates from samples containing 12 mycelial discs (55.5 mg dry wt.) of Rhizoctonia solani which were exposed to 10^{-4}M carboxin or deionized water after a 24 hour starvation period in deionized water.

Contact Period (Hrs)	Conductance (μ mhos)	
	10^{-4}M Carboxin	Water
0	23.3	25.0
6	23.3	23.8

indicate that 10^{-4}M carboxin had no effect on this RNA polymerase system. When the system was treated with 10^{-4}M carboxin for 30 min, 1210.2 pica moles of ^{14}C UTP were incorporated per 100 µg of soybean DNA. In control samples, using deionized water, 1255.0 pica moles were incorporated.

DISCUSSION

Mycelial discs of Rhizoctonia solani when treated with carboxin, demonstrated an efflux of cellular materials. The affect was likely a result of either alterations in the fungal plasma membranes or an inhibition of respiratory metabolism with the subsequent build up and leakage of intermediates. The leakage was similar to the response of a number of fungi to the polyene antibiotics although apparently of somewhat less magnitude (45).

Conductance data indicate that some of the materials coming from treated cells are ionic in nature. Of the inorganic ions checked, only potassium was present in sufficient amounts to increase conductivity, (Table III) although its contribution accounted for only a part of the total conductivity increase. Sutton (45) has reported a loss of 15.64 µg potassium/mg dry weight of yeast after a 30 min treatment with the polyene nystatin. Values reported in this paper (Table III) are approximately 2.0 µg/mg dry weight of R. solani. If potassium levels are nearly the same in yeast and R. solani and if potassium leakage can be considered a means by which to monitor membrane damage, then nystatin apparently alters plasma membranes more rapidly and to a greater extent than does carboxin.

The majority of organic materials exuded from cells after treatment with carboxin were negatively charged (Table IV). The negative charge indicates that these materials may be organic acids

and their presence in sample filtrates provides a plausible explanation for increases in conductivity although quantitative determinations to ascertain the extent to which they affect conductivity were not attempted. The presence of extracellular organic acids would substantiate the hypothesis that carboxin is inhibiting TCA respiration with the formation and leakage of intermediate acid pools.

When R. solani mycelia were starved in water for 24 hours respiration rates dropped considerably (Fig. 5). If carboxin inhibited respiration and leakage of respiratory intermediates was a result of this inhibition, then carboxin treatment of starved mycelium should not effect leakage if membranes were not being damaged. Such was found to be the case since discs starved for 24 hours prior to carboxin treatment exhibited the same amount of leakage as untreated starved discs (Table VI). Respiration, under conditions of starvation, proceeded so slowly that inhibition by carboxin was not observed. In this case, intermediate pools would not be formed and subsequent leakage would not be observed.

Phosphate was also released from carboxin treated cells (Fig. 4). Because of carboxin's interference with inorganic phosphate determinations using the phosphomolybdic acid test with reduction to molybdenum blue (1), the only test adaptable was that for total phosphate using a persulfate digestion. As a result it was impossible

to determine the nature of the released phosphate. However, the presence of either organic or inorganic phosphate could explain increases in conductivity. Mathre (27) and Ragsdale and Sisler (37) have suggested that carboxin inhibits aerobic respiration by inhibition of the TCA cycle. With such inhibition a reduction in electron transport and therefore oxidative phosphorylation would undoubtedly follow. This could result in an accumulation of adenosine diphosphate and inorganic phosphate both of which could be released from treated cells.

The effect of phosphate leakage was noticeable in growth resumption studies. The growth of mycelial discs which had been treated with carboxin, washed, and placed on media without a phosphate source was retarded as compared to similarly treated discs placed on media with phosphate. In control samples, without carboxin treatment, mycelial discs resumed growth equally well on both media regardless of phosphate content (Table V). It appears from this study, that carboxin treatment results in leakage of phosphate or phosphate containing materials which are necessary for growth, but that this leakage may be a secondary effect; the primary effect being an inhibition of the tricarboxylic acid cycle. The possibility also exists that the origin of released phosphate is the plasma membrane. However, replacement of membrane phosphate would seem to

be of lesser importance in growth resumption than replacement of phosphates necessary for metabolism. Also apparent in this study was the fungistatic nature of carboxin previously reported by Mathre (26).

A consideration that carboxin's metabolic interference is a secondary effect must be entertained. If carboxin were to disrupt mitochondrial membranes, then the disorganization of membrane bound enzymes and co-enzymes could conceivably result in respiratory inhibition. Implied here is an alteration in the spatial arrangement and not an inhibition of enzymes per se. Such a mode of action has been postulated for filipin by Shaw, Allam and Gottlieb (41). The ability of the polyenes to effect whole cell respiration and not cell free respiration suggests action on the plasma membrane.(14). The loss of essential metabolites, through membrane leakage after treatment of whole cells with filipin, could explain respiratory inhibition. In cell free systems all essential co-factors are necessarily added, apoenzymes remain active, and respiration is not affected. With carboxin treatment the situation is somewhat different. Mathre (personal communication) has demonstration respiratory inhibition in both whole cells and cell free extracts. In addition, cell free extracts from organisms which are not sensitive to carboxin under natural conditions also demonstrate respiratory

inhibition. Of suspected importance here is carboxin's inability to enter resistant cells, thus, cell membranes would appear to be the major penetration barrier. Gottlieb (13) has pointed to membrane sterols as the binding site for the polyenes and other workers have shown as association between antibiotics and membrane protein (18, 33). Possibly carboxin's mode of action is also explainable on the basis of cell membrane constituents. Bacteria which are unaffected by the polyenes do not contain sterols in their cell membranes and fungi which do not possess sterols are also polyene insensitive (25). Similar binding sites may be present in carboxin sensitive organisms and absent in resistant species. In resistant species, carboxin binding would not occur and membranes would remain intact. In cell free systems no plasma membrane barrier exists and resistant organisms become vulnerable. If this hypothesis is valid, differences must exist between the composition of plasma and mitochondrial membranes. Insensitive organisms which are sensitive in cell free systems might possess a site of action in mitochondrial membranes which does not exist in the plasma membrane.

The presence of extracellular organic acids after treatment with carboxin is somewhat difficult to understand unless some alteration first exists in the plasma membrane (3). As a comparison, Gottlieb (16) has demonstrated the leakage of tricarboxylic acids

after treatment of yeast with nystatin; which indicates that altered membranes will allow the passage of such materials.

With the data obtained to date, it is impossible to determine whether the release of materials from cells of R. solani treated with carboxin is due to plasma membrane damage or is a secondary effect as a result of interference elsewhere in the cell. The leakage phenomena in R. solani, as induced by carboxin, is similar in many respects to losses in other organisms as a result of treatment with materials known to alter membrane structure. In contrast to this, is the reversibility of carboxin's inhibition. The fungistatic nature of carboxin differs from materials, such as nystatin, in which binding and therefore inhibition is irreversible. In addition, no leakage was observed after carboxin treatment of starved cells. Alterations in mitochondrial membranes could explain TCA respiratory inhibition which is known to exist with carboxin treatment. However, this type of intervention implies structural differences between mitochondrial and plasma membranes of resistant organisms. Carboxin's inhibitory effects may be a result of more than one site of action; in which case, assignment of primary and secondary effects may be in error. In any case, the ability of carboxin to enhance the leakage of cellular materials must be completely understood before a mode of action is assigned.

Carboxin does not appear to inhibit DNA dependent RNA polymerase in a cell-free system from soybeans. Other workers have reported that such an inhibition does exist in whole cells of carboxin sensitive fungi (27, 37). A study using a polymerase system from a fungus was unsuccessful; however, Mathre (personal communication) has demonstrated carboxin's inhibitory effect on respiration of mitochondria from higher plants as well as from fungi. The possibility that polymerase systems in fungi and higher plants are appreciably different can not be overlooked and therefore definite conclusions await carboxin treatment of a cell free polymerase systems from susceptible fungi.

SUMMARY

Mycelial discs of Rhizoctonia solani, when treated with carboxin, demonstrate an efflux of cellular materials. The observed leakage is likely a result of either alterations in the fungal plasma membrane or an inhibition of respiratory metabolism with the subsequent build-up and leakage of respiratory intermediates.

Electrical conductance of sample filtrates was used as an assay for the presence of ionic exudates and therefore as an assay for carboxin induced leakage. Of the inorganic ions checked, only potassium was responsible for increases in conductance and was found to contribute about 7% to the total increase. Organic acids and phosphate materials were also released, although quantitative determinations to ascertain their affect on conductivity were not attempted.

Leakage stimulated by carboxin was similar, in many respects, to leakages induced in yeast by materials known to alter plasma membranes. In addition, the presence of extracellular organic acids is difficult to explain unless membrane damage exists, as weak acids do not readily cross intact plasma membranes. A possible source of organic acids does exist however, as a result of carboxin's inhibition of TCA metabolism. Electron transport systems, and therefore oxidative phosphorylation, are undoubtedly inhibited as a result of respiratory interferences. If phosphorylation of ADP is impeded, a

possible source of phosphate materials exists. Phosphate leakage was sufficient to retard further growth of the organism after fungicide removal and placement on a medium without phosphate.

When mycelial discs were starved for 24 hours respiratory 0_2 consumption dropped considerably. Carboxin treatment, after starvation, did not result in cell leakage indicating that membrane damage did not occur. If carboxin treatment is altering plasma membranes, the possibility exists that similar alterations could occur in other cell membranes. A disruption of mitochondrial membranes could alter the spatial arrangement of membrane bound enzymes and co-enzymes and respiratory inhibition could result.

Possibly carboxin's inhibitory effects may be a result of more than one site of action. Conceivably both respiratory inhibition and membrane damage could result with carboxin treatment. In sensitive organisms, membrane damage could provide a mode of entry for carboxin and respiratory inhibition could then follow.

Carboxin does not appear to inhibit DNA dependent RNA polymerase in a cell-free system from soybeans. Attempts to isolate such a polymerase system from a fungus were unsuccessful and therefore definite conclusions were not established.

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