Binding of 2-(2-oxazolyl) Benzimidazole by *Pithomyces chartarum* Conidia and Release during Germination

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(Received 6 March 1973)

Several benzimidazole derivatives, such as 2-(4'-thiazolyl) benzimidazole (TBZ) and methyl-*N*-benzimidazol-2-yl-*N*-(butylcarbamoyle) carbamate (MBC), are systemic fungicides effective against a wide range of fungi (Edgington, Khew & Barron, 1971). These fungicides do not inhibit spore germination but prevent germ-tube elongation (Gottlieb & Kumar, 1970). TBZ and MBC have been used successfully in the control of *Pithomyces chartarum*, the causative agent of facial eczema in grazing stock of New Zealand (Parle & di Menna, 1972).

Recently we reported that several benzimidazole derivatives, including TBZ, bound to the surfaces of ungerminated *Pithomyces chartarum* conidia (Stutzenberger & Parle, 1972). The binding capacity has been found to be undiminished by treatment with lipid solvents (methanol, acetone, benzene, and chloroform) at room temperature, by boiling for 10 min in weak acid or alkali (0.5 N-HCl or NaOH), by 4-h treatment with acid phosphatase (Sigma Type II 0.6 units/ml), and mild esterification procedures (0.1 N-HCl in methanol at –10 °C for one week). In contrast to this high and relatively stable binding capacity exhibited by ungerminated conidia, the mycelia of *Pithomyces chartarum* had a relatively low ability to remove benzimidazole derivatives from the medium, only about 1/700 that of conidia on an equal dry wt basis.

This marked difference in affinity for benzimidazole derivatives between conidia and mycelia, together with the relatively high stability of their binding sites on the conidial surfaces, prompted experiments to determine whether benzimidazole derivatives bound by dormant conidia were released during the germination process. The binding capacity of dormant conidia for fungitoxic benzimidazoles would be important in the field if the sites released the benzimidazoles during germination rather than holding them inert on the conidial surfaces. Binding during dormancy and release during germination (the time when benzimidazoles exert their toxicity) would provide a mechanism in the field for accumulating a potentially lethal concentration of fungicide even in the presence of competing materials such as soil (which we have found to adsorb TBZ).

METHODS

The use of TBZ or MBC was found to be impractical in *assay* experiments because of their low solubility at pH values optimal for germination (pH 5 to 7). 2-(2-Oxazolyl) benzimidazole (OB) was selected for use in these experiments for the following reasons: (i) greater water solubility than TBZ or MBC; (ii) high molar extinction coefficient (E_m of 3.2×10^4 at 313 nm in a solution of pH 0.5). This high E_m facilitated measurements of free concentrations without interference by spore exudates; (iii) less toxicity than TBZ or MBC, allowing greater germ-tube elongation at concentrations necessary to achieve a significant percent saturation of binding sites on the conidial surfaces.

Fig. 1. (a) Scatchard plot for OB adsorption data. Values for A are expressed as moles/l of reaction mixture. Inset illustrates the relationship of $\bar{v}$ to A . Points are collective data from duplicate experiments.

(b) Time course of OB binding by ungerminated conidia and release during germination. Inset illustrates the percentage of bound OB which is released during the germination process. Points are the mean values of data from duplicate experiments.

For determination of affinity constants and the maximal amount of OB bound/mg dry wt of conidia, 10 ml reaction mixtures contained 0.5 mg conidia/ml, 0.05 M-acetate buffer (pH 5.0), and concentrations of OB ranging from 5×10^{-2} to 2.1×10^{-3} M. The reaction mixtures were stirred at room temperature for 10 min simultaneously with control conidia suspensions containing no OB. Conidia were removed from suspension by filtration with Millipore filters. The clarified fluids were mixed with an equal volume of 1 M-HCl. After adsorption by conidia, residual OB was measured spectrophotometrically at 313 nm with control fluids as blanks.

In experiments involving the germination of spores, a medium consisting of 0.5 % glucose, 1 mM-alanine and 25 mM-phosphate (final pH 7.0) was employed. The OB concentration was 0.4 mM; conidia were usually added at a concentration of 5 mg dry wt/ml. After thorough mixing, chopped glass wool (low-lead type, B.D.H. Chemicals Ltd, Poole, Dorset) was added at the rate of 100 mg/ml to stimulate germination (Stutzenberger & Parle, 1973). These mixtures were aerated for 9 h at 26 °C by means of fritted glass spargers fitted at the bottoms of 40 ml test-tubes. Determination of free OB concentrations was made at intervals on appropriate dilutions of reaction mixtures and control fluids containing no OB in 0.5 N-HCl. Readings at each interval were corrected for evaporation which occurred in the aerated mixtures.

RESULTS AND DISCUSSION

Measurement of the decrease in the u.v. absorption spectrum of OB appeared to be reliable for determining the decrease in free concentrations of the compound after mixing in a suspension of conidia followed by filtration. The data for the binding of OB to the surfaces of dormant conidia, plotted by the method of Scatchard (1949), are shown in Fig. 1(a). The binding data for OB form a plot similar to those previously determined for TBZ and two other fungitoxic benzimidazole derivatives (Stutzenberger & Parle, 1972).

The constants for this adsorption system, computed by the method of Feldman (1972), indicate two classes of binding sites on the surfaces of *Pithomyces chartarum* conidia – one of relatively high affinity (association constant, K , of 15.3×10^4 l/mol), the other having lower affinity for OB (K of 0.22×10^4 l/mol). The maximal amount of OB bound at a theoretical infinite free concentration was calculated to be $0.139 \mu\text{mol/mg}$ dry conidia, 6.7 % being bound by the high-affinity class of sites and 93.3 % by the low-affinity class.

During the germination process much of the previously bound OB was released from the conidial surfaces (Fig. 1*b*). Conidia introduced into a medium containing 0.4 mM-OB reduced the free concentration to about 0.25 mM during the first 10 min of exposure. During the next 2 h the conidia swelled but no change in free OB concentration could be detected. Emergence of germ-tubes from some conidia (about 20 %) was noticed at 4 h; at 9 h, germ tubes were visible on about 60 % of the conidia and an average of 56 % of the OB bound at the initiation of the experiments had been released.

The morphological and biochemical changes which occur during germination of fungal spores have been the subjects of numerous studies (e.g. see review by Sussman, 1966). In *Pithomyces chartarum* the changes in the conidial wall which allow emergence of the germ tubes also appear to decrease the binding capacity for OB. If, in the field, the binding capacity for benzimidazole derivatives is similarly altered, the emerging germ tubes would be exposed to increased concentrations of the fungicide at a time when they are highly susceptible to its action.

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