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Mechanism of Action of the Fungicide Thiabendazole, 2-(4′-Thiazolyl) Benzimidazole

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Thiabendazole, 2-(4′-thiazolyl) benzimidazole (TBZ) inhibited the growth of *Penicillium atrovenetum* at 8 to 10 μg/ml. Oxygen consumption with exogenous glucose was inhibited at 20 μg/ml, but endogenous respiration required more than 100 μg/ml. TBZ inhibited completely the following systems of isolated heart or fungus mitochondria: reduced nicotinamide adenine dinucleotide oxidase, succinic oxidase, reduced nicotinamide adenine dinucleotide-cytochrome *c* reductase, and succinic-cytochrome *c* reductase at concentrations of 10, 167, 10, and 0.5 μg/ml, respectively. Cytochrome *c* oxidase was not inhibited. Antimycin A and sodium azide caused the usual inhibition patterns for both fungus and heart terminal electron transport systems. In the presence of antimycin, the fungicide inhibited completely succinate-dichloro-phenolindophenol reductase and succinate-2,2-di-*p*-nitrophenyl-(3,3-dimethoxy-4,4-biphenylene-5,5-diphenylditetrazolium)-reductase at 2 and 4 μg of TBZ per ml, respectively. Coenzyme Q reductase required 15 μg/ml. TBZ reduced the uptake by *P. atrovenetum* of glucose and amino acids and decreased the synthesis of various cell components. At 120 μg/ml, the incorporation of labeled carbon from amino acids-U-^{14}C was decreased: lipid, 73%; nucleic acids, 80%; protein, 80%; and a residual fraction, 89%. TBZ did not inhibit peptide synthesis in a cell-free protein-synthesizing system from *Rhizoctonia solani*. Probably the primary site of inhibition is the terminal electron transport system and other effects are secondary.

Thiabendazole, 2-(4′-thiazolyl)benzimidazole (TBZ) was first reported as an antihelmintic agent (2) and only later were its antifungal properties and potential for controlling fungus diseases recognized (16). Especially notable is the systemic distribution of this compound in plants which permits a more efficient use as a general disease control agent (17). The antifungal spectrum of TBZ is broad, yet selective, and several pathogens are resistant to it (P. M. Allen, Ph.D. Thesis, Univ. of Illinois, Urbana, 1969). TBZ controls a variety of plant diseases such as *Cercospora beticola* leaf spot of beets (4, 15), pear scab (5), crown rust of rye (7), verticillium wilt of cotton (6), and some transport and storage diseases of fruit.

In a previous paper, the effect of TBZ on spore germination was described (D. Gottlieb and K. Kumar, Phytopathology, *in press*). The compound inhibits spore germination but is more active after germination has begun than before this time. The action is fungicidal and causes stunting and malformation of the germ tubes once they have begun to emerge from the spore. TBZ is readily absorbed by spores, is distributed in small amounts among the various components and organelles, and is present in the greatest amount in the cytoplasmic fluid.

Two types of action have been attributed to TBZ: that it inhibits transamination which is partially counteracted by exogenous pyridoxine and biotin and that it interferes with the transfer of amino acids in protein synthesis (16). This report presents a general study of the mechanism of action of TBZ on various cellular metabolic processes.

MATERIALS AND METHODS

Penicillium atrovenetum Smith and *P. oxalicum* Currie and Thom were grown on potato-dextrose-agar from single spore isolates. A 5-ml amount of sterile GYE medium (glucose, 10 g; yeast extract, 10 g; distilled water, 1 liter) was added to agar slant cultures to make a spore suspension that was pipetted into 125 ml

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of GYE medium in a 500-ml Erlenmeyer flask. The flask was incubated on a reciprocal shaker at 26 C for 36 to 48 hr, and the contents were homogenized in a Waring Blendor for 30 sec. A 5-ml amount was transferred to another similar flask and grown for 24 hr. This culture was homogenized, and a 3-ml inoculum was added to a series of such flasks for growth studies. TBZ in ethanol was added in amounts to give concentrations described in Fig. 1, and the fungus was then grown for 48 hr. Mycelium was harvested through Whatman filter paper and dried under vacuum to constant weight.

The effect of fungicide on the inhibition of oxygen consumption in whole cells and cell-free preparation was measured by standard Warburg respirometry (11). The cells were grown for 24 hr as described above. Cell-free systems were made by breaking 20 to 25 g of mycelia in a French press maintained at 4,000 to 6,000 psi. The cell-free preparation was centrifuged at 5,000 × *g* for 10 min, the residue was removed, and the supernatant fluid was finally centrifuged at 30,000 × *g* for 30 min. Mitochondria in the pellet were then suspended in sucrose-Tris buffer [0.05 M tris (hydroxymethyl) aminomethane containing 0.8 M sucrose, *p*H 7.5], recentrifuged, and washed twice with buffer. The protein in the mitochondria was determined by the biuret method of Gornall et al. (8) with bovine albumin as a standard. Beef heart mitochondria were prepared as described by Crane et al. (3). Fungus mitochondria were used immediately after preparation, but beef heart mitochondria were frozen in a dry ice-acetone mixture and stored at −20 C until needed.

Succinic oxidase was determined manometrically in Warburg flasks containing phosphate buffer (*p*H 7.4), 200 μmoles; sodium succinate, 100 μmoles; cytochrome *c*, 2 mg; mitochondria, equivalent to 4 mg of protein; and TBZ in ethanol. Controls contained the same materials except for the absence of TBZ in the ethanol. Antimycin A (10 μg/ml) was added to other flasks containing no TBZ. Reduced nicotinamide adenine dinucleotide (NADH) oxidase was measured similarly except that 20 μmoles of NADH was added instead of succinate. The effect of TBZ on cytochrome oxidase was also determined by oxygen consumption, except that ascorbic acid was the hydrogen donor to cytochrome *c*. Sodium azide (10 mmoles) was substituted for TBZ to determine whether a typical cytochrome oxidase was present in the mitochondria.

Enzyme activities of mitochondria were also measured spectrophotometrically. Succinic-dichlorophenolindophenol (DPIP) reductase was determined by a decrease in absorbancy at 600 nm in a system containing phosphate buffer (*p*H 7.4), 50 μmoles; ethylenediaminetetraacetic acid (EDTA), 1 μmole; sodium succinate, 30 μmoles: DPIP, 0.05 μmole; mitochondria, equivalent to 600 μg of protein; and water, 1 ml. The change in optical density (OD) per minute per milligram of protein was calculated to determine the specific activity of the enzyme.

Succinate-cytochrome *c* reductase was measured by the increase in the absorbancy at 500 nm. The assay mixture contained sodium phosphate buffer (*p*H 7.4), 50 μmoles; sodium azide, 5 μmoles; cytochrome *c*, 0.7 mg; mitochondrial protein, 400 μg; sodium succinate, 0.05 ml (20 μmoles). A millimolar extinction coefficient of 19.2 was used for calculating the amount of reduced cytochrome *c* (10).

NADH oxidase was measured by the decrease in OD at 340 nm due to formation of nicotinamide adenine dinucleotide (NAD). The assay mixture contained sodium phosphate buffer (*p*H 7.0), 50 μmoles; mitochondrial protein, 300 μg; NADH, 0.1 μmoles; and water to make the volume 1 ml. NADH oxidase was also measured by oxygen uptake in Warburg flasks.

NADH-cytochrome *c* reductase assay was the same as for succinate-cytochrome *c* reductase, except that 0.2 μmole of NADH was substituted for succinate and the amount of mitochondrial protein was reduced to 100 μg.

Succinate-2,2-di-*p*-nitrophenyl-(3,3-dimethoxy-4,4-biphenylene)-5,5-diphenylditetrazolium (NBT)-reductase was measured by increases in OD, at 530 nm due to the formation of reduced NBT. The reaction mixture contained phosphate buffer (*p*H 7.4), 50 μmoles; sucrose (0.25 M), 25 μmoles: mitochondrial protein, 600 μg; sodium succinate, 30 μmoles; antimycin A, 10 μg; NBT, 1.0 μmole; and water to 1 ml.

Succinate-2-*p*-iodophenyl-3-*p*-nitro-5-phenyl tetrazolium chloride (INT)-reductase method was the same as for succinate-NBT-reductase, except that INT solution was added in place of NBT and the OD was measured at 570 nm. Succinate-coenzyme Q_6 reductase was assayed by the method of Ramasarma and Lester (14). Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer by standard techniques.

The uptake of ^{14}C-glucose and ^{14}C-amino acids was determined in the same experiments as those for the study of the effects of TBZ on metabolism. The radioactive compound and 1 g (wet weight) of cells were added to flasks containing GYE, and samples were removed at various times for assay. For leakage experiments, 9.4 μCi of glucose-*U*-^{14}C was added to a growing culture of *P. atrovenetum* in mid-log phase, and the mixture was incubated for 2 hr. The mycelium was washed free from radioactivity and then suspended in 0.2 M sodium phosphate buffer with and without TBZ for various times, and the ^{14}C in the buffer and that in the cells were assayed.

The metabolism of glucose-*U*-^{14}C and of an amino acid-*U*-^{14}C mixture was studied by adding these substrates to 100 ml of GYE medium in a 500-ml flask. TBZ was added to triplicate flasks as needed, and the radioactivity of the medium was determined. Mid-log-phase cells of *P. atroventum* (1.5 g) were placed in the medium and incubated for as long as 4 hr on a reciprocal shaker. The flasks were fitted with a carbon dioxide collecting device as described by Gottlieb and Tripathi (9). Radioactivity was determined in the cells, cell extracts, filtrates, and carbon dioxide.

Lipids were obtained by two extractions with 10 ml of chloroform-methanol (3:1) for 30 min each. The residue was twice suspended in 5 ml of 80% ethanol, and the combined filtrates were assayed by the biuret and anthrone procedures for amino acids and carbohydrates, respectively. The residue was suspended in 10 ml of 5% trichloroacetic ac'd and shaken for 1 hr at 4 C. OD was measured at 260 nm for soluble purine- and pyrimidine-containing compounds. The cold trichloroacetic acid residue was suspended in 10 ml of 10% trichloroacetic acid at 90 C for 1 hr and centrifuged; the supernatant fluid was neutralized with NaOH. The solution was assayed by the orcinol test for ribonucleic acid (2), the diphenylamine test for deoxyribonucleic acid (11), and OD at 260 nm for total base. The residue from this treatment was hydrolyzed with 6 N HCl in sealed ampoules at 121 C, and the solution was tested for amino acids with the ninhydrin and biuret reagents (8, 12). The residue was washed and dried in vacuum for 24 hr. Radioactivity in the various fractions was determined.

In vitro protein synthesis studied in *Rhizoctonia solani* systems were done as described by Obrig et al. (13).

Thiabendazole was provided by the Merck Chemical Division, Merck and Co., Inc., Rahway, N.J. Fresh solutions were prepared in absolute ethanol for each experiment. Reagent grade inorganic and organic chemicals were obtained either from Mallinckrodt Chemical Works, Fisher Chemical Co., or Allied Chemical Co. All biochemicals were from Sigma Chemical Co. Scintillation grade chemicals were from Packard Instrument Co. Radioisotopes were from New England Nuclear Corp.

RESULTS

Membrane function. TBZ has no important effect on the integrity of the cell membrane of *P. atrovenetum*. There was no markedly increased leakage of ^{14}C-labeled materials from cells that had first been incubated in glucose-U-^{14}C, washed, and placed in phosphate buffer containing TBZ. Labeled materials in the buffer increased with times of incubation from 30 min to 48 hr even in the absence of fungicide but were not stimulated by the presence of as much as 40 μg of TBZ per ml. However, the fungicide interfered slightly with the uptake of glucose-U-^{14}C; between 5 and 40 μg/ml, inhibition of uptake varied from 5 to 35%.

Growth and cellular respiration. TBZ inhibited the growth of *P. atrovenetum* 45, 90, and 100% at concentrations of 0.5, 8.0, and 10 μg/ml, respectively (Fig. 1). With glucose as the substrate, TBZ completely inhibited total respiration at 13 μg per mg (dry weight) of fungus mycelium or at 50 μg per ml of medium. It reduced oxygen consumption of endogenous substrates 10, 64, and 100% at 20, 50, and 100 μg/ml, respectively. Exogenous respiration was more sensitive; 10 μg/ml or less did not inhibit oxygen consumption but 20 μg/ml was completely effective.

Interference with aerobic respiration could be attributed to the action of TBZ on fungal mitochondria. The succinate oxidase system of mitochondria from *P. atrovenetum* was too unstable to give satisfactory measurements, but those from

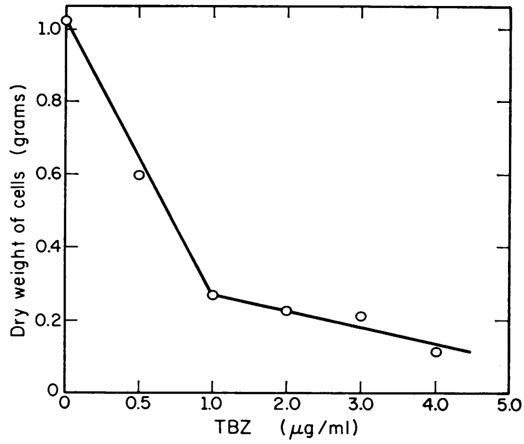

FIG. 1. *Inhibition of growth of Penicillium atrovenetum by thiabendazole.*

beef heart were stable and consumed oxygen linearly at 53 μliters of oxygen per ml of protein per hr. The fungicide decreased this respiration 23, 45, 51, and 100% at 2, 10, 20, and 167 μg of TBZ per ml, respectively. Antimycin and sodium azide, which inhibit the terminal electron system, also inhibited succinic oxidase.

Low concentrations of TBZ decreased NADH oxidase in fungus and beef heart mitochondria when measured either spectrophotometrically or manometrically (Table 1). Inhibition of oxygen consumption was complete at 10 μg/ml by the manometric method. Similar results were obtained with the beef heart mitochondria.

To determine whether the inhibition of oxidation was before or after cytochrome *c* in the electron transport system, the oxidation of reduced cytochrome *c* was blocked by the addition of 10^{-3} M sodium azide and the reduction of cytochrome *c* was measured. NADH-cytochrome *c* reductase was inhibited in both fungal and beef heart mitochondria, the inhibition increasing with increasing concentrations of TBZ (Table 2, Fig. 2). The fungicide inhibited the reductase in *P. atrovenetum* mitochondria 25, 65, and 100% by 2, 6, and 10 μg of TBZ per ml, respectively. Activity of this enzyme was also completely inhibited by 10^{-6} M antimycin A (Calbiochem, Los Angeles, Calif.), indicating the typical antimycin-sensitive terminal electron transport pathway was present which could be inhibited by TBZ. Since *P. atrovenetum* mitochondria did not oxidize succinate, the activity of its succinate-cytochrome *c* reductase could not be measured. Beef heart mitochondria contained only a negligible endogenous substrate for NADH-cytochrome *c* reductase, but exogenous NADH readily reduced cytochrome *c*

TABLE 1. *Inhibition by TBZ of NADH oxidase[a] in Penicillium atrovenetum mitochondria[b]*

TBZ (µg/ml)	Absorbance at 340 nm	Per cent inhibition (QO_2)
0	0	0
3	25	52
6	86	60
10	100	84
20	100	100

[a] NADH oxidase activity was measured by the absorbance at 340 nm, and oxygen consumption was determined by using standard manometric techniques.

[b] With antimycin A (10^{-6} M, 10 µg/ml), absorbance at 340 nm was 100 and per cent inhibition was 100. With sodium azide (10^{-3} M), absorbance at 340 nm was 100 and per cent inhibition was 100.

TABLE 2. *Inhibition by TBZ of NADH-cytochrome c reductase[a] in Penicillium atrovenetum mitochondria[b]*

TBZ (µg/ml)	Absorbance at 550 nm	Per cent inhibition
0	0.70	0
2	0.52	25
6	0.42	65
10	0.00	100
20	0.00	100

[a] In the presence of sodium azide (10^{-3} M).

[b] With antimycin A (10^{-6} M, 10 µg/ml), absorbance at 550 nm was 0 and per cent inhibition was 100.

without any lag period (Fig. 2). The specific activity of the enzyme in the absence of TBZ was 0.01 µmole of cytochrome *c* reduced per ml of protein per min. TBZ concentrations of 4, 6, 10, and 50 µg/ml inhibited NADH-cytochrome *c* reductase 0, 12, 17, and 100%.

Succinate-cytochrome *c* reductase was present in beef heart mitochondria and had a specific activity of 22 µmoles of cytochrome *c* reductase per mg of protein per min. The reductase was completely inhibited by 10^{-6} M antimycin A. TBZ was very active in this system, and a concentration of 0.5 µg/ml prevented the reduction of the cytochrome *c* by succinate (Fig. 3).

In contrast to the interference of TBZ with the chain of reactions from the substrate hydrogen donor to the reduction of cytochrome *c*, the compound did not interfere with the next step, the oxidation of reduced cytochrome *c*. TBZ (10 µg/ml) was entirely inactive in a Warburg flask system in which 37 µliters of oxygen per mg of protein was consumed in 30 min. That the cytochrome *c* oxidase was reacting normally was indicated by its inactivation at 10^{-3} M sodium azide.

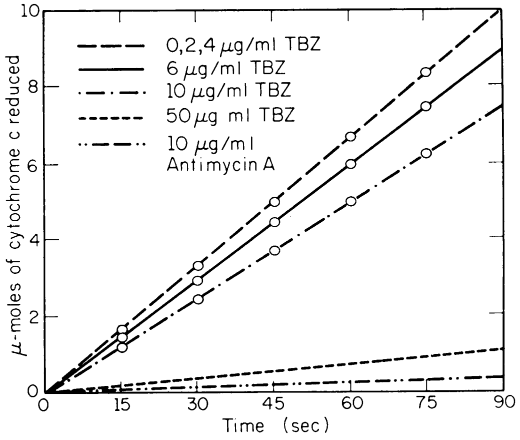

FIG. 2. *Inhibition by thiabendazole of beef heart mitochondrial NADH-cytochrome c reductase.*

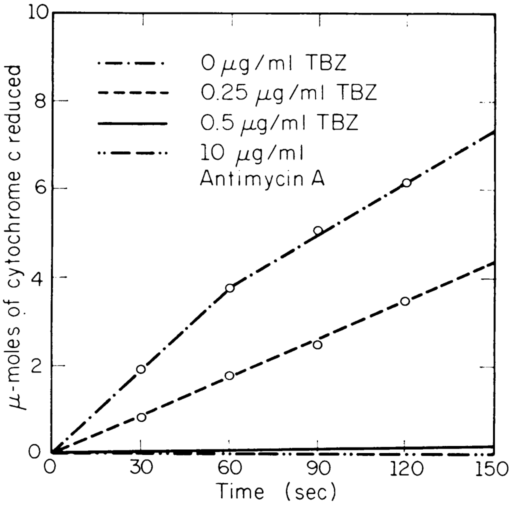

FIG. 3. *Inhibition by thiabendazole of beef heart mitochondrial succinate-cytochrome c reductase.*

It was apparent that the action of TBZ on the respiratory system was somewhere between the substrate and the enzymes which reduced cytochrome *c*. The interference by TBZ with the reduction of various artificial hydrogen acceptors could help point to the steps in the terminal electron transport system that were being inhibited. Beef heart mitochondria reduced DPIP with succinate as the hydrogen donor. This reduction was not prevented by the addition of antimycin A at 10 µg/ml, indicating that the reduction took place before the antimycin-sensitive site. The addition of TBZ to this system increasingly in-

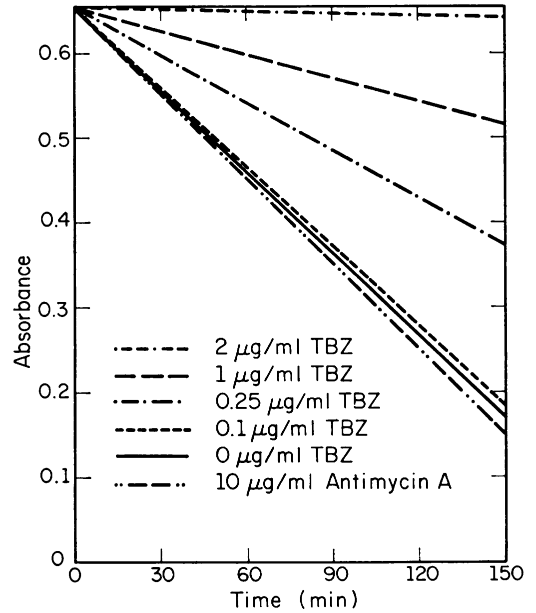

FIG. 4. *Inhibition by thiabendazole of dichlorophenolindophenol reductase from beef heart mitochondria as measured by absorbance of reduced product at 600 nm.*

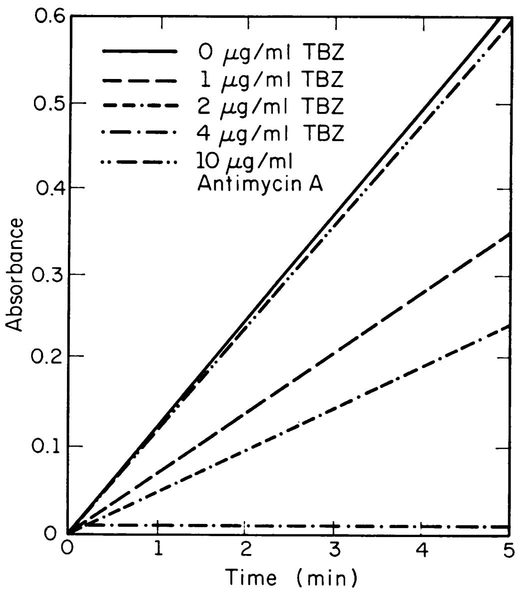

FIG. 5. *Inhibition by thiabendazole of succinate-NBT reductase from beef heart mitochondria as measured by absorbance of the reduced product at 530 nm.*

hibited the reduction of DPIP as the concentration of the fungicide was raised (Fig. 4). The system was very sensitive and was inhibited 20, 56, and 100% by 0.25, 1.00, and 2 μg/ml, respectively. The addition of antimycin A did not prevent the reduction of DPIP, indicating that it occurred before the antimycin-sensitive site. Similar interference with reduction of DPIP occurred with NADH as the hydrogen donor. The tetrazolium dye NBT was also reduced by beef heart mitochondria, and its reduction was not decreased by the addition of antimycin A. The reduction of NBT also must have occurred somewhere between the substrate and the antimycin-sensitive site of the terminal electron transport system. TBZ prevented the passage of electrons somewhere before the antimycin-sensitive site (Fig. 5). The inhibition was 50, 63, and 100% at 1, 2, and 5 μg/ml, respectively. TBZ also inhibited the reduction of INT by 32, 50, and 100% at 0.25, 0.5, and 1.0 μg/ml, respectively, but sodium azide had no effect on this system.

The electrons from succinate flow via some unknown nonheme intermediates to coenzyme Q and reduce this intermediate. TBZ decreased the reduction of exogenous coenzyme Q_6 by the beef heart mitochondria linearly with increasing concentration and prevented it at 15 μg/ml (Table 3). Thenoyltrifluoroacetate also inhibited the reduc-

TABLE 3. *Inhibition by TBZ of succinate-coenzyme Q_6 reductase of beef heart mitochondria*[a]

TBZ (μg/ml)	Absorbance at 518 nm	Per cent inhibition
0.00	0.187	0
0.25	0.178	9
1.00	0.156	28
5.00	0.126	55
10.00	0.102	77
15.00	0.078	100

[a] With thenoyltrifluoroacetone (10 mM), absorbance at 518 nm was 0.128 and per cent inhibition was 54. With the blank, absorbance at 518 nm was 0.077.

tion of coenzyme Q_6 by succinate. These results indicate that the reduction of the electron flow is inhibited early in the terminal electron transport system, somewhere between succinate and coenzyme Q.

Effect of TBZ on synthetic cellular metabolism. Carbon dioxide production was inhibited only 10% at 5 to 40 μg of TBZ per ml. The fungicide also had a depressing effect on synthetic activities from glucose of *P. atrovenetum* and, as the concentrations of TBZ were increased, the inhibition also increased. At 20 μg/ml, the per cent inhibitions for the various cell fractions were lipids, 33; nucleic acids, 40; protein, 32; and residue (presumably cell wall), 45%. The nucleic acid fraction gave positive tests by the orcinol method for RNA and the diphenylamine tests for DNA. The rates of inhibition of incorporation into the 80% ethanol and 5% cold trichloroacetic acid extracts were 31 and 21%, respectively. There was also a decrease of ninhydrin-positive material in the ethanol and of anthrone-positive materials in the cold trichloroacetic acid.

Experiments with a mixture of ^{14}C-amino acids as substrate gave similar data, but since higher concentrations of TBZ were used there was a greater inhibition both of uptake of substrate and of carbon dioxide production (Table 4). At 120 μg/ml, TBZ inhibited the incorporation of labeled carbon into the various cell fractions from 56 to 89% (Table 5). In most cases, the maximum inhibition was reached at 80 μg of TBZ per ml (Table 6).

The effect of TBZ on protein synthesis was also studied in a cell-free in vitro system from *R. solani* (13). Since there were no cell membrane barriers, the direct effect of TBZ on protein synthesis could be determined. The complete system incorporated 2,600 counts per min per assay per hr, and the addition of 50 μg of fungicide per tube resulted in 2,500 counts/min in the same period. Puromycin decreased the counts per minute to 558 and ribonuclease to 478. Absence of poly U, of ribosomes, and of the supernatant fraction resulted in 157, 389, and 257 counts/min, respectively. Obviously TBZ did not inhibit protein synthesis (Table 7).

Antagonists to the action of TBZ. Reversal of inhibition of growth of fungi by TBZ (16) has been reported for pyridoxal hydrochloride and guanine. The inhibition of *P. atrovenetum* by 2 μg of TBZ per ml was not reversed either by 10 μg of pyridoxal hydrochloride per ml or by 100 μg of guanine per ml.

TABLE 4. *Effect of TBZ on the uptake of ^{14}C-amino acids in Penicillium atrovenetum*

TBZ (μg/ml)	Uptake		
	No. of dpm per mg of cells[a]	Per cent uptake of amino acids[b]	Per cent inhibition
0	5,629	48	0
20	4,848	43	10
40	4,319	36	25
80	1,547	12	75
100	1,272	9	81
120	988	7	85

[a] An average of 3.4×10^6 dpm of ^{14}C was initially added in the media.

[b] Based on the total amount of ^{14}C taken up by the cells.

TABLE 5. *Effect of TBZ on the production of $^{14}CO_2$ in Penicillium atrovenetum*

TBZ (μg/ml)	CO_2 production	
	No. of dpm/mg of cells	Per cent inhibition
0	193	0
20	148	23
40	138	29
80	79	59
100	76	61
120	67	65

TABLE 6. *Effect of TBZ on the incorporation of ^{14}C from ^{14}C-amino acids into various cell components of Penicillium atrovenetum*

TBZ (μg/ml)	Per cent inhibition					
	Lipid	80% Ethanol	5% Cold trichloroacetic acid	10% Hot trichloroacetic acid	Protein	Residue
0	0	0	0	0	0	0
20	22	12	3	23	26	38
40	51	24	21	39	40	54
80	75	53	57	73	80	77
100	73	55	64	80	87	85
120	73	56	66	80	88	89

TABLE 7. *Effect on TBZ on in vitro protein synthesis in Rhizoctonia solani*

Assay condition	Counts per min per assay[a]
Complete system, 0 min	68
Complete system, 60 min	2,600
Plus TBZ (50 μg)	2,500
Plus puromycin (50 μg)	558
Plus ribonuclease (30 μg)	478
Minus ribosomes	389
Minus supernatant fraction	257
Minus poly U	157

[a] All of the data are from 60 min of incubation, unless otherwise indicated.

DISCUSSION

The limitation of growth of fungi by TBZ can be ascribed to an interference with many cellular metabolic activities. Although the reduction of the synthetic capacities of *P. atrovenetum* results from the action of this compound and the effect is concentration-dependent, it is extremely doubtful that such inhibitions reflect primary sites for the action of TBZ. One standard for judging which of various metabolic systems is most directly affected is the concentration that is required to prevent such cellular functions. The process that is most easily inhibited is assumed to be the primary site of action. Growth of *P. atrovenetum* was inhibited 90% by 8 μg of TBZ per ml. At such concentrations, the inhibition of synthesis of protein, nucleic acids, nucleotide, lipids, and probably carbohydrates was very low. Even concentrations 10 times greater were usually ineffective for inhibiting these processes. Protein synthesis was not the prime target of the fungicide because cell-free systems from *R. solani*, which was very sensitive to the fungicide, were not inhibited from incorporating amino acids-*U*-^{14}C. Puromycin, on the other hand, readily inhibited the synthesis

An inhibition of protein synthesis by TBZ has also been observed by Staron et al. (17), who believed that the fungicide interferes with the transfer of amino acids for peptide synthesis. They also attributed part of the growth inhibition to a decrease in the transaminating properties of the TBZ-treated cells because the inhibiting effect could be partially offset by the addition of pyrodoxine and biotin. This type of reversal was not found in the current studies, so that probably decreases in protein synthesis are not primary effects of TBZ.

The inhibition of oxygen consumption of *P. atrovenetum* furnished the best clue to the primary site of action of TBZ. Although 50 μg of TBZ per ml was required to stop total oxygen consumption, this high requirement could be attributed to the relative insensitivity of the endogenous portion of the respiration. TBZ at 50 μg/ml completely inhibited endogenous respiration, whereas at 20 μg/ml it inhibited exogenous respiration. The limitation on exogenous respiration almost certainly is a result of a fungicide-produced lesion in the mitochondria from *P. atrovenetum* and beef heart. NADH and succinic oxidase as well as the respective cytochrome *c* reductases were inhibited by low concentrations of TBZ, but cytochrome *c* oxidase was unaffected. The block to electron flow is thus before cytochrome *c*. In these mitochondrial systems, both DPIP and NBT were reduced in the presence of antimycin A, and low concentrations of TBZ prevent these reductions. The fungicide could thus inhibit electron flow before the antimycin-sensitive site. Finally, interference by low concentrations of TBZ in the reduction of coenzymes Q_6 by succinate indicates that the action of the fungicide is somewhere between the substrate and coenzyme Q. TBZ resembles another fungicide, Dexan, in its action on the mitochondrial electron transport (18).

Inhibiting the terminal electron transport system would prevent the formation of energy by this system and its subsequent storage in adenosine triphosphate. Since the synthetic activities of the fungus are dependent on the availability of energy for such processes, the decreased formation of protein, nucleic acid, and other essential cell components would be expected. Similarly, the permeability of the cell membrane to nutrients such as amino acids and sugar are usually energy-dependent and a decreased energy supply would reduce their uptake from the medium. The inhibition of the terminal electron transport system of the mitochondria probably is the primary site of action of TBZ, and other decreases in other metabolic function are secondary and follow from the unavailability of energy.

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