Effect of 2-Substituted Benzimidazoles on the Fungus *Pithomyces chartarum*

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SUMMARY

The ability of a variety of 2-substituted benzimidazoles to inhibit germ-tube elongation in *Pithomyces chartarum* conidia was determined. Contact with a solid surface, such as agar or glass-wool strands, was an essential requirement for germination. The degree of inhibition which benzimidazole derivatives exerted on germ-tube elongation varied markedly with the substituted group; 2-(4'-thiazolyl) benzimidazole (TBZ) was most active. Germ-tube elongation proceeded linearly on agar media and was unaffected by benzimidazole derivatives existing as cations. Inhibition occurred only when the pH of the medium neared, or exceeded, the pK_a of the benzimidazole derivative being tested. Inhibition of respiration with exogenous glucose after 3 to 4 h of exposure of germinating spores to TBZ appeared to be only an indirect effect of TBZ action; TBZ, in concentrations 100 times that necessary to inhibit growth, failed to affect oxygen uptake or respiratory control of isolated mitochondria with a variety of substrates. TBZ inhibition of germ-tube elongation was partially eliminated by high concentrations of vitamin B₁₂. TBZ treatment produced distorted mycelial growth with increased vacuolation and elevated RNA/DNA ratios. These data suggested a condition of unbalanced growth similar to that caused by vitamin B₁₂ deficiency in bacterial and mammalian cells. TBZ may act as a precursor (replacing 5,6-dimethyl benzimidazole) in the formation of an inactive vitamin B₁₂ coenzyme analogue.

INTRODUCTION

A broad spectrum of biological activity has been attributed to benzimidazole and its derivatives. Wooley (1944) presented evidence that benzimidazole acts as a purine analogue in some yeasts and bacteria. Bucknall & Carter (1967) and Skehel, Hay, Burke & Cartwright (1967) found that 2-mercapto-1-(β -4-pyridethyl) benzimidazole inhibited the incorporation of [³H]uridine into the RNA of mammalian cells in tissue culture. This substituted benzimidazole was also shown to be a reversible inhibitor of interferon activity by Friedman & Pastan (1970). Paget, Kisner, Stone & De Long (1969) reported that a series of 1-(benzimidazol-2-yl)-3-substituted ureas and their analogues acted as immuno-suppressive agents in mice and antiviral agents in several viral diseases. Allen & Gottlieb (1970) reported that 2-(4-thiazolyl) benzimidazole (TBZ) inhibited several enzyme systems in fungal and beef-heart mitochondria. Clemons & Sisler (1971) published data which suggested that methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate (BCM) interfered with DNA synthesis (or some closely related process) in fungi, and Seiler (1972) demonstrated the mutagenicity of benzimidazole and its derivatives in *Salmonella typhimurium*.

In a previous report (Stutzenberger & Parle, 1972), we described the binding of 2-substituted benzimidazoles to the surfaces of ungerminated *Pithomyces chartarum* conidia. The

inhibitory effect which a variety of these compounds have on germ-tube elongation has now been studied, with particular emphasis on the action of TBZ, the fungistatic agent used for the control of *P. chartarum* growth on New Zealand grazing lands.

METHODS

Organisms. *Pithomyces chartarum* strain C spores were produced, harvested, and washed as previously described (Stutzenberger & Parle, 1972).

Germination of spores. In the preliminary studies on the chemical and physical requirements for rapid and synchronous germination of these spores, a medium of the following composition was used (ml): 10^{-1} M-glucose, 5; 10^{-2} M-alanine, 5; 10^{-1} M-phosphate buffer (pH 6), 5; mineral salts solution (10^{-4} M-FeSO₄, 10^{-4} M-CaCl₂, 10^{-4} M-ZnSO₄, and 10^{-2} M-MgSO₄), 5; distilled water to 45. This medium was either solidified in Petri plates with 1% agar or placed in 125 ml Erlenmeyer flasks containing 2 g of chopped, low-lead-content glass wool (BDH Chemicals Ltd, Poole, Dorset). A spore suspension (about 15 mg dry wt of spores/ml) was either spread on the agar surface in amounts of 0.1 ml or added to the flasks (5 ml) and incubated at 26 °C during aeration by bubbling air. During routine measurements of germ-tube elongation, spores were spread on Potato Dextrose Agar (PDA) and incubated at 26 °C.

Effect of 2-substituted benzimidazoles on germ-tube elongation. Benzimidazole and 15 of its 2-substituted derivatives were dissolved as stock solutions in 10 mM-HCl or in 95% ethanol depending on solubility. The compounds were added in various concentrations to molten PDA and plates prepared. Four discs (1 cm in diam.) were cut from each plate, placed on glass slides, and inoculated with spores. These, together with control discs, were incubated at 26 °C for 9 h. All discs were then stained with lactophenol cotton blue and examined at $\times 400$ magnification in a Reichert Diapan microscope. The length of 30 germ tubes was measured with a micrometer eyepiece and averaged for each slide of 4 discs.

Determination of pK_a value for TBZ. No pK values for TBZ were found in the literature, and its low solubility ($< 25 \mu\text{g/ml}$) at pH values over 5 (Weinke, Lauber, Greenwald & Preiser, 1969) precluded a pK_a determination by titration. However, the pK_a for TBZ could be determined spectrophotometrically by measuring the decrease in peak extinction with increased pH in the u.v. range. The extinction peak shifts from 302 nm at low pH to 298 nm at high pH. This shift is accompanied by a decrease in peak extinction amounting to 0.165 unit at a TBZ concentration of $15 \mu\text{g/ml}$. Measurement of the change in peak extinction with small changes in pH over the range 3 to 7 produced data from which the pK_a could be calculated by using the following equation:

$$pK_a = \text{pH} + \log_{10} \frac{E - E_m}{E_i - E},$$

where E_i , E_m and E are the extinction coefficients of the ionized molecule, the unionized molecule, and a mixture of the two respectively at the analytical wavelength (Albert & Serjeant, 1962). The average pK_a value, calculated from data at 10 pH values, was 4.74.

Respiratory studies. Spore respiration was measured by monitoring oxygen uptake polarographically at 26 °C in a YSI Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.). The reaction mixtures normally contained: 4 to 6 mg dry wt of spores, 0.4 ml of 1 M-phosphate buffer (pH 6), and water to 3.8 ml. During respiration measurements on germinating spores, 0.1 g of chopped glass wool was

also included. Various concentrations of carbohydrate were added (0.2 ml) after measurement of endogenous respiration rates.

Isolation of mitochondria. Cultures used for the isolation of mitochondria were produced in 4 l quantities of Sabouraud Dextrose Broth at 26 °C and vigorously aerated by bubbling air. When mycelial dry wt reached 0.8 to 1.0 g/l (usually at 48 to 72 h), the mycelia were harvested by filtration, washed twice with distilled water, and once with 0.05 M-tris buffer (pH 7.4). The washed mycelia were mixed with an equal weight of acid-washed glass sand (particle size approx. 100- to 200-mesh) in an extraction medium containing 0.4% bovine serum albumin (BSA), 0.3 M-mannitol, 5 mM-EDTA, and 0.05 M-tris buffer (pH 7.1). The mycelial paste was ground by one-second passes in a motor-driven mortar and pestle at 1 °C. After a sequence of five passes, the fluid containing liberated mitochondria was pressed through a 100-mesh stainless steel screen and the remaining paste was reground. This procedure, repeated six times, allowed higher recovery of intact mitochondria than one continuous grinding period. The crude cell extract was centrifuged at 800 g for 10 min at 4 °C. The sediment was discarded and the supernatant fluid centrifuged at 8800 g for 30 min to sediment the mitochondria. The light tan pellet was resuspended in 2 to 3 ml of extraction medium at 1 °C.

Oxygen uptake of isolated mitochondria was measured polarographically at 26 °C in a reaction mixture similar to that recommended by Hunter (1955) as modified for use with fungal mitochondria (Mattoon & Balcavage, 1967). The constituents were (ml): 0.4% BSA in 0.3 M-mannitol, 1.8; 0.2 M-phosphate buffer (pH 7.1), 0.3; 0.2 M-NaF, 0.2; 0.01 M-NAD⁺, 0.2; 0.1 M-MgCl₂, 0.1; 0.25% horse heart cytochrome *c* (Sigma, London), 0.1; mitochondrial suspension containing 0.45 to 1.2 mg protein, 0.3; and 0.2 M-substrate, 0.2. The final phosphate acceptor, used in the determination of the respiratory control ratio, consisted of (ml): Sigma yeast hexokinase (10 mg/ml), 0.1; 0.8 M-glucose, 0.1; and 10⁻¹ M-ADP (pH 6.8), 0.1. NADH-linked cytochrome *c* reductase activity was measured by the method of King & Howard (1967).

Extraction and determination of mycelial RNA and DNA. Extraction of nucleic acids from mycelia was performed in a manner similar to that advised by Friesen (1968). Mycelia (25 ml samples) were harvested from culture fluids by centrifugation (10⁴ g, 15 min, 4 °C). The pellets were resuspended in 10 ml of 5% trichloroacetic acid at 1 °C, and recentrifuged. This procedure eliminated interfering substances without detectable loss of nucleic acids. Pellets were drained of all supernatant fluid, washed once with distilled H₂O, recentrifuged and dispersed in 5 ml of 1 N-HClO₄. The mixtures were heated at 70 °C for 5 min and allowed to stand at room temperature overnight. The extracted cells were removed by centrifugation and the nucleic acid content of the HClO₄ extract was determined. The DNA concentration was measured by the diphenylamine method of Burton (1968) and RNA by the orcinol method as described by Schneider (1957). Calf thymus DNA (Sigma Type I) and yeast RNA (Sigma Type VI) were used as standards in these assays. Total nucleic acid was also estimated by the ultraviolet absorption peak at 260 nm and by the phosphorus content of the extracts (measured by the method of Sherman (1942), as modified by F. D. Dorofaeff for autoanalysis in our laboratory). The extracted nucleic acids (totals calculated from the diphenylamine and orcinol tests) had an extinction at 260 nm which averaged 0.88 that of equivalent concentrations of the nucleic acid standards. The average phosphorus content of the extracted nucleic acids was 7.9% with a standard deviation of $\pm 0.9\%$.

RESULTS AND DISCUSSION

Spore germination. Benzimidazole derivatives such as BCM and TBZ do not prevent fungal spore germination, but rather inhibit germ-tube elongation (Gottlieb & Kumar, 1970). Therefore, in our studies it was necessary to obtain spore germination as rapidly and as synchronously as possible, often while maintaining them under conditions which were compatible with respirometric and other quantitative determinations. Difficulties were encountered, for spores maintained either in stationary flasks or kept in suspension by stirring or bubbling air failed to form germ tubes even after incubation for 48 h at 26 °C in distilled water, phosphate buffer, or complex media (Sabouraud Dextrose Broth, or yeast extract supplemented with glucose). Removal of these spores to water agar or Sabouraud Dextrose Agar resulted in germ-tube formation within 1 to 2 h. Self-inhibition of germination (such as reported by Allen, 1955 and Yarwood, 1956) was initially considered, but dilution of spore suspensions to low density (less than 50 spores/ml of suspending fluid), removal of low mol. wt substances by dialysis of spore suspensions, and repeated washing and transfer to fresh media failed to induce germination. A variety of other treatments, reported to stimulate germination of fungal spores, was also tested. Exposure of *Pithomyces chartarum* spores to various concentrations of organic solvents and furans (Sussman, Lowry & Tyrrell, 1959), glycocholate or taurocholate (Elliott, 1949), or indoleacetic acid (van Sumere, van Sumere-de Preter, Vining & Ledingham, 1957) was ineffective. Heat shocking at 40 °C (the highest temperature at which the spores remained viable) was unsuccessful.

It was then discovered that an effective stimulant of spore germination was contact with a solid surface. Fig. 1(a) illustrates the time course of spore germination in two systems which provide contact with a surface. When spores were placed in a fluid medium containing chopped glass wool, attachment to the glass strands occurred within 60 to 90 min and the surrounding medium became almost spore-free. The effect of glass wool was comparable to that of the agar medium in providing a germination stimulus. After 4 h of incubation, the average percentages of germinated spores were 0, 81 and 94 respectively for spores in the fluid alone, in the fluid with glass wool, and on the agar. Spores could also be induced to germinate by contact with acid-washed sand or polyvinyl chloride plastic shavings, but the rate and per cent of germination were low and erratic compared to that on glass wool. The basis of this requirement for a solid surface during germination is unknown, although fungal thigmotropism toward solid surfaces has been studied (Corner, 1935; Dickinson, 1972). Glass wool was used as a stimulant to germination in our respirometric studies on *Pithomyces chartarum* spores. Germination on an agar medium was used in the testing of inhibitory action of benzimidazoles on germ-tube elongation.

Effect of benzimidazoles on germ-tube elongation. Germ-tube elongation on PDA at 26 °C proceeded at a linear rate after the first 5 h of incubation (Fig. 1b). Parle & di Menna (1972a) reported an inverse relationship of germ-tube elongation to concentration of TBZ or BCM in PDA. We used measurement of germ-tube elongation as a sensitive method for testing the toxicity of a variety of 2-substituted benzimidazoles (Table 1). Benzimidazoles substituted with thiazole, thienyl, pyrazole, furan, phenyl, or the carbamic acid methyl ester groupings were effective inhibitors of germ-tube elongation, while other substituted groups conferred little or no toxic activity on the benzimidazole molecule. Molecules with rather similar chemical structure differed widely in their ability to inhibit elongation (e.g. compare the activities of 2-phenyl benzimidazole and 2-OH-benzyl benzimidazole, and the activities of 2-benzimidazolylurea and BCM).

Fig. 1. (a) Time course for germination of *Pithomyces chartarum* spores in a fluid medium without glass wool (○), in fluid medium with glass wool (△), and on medium solidified with 1 % agar (□). Each point is the mean of at least two experiments. (b) Time course of germ-tube elongation on PDA at 26 °C. Each point gives the average and standard deviation for 30 measurements.

Table 1. *Inhibition of germ-tube elongation on PDA by various 2-substituted benzimidazoles*

Spores were incubated for 9 h on PDA at 26 °C. Thirty germ tubes were measured and averaged in each test. The average germ-tube length of the controls (no benzimidazole added) was arbitrarily assigned a value of 100 %. The standard deviation of quadruplicate tests was ± 5.8 %.

Substituted group	Per cent of maximal germ-tube length	
	0.2 µg/ml	2.0 µg/ml
None	—	101.4
-OH	—	99.0
NH ₂	—	103.9
-CH ₃	—	102.3
α -Hydroxybenzyl	—	104.5
Urea	—	105.4
γ -Hydroxypropyl	—	100.8
<i>p</i> -Benzenesulfonic acid	—	99.5
2-Oxazolyl	89.0	82.5
2-Thienyl	80.1	17.5
3-Thienyl	87.6	23.2
1-Pyrazolyl	80.8	31.6
Phenyl	98.0	29.6
4-Furyl	91.0	14.8
Carbamic acid methyl ester (BCM)	68.5	15.0
4-Thiazolyl	19.7	18.4

The ionization of the substituted benzimidazoles was also important in their toxicity. Unsubstituted benzimidazole, with a pK_a of 5.5, possesses both acid and base characteristics, existing in the cationic form at low pH and as an unionized molecule at alkaline pH. Substitution of groups at the 2-position alters the pK_a significantly. For example, the pK values for 2-methyl benzimidazole and 2-phenyl benzimidazole (PBIZ) are 6.2 and 5.2 respectively (*Handbook of Chemistry and Physics*, 1968). Substituted benzimidazoles inhibitory to germ-tube elongation were only effective when the pH of the test system was

Fig. 2. Effect of pH on the toxicity of TBZ (0.2 µg/ml) in PDA. Germ-tube elongation of controls (○) and on agar containing the fungicide (△) was measured after 9 h at 26 °C.

Fig. 3. (a) Increase in oxygen uptake with increasing concentrations of glucose (△) compared to the endogenous rate (○). Points are collective data from two experiments. (b) Effect of TBZ concentration on the rate of oxygen uptake due to addition of exogenous glucose. The endogenous respiration rate remained unaffected. Points are the means of two determinations.

sufficiently high to maintain the compounds mainly in their unionized forms. An example of this is illustrated in Fig. 2. The effect of TBZ became apparent in the pH range 4.7 to 5.0; these data correlated with the pK_a value of 4.74 for TBZ.

It appears therefore that substituted benzimidazoles are inhibitory to germ-tube elongation only when they are in the unionized state. This is probably because of their inability when ionized to penetrate the cell membrane, for it is well known that certain metabolic inhibitors, such as dinitrophenol and fluoroacetate, can gain access to the interior of fungal cells only in the unionized forms (Rothstein, 1965).

Effect of TBZ on respiration. Our efforts in further studies were concentrated on the

Table 2. *Oxygen uptake of Pithomyces chartarum mitochondria with various substrates*

Each value is the average of two to five determinations.

Substrate	Respiratory rate*	Acceptor control ratio†
Isocitrate	0.163	1.6
Succinate	0.193	1.9
α -Ketoglutarate	0.043	1.3
Malate plus pyruvate	0.041	2.1
NADH	0.915	1.1
β -Hydroxy butyric acid	0.026	1.4
Lactate	0.089	1.1

* Respiratory rate is expressed as μ A of oxygen consumed/min/mg of mitochondrial protein.

† Acceptor control ratio is expressed as the respiratory rate in the presence of added ADP compared to the rate in the absence of ADP.

action of TBZ, since it was found to be the most active of the 2-substituted benzimidazoles against *Pithomyces chartarum*, and has been successfully used in the field to control its growth (Parle & di Menna, 1972*b*). The report (Allen & Gottlieb, 1970) that TBZ inhibited the oxygen consumption of *Penicillium atrovenetum* with exogenous glucose, and inhibited mitochondrial functions at concentrations of 0.5 to 167 μ g/ml, prompted us to test the effect of TBZ on the respiration of *P. chartarum*. In initial experiments, it was found that freshly harvested spores had a high endogenous respiration rate and did not respond to added glucose. However, storage of spores for 4 to 6 weeks at 2 °C allowed diminution of the endogenous rate; responses to added glucose were then attained. Fig. 3(*a*) illustrates the endogenous respiration rate of germinating spores and the response to various glucose concentrations. Addition of TBZ in concentrations up to 10 μ g/ml had no immediate effect on oxygen uptake in the presence of glucose. However, on prolonged exposure (3 to 4 h) of germinating spores to TBZ, oxygen uptake with exogenous glucose was reduced as much as 89% $\pm$ 7% depending on the concentration of TBZ added (Fig. 3*b*). The endogenous respiration was not affected even at TBZ concentrations of 20 μ g dry spores/ml.

The TBZ inhibition of oxygen uptake with exogenous glucose and the finding of Allen & Gottlieb that TBZ inhibited functions of fungal and beef-heart mitochondria (particularly those involved in the oxidation of succinate and NADH) led us to experiments with tri-carboxylic acid cycle intermediates as substrates. Neither spores nor actively growing mycelia of *Pithomyces chartarum* were stimulated by the addition of malate, succinate, citrate, isocitrate, fumarate, or pyruvate. These negative results were not considered unusual in view of similar reports on other fungi (reviewed by Niederpruem, 1965) which indicated permeability barriers to TCA cycle intermediates in whole cells. Mitochondria liberated from *P. chartarum* mycelia, however, actively consumed oxygen with a variety of substrates (Table 2) and exhibited a relatively high degree of respiratory control considering the inherent difficulties in isolation of fungal mitochondria (Niederpruem, 1965). The presence of TBZ in concentrations to 20 μ g/ml had no effect on oxygen uptake with the TCA cycle intermediates, nor did it affect the degree of respiratory control. The NADH-linked cytochrome *c* reductase system was likewise unaffected by TBZ. Thus, it appeared unlikely that the primary site of TBZ action in *P. chartarum* was located in respiration or in oxidative phosphorylation. The decrease in respiration with exogenous glucose on prolonged exposure to TBZ was probably only an indirect effect. This conclusion was compatible with the

Fig. 4. (a) Inhibition of germ-tube elongation as a function of TBZ concentration. (b) Reversal of that inhibition by increasing concentrations of B_{12} . TBZ concentration was held constant at $0.15 \mu\text{g/ml}$ ($0.75 \mu\text{M}$); B_{12} concentration was varied from 0 to $2 \mu\text{g/ml}$ ($1.47 \mu\text{M}$). Each point is the mean of two determinations.

report of van der Kerk & Sijpesteijn (1969) that TBZ and BCM did not act as respiratory inhibitors at concentrations 100-fold that required to stop growth of *Aspergillus niger*.

Vitamin B_{12} and TBZ activity. Growth of *Pithomyces chartarum* was inhibited at TBZ concentrations as low as 0.05 to $0.1 \mu\text{g/ml}$. The effectiveness of TBZ in preventing growth at such low concentrations indicated that its action was site-specific in some essential biosynthetic process. A variety of compounds was tested to determine if any were effective in reducing TBZ inhibition of germ-tube elongation.

The following compounds were tested in various concentrations: adenine and guanine, biotin, pyridoxine, thiamine, riboflavin, ascorbic acid, unsubstituted benzimidazole, thiazole, glutathione, and 5,6-dimethylbenzimidazolylcyanocobamide (vitamin B_{12}). A statistical analysis of germ-tube elongation on PDA indicated that a difference of $\pm 10\%$ was significant in evaluating the protective capacities of these compounds against the action of TBZ. The ability of each of these compounds to antagonize TBZ action was calculated as follows:

$$\frac{L_c - L_t}{L_c} \times 100 = \% \text{ inhibition of germ-tube elongation in absence of compound being tested,}$$

$$\frac{L_x - L_{x+t}}{L_x} \times 100 = \% \text{ inhibition in presence of compound,}$$

where L_c = germ-tube length of control (no TBZ or compound); L_t = germ-tube length in presence of TBZ only; L_x = germ-tube length in presence of compound only; L_{x+t} = germ-tube length in presence of TBZ and compound. Evaluated in this manner, only vitamin B_{12} consistently reversed TBZ inhibition. This reversal was studied further by replacing PDA with a 1.5% agar medium containing 2% glucose, 0.4% vitamin-free Casamino acids (Difco), and 0.1 M -phosphate buffer, pH 6. Fig. 4 illustrates the inhibition of germ-tube elongation as a function of TBZ concentration and partial reversal of this

Table 3. Inhibition of germ-tube elongation by various concentrations of PBIZ action by several concentrations of vitamin B₁₂ *

Determination of germ-tube length was made after 9 h at 26 °C on the Casamino acids–glucose–phosphate agar.

PBIZ concentration (μM)	Per cent inhibition	
	PBIZ alone	PBIZ + (B ₁₂)
1.55	26	0 (0.368 μM -B ₁₂)
5.15	40	20 (0.368 μM -B ₁₂) 11 (0.736 μM -B ₁₂)
7.75	58	35 (0.368 μM -B ₁₂)
10.30	73	62 (0.184 μM -B ₁₂) 54 (0.368 μM -B ₁₂) 31 (0.736 μM -B ₁₂)

* Germ-tube elongation was inhibited an average of 9.5 % by 0.736 μM -B₁₂ in the absence of PBIZ.

inhibition by various concentrations of vitamin B₁₂. In these experiments, we found that B₁₂ itself was somewhat inhibitory to germ-tube elongation at relatively high concentrations. For example, at concentrations of 3 to 4 $\mu\text{g}/\text{ml}$, germ-tube elongation was reduced 16 to 30 %, and therefore concentrations in excess of 2 $\mu\text{g}/\text{ml}$ were not employed. This inhibition of germ-tube elongation may be a reflexion of a state of 'unbalanced growth' caused by an excess of B₁₂ similar to that observed in *Lactobacillus leichmannii* (Beck, Hook & Barnett, 1962). The possibility also exists that a B₁₂-related compound (produced endogenously) is required for activity in *Pithomyces chartarum* and that exogenous B₁₂ may act as competitor; a variety of B₁₂-related compounds are synthesized and serve as specific co-factors in living systems (see review by Beck, 1962).

Vitamin B₁₂ was also protective against the less toxic substituted benzimidazoles. An example of this is shown in Table 3. The inhibitory effect of PBIZ on germ-tube elongation was tested at various PBIZ and B₁₂ concentrations. At low PBIZ concentrations, B₁₂ afforded complete protection against inhibition; at high PBIZ concentrations, B₁₂ was partially protective. This protection was apparently not the result of direct chemical interaction of B₁₂ and PBIZ. Incubation of PBIZ (10 $\mu\text{g}/\text{ml}$) together with B₁₂ (50 $\mu\text{g}/\text{ml}$) at room temperature for 2 h or 99 °C for 5 min in 0.1 M-phosphate buffer (pH 6) did not alter the u.v. spectrum of either compound when controls containing only PBIZ or B₁₂ were used.

The partial reversal of TBZ inhibition by vitamin B₁₂ suggested the possibility that TBZ may create a deficiency in active B₁₂ coenzyme. This view is not without precedent; substituted benzimidazoles have been shown to act as specific inhibitors of vitamin B₁₂ activity in bacterial mutants (Scott, Rogers & Rose, 1958). The inhibitory action of some of these substituted benzimidazoles could be reversed by exogenous B₁₂ while that of others allowed little or no reversal.

A B₁₂ coenzyme (5,6-dimethylbenzimidazolyl 5'-deoxyadenosine cobamide or DBCC) is required for ribonucleotide reductase activity in *Lactobacillus leichmannii* and mammalian cells (Beck, 1968). Inhibition of the reactions which reduce ribonucleotides to deoxyribonucleotides results in abnormal growth, elevated RNA/DNA ratios, and impaired cell division in B₁₂-starved *L. leichmannii* (Beck, 1962). We observed similar characteristics in the growth of *Pithomyces chartarum* cultures when exposed to TBZ (0.2 to 0.4 $\mu\text{g}/\text{ml}$) in a medium consisting of 0.4 % Casamino-acids (vitamin-free), 2 % glucose, and 0.1 M-phosphate

Fig. 5. *Pithomyces chartarum* mycelia grown in the vitamin-free Casamino acids medium in (a) the absence of TBZ; (b) 0.2 µg TBZ/ml. Bar markers represent 5 µm.

buffer (pH 6). After 24 h of exposure to TBZ at 26 °C, mycelial structure was markedly distorted (Fig. 5), and a great degree of vacuolation was apparent. Determination of the RNA/DNA ratios revealed that TBZ treatment induced significantly increased ratios (Fig. 6). The RNA/DNA ratios for the controls remained quite constant during the growth period, averaging about 18 µg RNA/µg DNA. The RNA and DNA contents on controls averaged 69.5 µg RNA and 3.9 µg DNA respectively/mg dry mycelial wt. On exposure to TBZ, cells increased their RNA content about 10% while decreasing their DNA content about 30%. This change elevated the RNA/DNA ratios to a value averaging about 28 after 24 h of exposure.

The presence of a deoxyribonucleoside allowed growth of vitamin B₁₂-starved *Lactobacillus leichmannii* (Beck *et al.* 1962). This, together with the decrease in DNA synthesis by TBZ-treated *Pithomyces chartarum* mycelia, prompted studies to determine whether deoxynucleosides could reduce inhibition of germ-tube elongation by TBZ. The following deoxynucleosides were tested in various concentrations: 2'-deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine, thymidine, and 2'-deoxyuridine. These deoxynucleosides alone or in combination inhibited germ-tube elongation at high concentrations (e.g. 29% inhibition when concentrations were 20 µg each/ml) and did not protect against the action of TBZ. Perhaps *P. chartarum* cannot utilize deoxynucleosides as precursors for DNA synthesis, as the usual pathway for deoxynucleotide production is by reduction of the corresponding ribonucleotides.

Partial reversal of TBZ inhibition by exogenous vitamin B₁₂, the elevated RNA/DNA ratios in cells treated with TBZ, and the fact that B₁₂ contains a benzimidazole grouping suggest that TBZ, a thiazolyl-substituted benzimidazole, may act as a precursor for an

Fig. 6. Increase in the RNA/DNA ratios of *Pithomyces chartarum* cells exposed to TBZ (Δ) compared to control ($\circ$). Inset illustrates increase in dry cell wt (mg/ml) of both cultures. The average and standard deviation of three determinations are shown at 72 h. Arrows indicate time of TBZ addition ($0.4 \mu\text{g/ml}$).

inactive analogue of a vitamin B_{12} -related coenzyme in which 5,6-dimethylbenzimidazole is replaced by a portion or all of the TBZ molecule. The ability of 5,6-dimethylbenzimidazole (DMB) to antagonize TBZ action was tested. The presence of DMB ($1 \mu\text{g/ml}$) reduced inhibition by TBZ ($0.2 \mu\text{g/ml}$) by about one-third, but higher DMB concentrations were quite inhibitory in themselves and precluded further studies.

These data on the effects which TBZ and other substituted benzimidazoles cause in *Pithomyces chartarum* are consistent with the possibility that such molecules may act as precursors of inactive vitamin B_{12} -related coenzymes. A ribonucleotide reductase system dependent on a B_{12} -related coenzyme could account for the elevated RNA/DNA ratios in TBZ-treated cells. We are now starting to isolate and characterize the ribonucleotide reductase system in *P. chartarum* to evaluate further this possibility.

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