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Mechanism of action of carboxin and the development of resistance in yeast

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Abstract. Carboxin prevents the growth of yeast by inhibiting protein synthesis; the resumption of growth in the presence of this chemical appears to be primarily due to a cellular alteration affecting carboxin entry into the cells.

Keywords. Carboxin; macromolecular synthesis; resistance development.

Introduction

Carboxin (2,3,dihydro-5-carboxanilido-6-methyl l,4,oxathiin, vitavax) is one of the several systemic fungistatic chemicals used in agriculture to control pathogenic fungi. In recent years, there are reports of resistance development to this chemical (Ogawa *et al*., 1977) and the mechanism of this resistance has been attributed to the alteration of the target site. For example, in *Ustilago* sp. This chemical is reported to inhibit growth by affecting mitochondrial succinic dehydrogenase and in resistant strains the target site has been found to be altered (Gunatilleke *et al*., 1976). The use of mycelial fungi which are obligate aerobes, to understand the mechanism of action of fungistatic chemicals and resistance development has certain disadvantages, whereas the ascomycetous yeast *Saccharomyces cerevisiae* provides an ideal test system. The latter is sensitive to most fungistatic chemicals, is a facultative anaerobe and is also amenable to both genetic and biochemical analysis. Using this organism, we have tried to understand the mechanism of action of carboxin and the development of resistance to carboxin. In this paper, we report that in yeast the target site is not the respiratory system and that the development of resistance involves increased entry of the chemical into the cells.

Materials and methods

The haploid yeast strain *S. cerevisiae,* 2180-1B (α ρ+) was obtained from the yeast stock culture centre, University of California, Berkeley, California, USA. Strain 9B (α, ρ−), a respiratory deficient strain lacking mitochondrial protein synthesis

was isolated in this laboratory (Tauro, unpublished). These cultures were maintained on yeast extract—agar slants (yeast extract, 0.5%; peptone 1%; dextrose 2% and agar 2%) by regular transfers. *Aspergillus niger* 3/1 was from the culture collection of this department and was maintained on potato dextrose agar slants. Carboxin (97% W.P.) was from M/s Uniroyal Chemical Co., Connecticut, USA. Stock solutions of this chemical were prepared in acetone and diluted as and when required. [^{14}C]-L-thereonine (sp. act 80 mCi/mmol) and [^{14}C]-uracil (sp. act. 46.7 mCi/mmol) were from Bhabha Atomic Research Centre, Bombay. For growth measurement, cells from a 12 h old slant culture were transferred to 50 ml of synthetic medium (Rose and Nickerson, 1956) in 250 ml conical flasks and incubated at 30°C on a rotary shaker (230 rpm). At intervals, 5 ml.samples were withdrawn and the growth was measured by determining the absorbance at 620 nm using a Bausch and Lomb Spectrophotometer.

For the measurement of DNA synthesis, a 12 h old culture was diluted to 0.2 absorbance units in 50 ml of synthetic medium in multiple flaks with or without carboxin (200 μg/ml) and incubated at 30°C on a rotary shaker. At intervals of 30 min duplicate flasks were withdrawn and the cells were collected by centrifugation at 1500 *g* for 10 min at 4°C. The pellet was washed twice with ice cold water and the DNA extracted by the method of Clemons and Sisler (1971). The DNA content of the extract was determined colorimetrically using diphenyl amine reagent (Burton, 1956). For the measurement of RNA and protein synthesis, a 12 h old culture was diluted to 0.2 absorbance with fresh medium and 20 ml of this was treated with either 20 μCi of [^{14}C]-uracil or 20μCi of [^{14}L]-threonine (for RNA and protein synthesis respectively) in the presence or absence of carboxin (200 μg/ml) and incubated at 30° C on a rotary shaker. Samples (1 ml) were withdrawn at intervals of 10 min into tubes containing 1 ml of 10% cold tricholoroacetic acid and incubated at 4°C for 6 h. The tubes were then centrifuged at 1500 *g* for 10 min at 4°C and the pellet was washed thrice with a total of 25 ml of 5% cold trichloroacetic acid containing either excess of cold uracil or L-threonine. The pellets were finally suspended in 1 ml of 10% trichloroacetic acid and transferred to scintillation vials containing 10 ml of Bray's scintillation fluid (Hash, 1972). The radioactivity of the sample was determined using an automatic Beckman Liquid Scintillation counter.

Residual carboxin in the growth medium was assayed colorimetrically using the method of Lane (1970) and by bioassay using *A. niger* 3/1. For bioassay, spores were inoculated into cell-free spent medium after removal of yeast cells and determining growth by visible observations. The ultraviolet and infrared spectra of the residual chemical in the growth medium were determined by extracting with chloroform and using a Beckman automatic recording spectrophotometer (Model 25) or an infrared spectrophotometer respectively.

Results

Effect of carboxin on yeast growth

Figure 1 shows the growth of a respiratory component(ρ^+) and a respiratory deficient (ρ^-) strain of *S. cerevisiae* in the presence or the absence of carboxin.

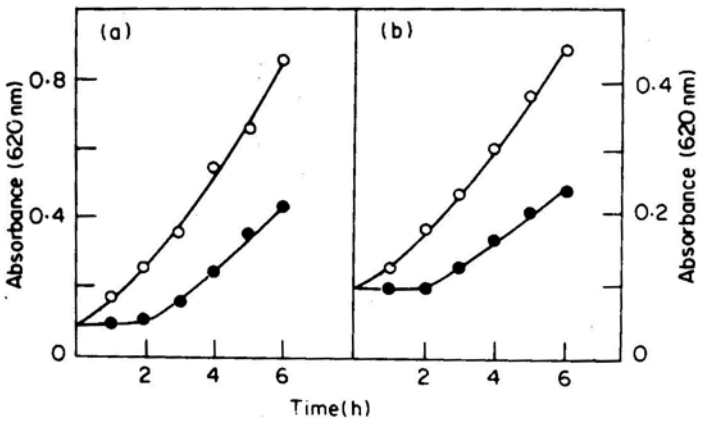

Figure 1. Effect of carboxin on the growth of *S. cerevisiae.* a. *S. cerevisiae* 2180-1B ρ+; b. *S. cerevisiae* 9 Bρ⁻. Control (○); + carboxin (200 µg/ml) (●).

At a concentration of 200 µg/ml carboxin (maximum water solubility level) growth was inhibited in both strains for about 120 min, after which it resumed at a slower rate than in control. The pattern of inhibition was similar both in the respiratory deficient and competent cells suggesting that the respiratory site might not be the target for carboxin in yeast. Therefore in subsequent studies, only strain 9 Bρ⁻ was used.

Effect of carboxin concentration on the duration of growth inhibition

To determine if the duration of inhibition was dependent on the concentration of the chemical, cells were inoculated into media containing 0, 50, 100 and 200 µg carboxin/ml and incubated as before (figure 2). It was found that the duration of

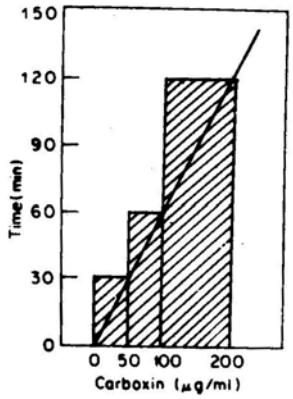

Figure 2. Effect of carboxin concentrations on the period of growth inhibition.

growth inhibition was dependent on the concentration of the chemical in the medium suggesting that the transport of this chemical into the cells occurred perhaps through a passive diffusion process which was concentration dependent.

In later experiments, unless otherwise stated, a concentration of 200 μg of carboxin/ml was used.

Effect of carboxin on macromolecular synthesis

To determine the mechanism of growth inhibition and the exact site of action, the synthesis of DNA, RNA and proteins in the presence and absence of carboxin was determined (figure 3). During the first 60-120 min of exposure to carboxin, DNA

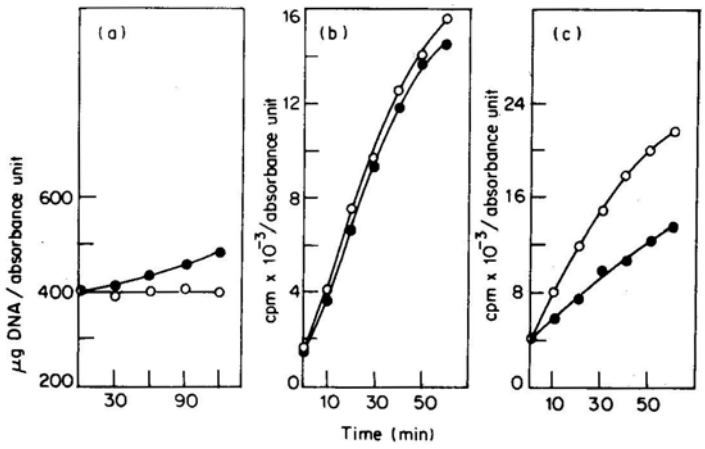

Figure 3. Effect of carboxin on macromolecular synthesis in *S. cerevisiae* 9B ρ⁻; a. DNA synthesis; b. RNA synthesis; c. Protein synthesis. Control (O); +carboxin (200 μg/ml) (●).

synthesis continued without an increase in absorbance (growth); RNA synthesis was normal while protein synthesis was reduced by about 40-50%. It therefore appeared that the site of action of carboxin might be protein synthesis and the inhibition of growth could have been mainly due to the temporary inhibition of protein synthesis.

Mechanism of resumption of growth

In the presence of 200 μg carboxin/ml growth was inhibited for about 120 min after which it was resumed at a slower rate. The resumption of growth in the presence of carboxin could occur (i) as a result of autodegradation or biodegradation of the chemical or (ii) structural alteration leading to the formation of a nontoxic compound or (iii) due to the development of cellular impermeability (Dekker, 1977). To determine as to which of the above mechanisms was responsible to allow growth in the presence of carboxin, a variety of tests were conducted.

To test for autodegradation, uninoculated flasks containing the chemical were incubated for varying periods of time beyond 2 h and later inoculated with yeast culture and the pattern of growth determined. It was found that the growth was similar to that reported in figure 1 indicating that the chemical does not undergo autodegradation (data not given).

To test for biodegradation or structural alterations leading to detoxification, the culture was centrifuged after the resumption of growth and the clear supernatant was used for determining the level of carboxin colorimetrically or by bioassay. It was found that about 95.5% of the chemical was still present in the growth medium even after 4 h of growth. Further, the ultraviolet and infrared spectra of the chloroform extracted chemical were identical with that of the authentic sample. Also, inoculation of the spent medium with a fresh yeast culture after 3 h of growth showed a pattern of growth similar to that in figure 1 (data not presented). All these results allow us to conclude that the chemical does not undergo any change but that the resumption of growth apparently is due to cellular alteration leading to a decreased permeability to this chemical which allows resumption of protein synthesis.

Specificity of resistance

To determine if the resistance developed against carboxin is specific or nonspecific, cells grown in medium containing carboxin for 4 h were centrifuged and transferred to a medium containing oxycarboxin, a close analogue of carboxin (figure 4). It was seen that cells exposed previously to carboxin were unable to resume growth immediately (figure 5) suggesting that the resistance was specific only to carboxin.

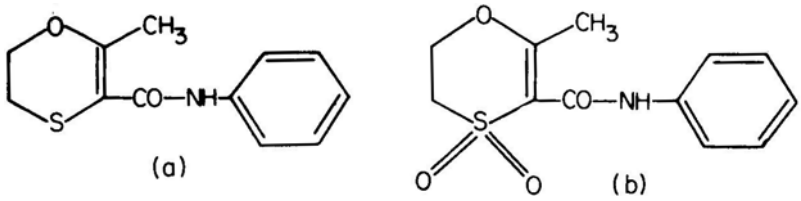

Figure 4. Structure of carboxin and oxycarboxin; a. Carboxin (vitavax): 2,3, dihydro-5-carboxanilido-6-methyl l,4 oxathiin. b. Oxycarboxin (plant vax): 2,3-dihydro-5-carboxanilido-6-methyl 1,4 oxathiin 4,4-dioxide.

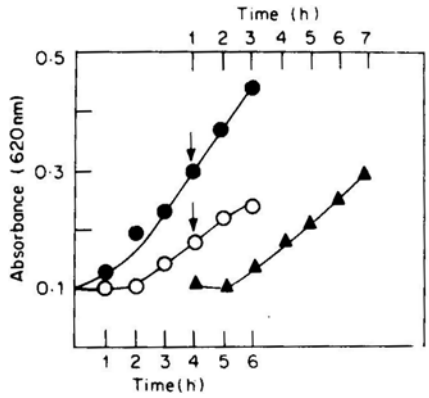

Figure 5. Test for cross resistance against oxycarboxin. Control (○); +carboxin (200 µg/ml) (●); cells transferred from either control or plus carboxin to fresh medium containing oxy-carboxin (the arrows indicate the time of transfer), (▲).

Induction of resistance

If the resumption of growth in the presence of carboxin is due to cellular alteration induced by carboxin, then cells exposed to carboxin for various time intervals and retransferred to fresh carboxin medium should show a proportionate reduction in the period of the growth inhibition. To test this, cells from carboxin medium were centrifuged after 1 and 2 h of exposure and resuspended in fresh carboxin medium (figure 6). As expected, cells exposed to carboxin previously showed a

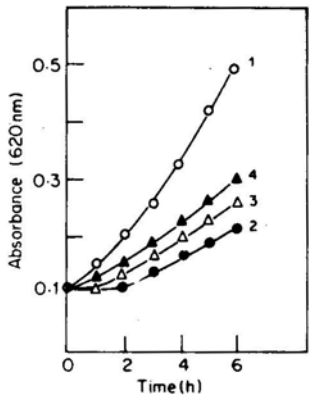

Figure 6. Induction of resistance to carboxin. Control (○); +carboxin (●); cells exposed to carboxin for 1 h and transferred to fresh carboxin medium (Δ); similar to 3 but cells exposed to carboxin for 2 h (▲). The concentration of carboxin was 200 µg/ml.

proportionate decrease in the duration of inhibition before growth resumed, indicating that the mechanism which allows growth is induced within the first 120 min of exposure to the chemical.

To determine the period for which the induced cells are resistant to carboxin, the cells were grown in carboxin medium for 12 h and then transferred to carboxin free medium. At various intervals the samples were withdrawn, centrifuged and retransferred to carboxin medium. As a control, the carboxin grown cells were directly transferred to fresh carboxin medium without exposing to carboxin free medium (figure 7). It was found that the ability to grow in the presence of carboxin

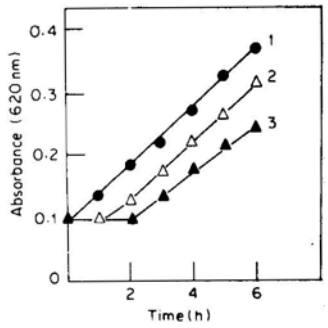

Figure 7. Test for the nature of induced resistance. Carboxin grown cells were retransferred to fresh carboxin medium (●); carboxin grown cells were exposed to carboxin free medium for 30 min and retransferred to fresh carboxin medium (Δ); similar to 2 but cells were exposed to carboxin free medium for 1 h (▲). The concentration of carboxin was 200 µg/ml.

was lost within 60 min of growth in its absence suggesting that the cellular alteration is conditional and transient.

Discussion

Our studies using respiratory deficient yeast mutant demonstrate that carboxin inhibits growth in ascomycetous yeast primarily by affecting protein synthesis. Alternate mechanisms in other mycelial fungi cannot be completely ruled out.

The resumption of growth in the presence of carboxin was apparently due to a specific alteration that affected the cell permeability to carboxin. Estimation of residual carboxin in growth medium indicated that more than 95% of the chemical was not metabolised. The rate of transport was altered after about 2-3 h of yeast growth in the carboxin medium (Tauro, unpublished data). Examples of such cellular alterations induced by chemicals were not reported in fungi. In *Staphylococcus aureus,* growth in the presence of tetracycline was reported to occur by a similar mechanism involving the synthesis of specific proteins (Levy *et al.*, 1978). Such mechanisms may be quite common in pathogenic fungi and may be induced by both fungistatic and fungicidal chemicals. We have preliminary evidence to show that such cellular alterations leading to decreased permeability to the chemical occur at the level of the cytoplasmic membrane and such mechanisms are under genetic control.

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