same, structural elucidation is necessary to establish that the active factors(s) of both sexes are identical substances.

It is known that maturation of surf clam oocytes can be induced with higher concentrations of KCl[3]. However, the concentration of KCl necessary for inducing 100% GVBD was 45 mM both in the presence and absence of the body fluid. Thus, it is evident that the body fluid factor specifically potentiates serotonin action but not KCl action in the induction of GVBD.

The activity of body fluid in enhancing the serotonin-induced GVBD remained stable after exposure to heat, acid or base. No loss of the activity was detected after heating at 100 °C for 30 min in NSW, in 0.1 N HCl or in 0.1 N NaOH. Activity was retained after treatment with proteolytic enzymes, 0.07% trypsin or 0.2% pronase, at 30 °C for 1 h. The activity was stable in 95% ethanol.

The factor was retained after dialysis in tubing with a molecular weight cutoff of 1000 daltons and was partially lost after dialysis in tubing with a molecular weight cutoff of 10,000 daltons. The activity was completely adsorbed on active charcoal and partially recovered from the charcoal by treatment with acetone or 95% ethanol containing 1 N HCl. This finding indicates the highly hydrophobic nature of the body fluid factor. The present evidence suggests that the serotonin-potentiating factor in the surf clam body fluid is neither a peptide nor an inorganic ion.

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Protective effect of vitamins against trichothecene toxicity towards *Saccharomyces cerevisiae*

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Summary. Several trichothecene mycotoxins were shown to inhibit the growth of *Saccharomyces cerevisiae*. This effect was most pronounced with the macrocyclic trichothecenes, especially verrucarin A. Much less growth inhibition was observed with T-2 toxin. Verrucarol, diacetoxyscirpenol, acetyl T-2 toxin, HT-2 toxin, T-2 tetraol and neosolaniol were inactive at a concentration of 75 µg of toxin per disc. Incubation of *S. cerevisiae* with verrucarin A together with vitamins resulted in a decrease in toxicity. Pyridoxine-HCl, Ca-pantothenate, thiamine-HCl and α-tocopheryl acetate were amongst the most potent of the vitamins tested which reversed growth inhibition, overcoming the inhibitory potential of the toxins.
Key words. Saccharomyces cerevisiae; trichothecenes; mycotoxins; vitamins.

Trichothecenes are a group of mycotoxins, produced by soil fungi, which contain 12, 13-epoxytrichothec-9-ene[3]. These toxins, widely distributed in nature, can cause tremendous damage to humans, animals and plants[3-6]. Their principal mode of action in eucaryotes is inhibition of protein synthesis[7]. Recent studies in our laboratory have suggested that these toxins are also active at the level of the cell membrane. T-2 toxin inhibited platelet aggregation[8], and phagocytosis by polymorphonuclear cells[9], and induced hemolysis in human red blood cells[10]. Acetyl T-2 toxin markedly reduced the intracellular pool size of soluble low molecular weight precursors in *Mycoplasma gallisepticum* during biosynthesis of macromolecules[11].

Schoental suggested that alkylation of coenzymes by the trichothecenes may be the biochemical basis of the effect of these hepatotoxins[12]. It was further suggested that B-vitamins may prevent acute liver lesions induced by these agents[12, 13]. Recently, we showed that, in vitro, α-tocopherol is effective in protecting erythrocytes against the hemolysing effect of T-2 toxin[10]. As vitamins B_1, B_3 and B_6 are essential constituents of coenzymes, their concentration may be of crucial importance in determining the toxicity of xenobiotic agents. This is supported by observations that the effects of the trichothecene mycotoxins are more acute in individuals suffering from malnutrition and B-vitamin deficiency[4].

Because of the severe health hazards posed to humans and animals by the trichothecenes, we thought it important to examine the influence of potential protective agents against their toxic actions. Trichothecenes are cytotoxic and cytostatic to several types of normal and malignant cells[5]. In view of their extreme toxicity, we selected a model system (the yeast *S. cerevisiae*) which would be extremely sensitive to the toxic effect of trichothecenes. Our main purpose was to screen for compounds that would inhibit the effect of these toxic agents. The information provided by such a study should prove of value in the planning of a rational treatment program for trichothecene intoxication. To obtain some insight regarding the beneficial effects of vitamins and other reducing agents against verrucarin A toxicity, we tested their influence on the growth initiation period in *S. cerevisiae*.

Materials and methods. Strains. Saccharomyces cerevisiae (bakers yeast, listed as cat. No 15-6250), and *Crithidia fasciculata* LPS 295 were obtained from the Carolina Biological Supply Company, Barlington, N.C., USA. *Ochromonas danica* (listed as cat. No 933/2) came from the Culture Collection of Algae and Protozoa, University of Cambridge, U.K. *Media. S. cerevisiae* was grown in a Sabouraud dextrose medium, (g per 100 ml): neopeptone, Difco, 1; dextrose 4; with or without agar 1.5. *C. fasciculata* was grown in the medium described by Wertlieb and Guttman[14], with or without 1.5%

agar. *O. danica* was grown in the following medium (g per 100 ml): lactalbumin hydrolysate, 0.3; glucose, 0.1; yeast extract, 0.2; pH 6; with or without agar, 1.2.

Materials. Vitamins were purchased from the Nutritional Biochemical Co., Cleveland, Ohio, USA; reduced glutathione from Merck, Darmstadt, Germany; 2-mercaptoethanol from Fluka AG, Buchs, Switzerland; yeast extract from Biolife Italiana, Milano, Italy; heart infusion broth from Difco, Detroit, Michigan, USA. Verrucarin A and roridin A were obtained as a gift from Makor Chemicals, Israel.

Preparation of the trichothecenes. T-2 toxin (4β,15-diacetoxy-3α-hydroxy 8α-(3-methylbutyrloxy)-12, 13-epoxytrichothec-9-ene), diacetoxyscirpenol (DAS) (4β,15-diacetoxy-3α-hydroxy-12,13-epoxytrichothec-9-ene) and neosolaniol (8-hydroxy-diacetoxyscirpenol) were isolated from *Fusarium sporotrichioides* grown on wheat, as previously described[6]. Its derivatives, HT-2 toxin (15-acetoxy-3α, 4β-dihydroxy-8α (3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene), T-2 tetraol (3α, 4β, 8α, 15-tetrahydroxy-12,13-epoxytrichothec-9-ene) and acetyl T-2 toxin (3α-4β,15-triacetoxy-8α- (3-methylbutyryloxy) -12,13-epoxytrichothec-9-ene) were synthesized from T-2 toxin in our laboratory according to the published procedure[15]. Verrucarol (4β 15-dihydroxy-12,13-epoxytrichothec-9-ene) was prepared by hydrolysis of verrucarin A[16].

Test of growth inhibition. a. *Agar diffusion method.* The 24-h culture of *S. cerevisiae* was diluted with physiological saline solution to a slight turbidity (10 Klett units) and thereafter used for inoculation. The 72-h *C. fasciculata* culture was diluted to 50 Klett units turbidity and used for inoculum. The 5-day culture of *O. danica* was used undiluted for the inoculum. The agar plates were flooded with the appropriate inoculum and after 5 min, the excess liquid was withdrawn. The toxins were dissolved in ethyl alcohol (EtOH, 1 mg/ml). In each experiment, 13-mm Whatman AA discs were impregnated with 75 µg of a toxin and placed on the agar, and the plates were incubated at 27°C. Zones of growth inhibition were monitored between 24 and 72 h[17].

b. *Tube method (liquid medium).* Verrucarin A, roridin A and T-2 toxin were dissolved in EtOH 1 mg/ml and added to the tubes with *S. cerevisiae*. For determining the minimal inhibitory concentration (MIC) 2-fold serial dilutions were carried out[17]. The tubes were incubated for 20 h at 27°C and the growth was determined. The incubation volume was 2 ml and the final concentration of EtOH in the medium fluctuated between 1 and 5%. Ethanol itself at this concentration was inactive.

For testing the protective effect of various vitamins and other substances, against growth inhibition of *S. cerevisiae*, the following method was used:

To the Klett tubes with 1 ml of *S. cerevisiae* in liquid medium, 10 µg of verrucarin A in 10 µl of EtOH was introduced. One of the following protective substances was added to each tube (mg per tube): pyridoxine, 2; Ca-pantothenate, 2; thiamine, 10; d,l-α-tocopheryl acetate, 0.25; nicotinamide, 2; inositol, 10; ascorbic acid, 10; heart infusion broth, 2; yeast extract, 2; cystein, 1; glutathione, 2; 2-mercaptoethanol, 0.1. The tubes were incubated at 27°C for 10 days. They were inspected every day. The first day of the appearance of growth (above 10 Klett units) was recorded as the day of growth initiation. Media with ethanol and the above protective agents served as controls. All experiments were carried out in duplicate. The experiments were repeated three times.

Growth measurements. Cultures were grown in Klett test tubes. Growth was determined each day by reading the turbidity at 640–700 nm on a Klett-Summerson Photoelectric Colorimeter, using filter 66. After take-off (10 Klett units) the cultures generally got to their maximal growth (190–220 Klett units) within 3–4 days. Turbidity of the controls after the same period was 240–250 Klett units.

Results. The table shows that verrucarin A was the most potent inhibitor of *C. fasciculata* and *S. cerevisiae* growth. Roridin A and T-2 toxin were much less effective. Verrucarol, diacetoxyscirpenol, acetyl T-2 toxin, HT-2 toxin, T-2 tetraol and neosolaniol were inactive at a concentration of 75 µg of toxin per disc. None of the above-tested toxins affected the growth of *O. danica*.

In order to establish more precisely the effects of verrucarin A, roridin A and T-2 toxin on the growth inhibition of *S. cerevisiae*, the tube method was used. The MIC values of these toxins in µg/ml were 1.5, 8 and 50, respectively (table).

The most active vitamins in overcoming growth inhibition of *S. cerevisiae* by verrucarin A, were pyridoxine, after 5 days, and Ca-pantothenate, thiamine and d,l-α-tocopheryl acetate, after 6 days. Nicotinamide overcame the growth inhibition after 7 days, inositol after 8 days, and ascorbic acid was inactive. Two extracts containing a mixture of vitamins were tested as well. Heart infusion broth initiated growth after 7 days, and yeast extract after 9 days. Cysteine and glutathione initiated growth after 8 days of incubation. 2-mercaptoethanol was inactive at the concentration used in our experiment.

Discussion. S. cerevisiae shows a remarkable sensitivity to the inhibitory effect of verrucarin A; concentrations of 1.5 µg/ml completely suppressed its growth. The inhibitory effects of the macrocyclic trichothecenes verrucarin A and roridin A are much more pronounced than that observed with the non-macrocyclic trichothecenes (table). Recently, it was reported that the growth of *S. cerevisiae* was inhibited by 100 µg/ml of T-2 toxin[18]. However, this toxin was reported as not being bacteriostatic at a concentration of 50 µg per disc when tested on 54 bacterial strains[19]. In the present study, only verrucarin A had a significant inhibitory effect on *C. fasciculata* and *S. cerevisiae* (table). Verrucarin A and the other tested trichothecenes were ineffective on the algae *O. danica* in concentrations of up to 75 µg/disc.

The amounts of toxin required for complete growth inhibition of *S. cerevisiae* were found to be: 1.5 µg/ml for verrucarin A, 8.0 µg/ml for roridin A and 50 µg/ml for T-2 toxin, giving a ratio of 33:6:1 for their irrespective inhibitory potencies (table). The greater inhibitory effect of the macrocyclic trichothecenes could be related either to their rapid penetration through the cell wall and membrane or to their accumulation within the cell at a concentration sufficient to exhibit toxic effects.

Certain vitamins, like tocopheryl acetate, pyridoxine or the other B-complex vitamins, enhance the initiation of yeast growth in the presence of verrucarin A. These data are in line with the previously suggested hypothesis that the toxins destroy certain coenzymes and that the addition of excess vitamin enables the cells to reverse this damage[4,12,18]. The fact that different vitamins differ in their overall effectiveness

Effect of various trichothecene mycotoxins on the growth of *S. cerevisiae* and *C. fasciculata*[a]

Microorganism[b]	Toxin[c]	Zones of growth inhibition (mm)[c]	MIC[d] (µg/ml)
S. cerevisiae	Verrucarin A	21 (± 1.4)	1.5 (± 0.3)
	Roridin A	15 (± 0.5)	8.0 (± 2.8)
	T2-toxin	14 (± 0.5)	50.0 (± 4.8)
C. fasciculata	Verrucarin A	22 (± 1.4)	-
	Roridin A	15 (± 1.4)	-
	T2-toxin	14 (± 0.3)	-

[a] Zones of growth inhibition were monitored (disc method) after incubation at 27°C during 24 h for *S. cerevisiae* and during 24–48 h for *C. fasciculata*. [b] *O. danica* was not affected by the tested toxins at 75 µg/disc. [c] All 13-mm discs were impregnated with 75 µg toxin in each experiment. Verrucarol, DAS, acetyl T-2 toxin, HT-2 toxin, T-2 tetraol, neosalaniol were ineffective at this concentration. [d] MIC-minimum inhibitory concentration.

may imply that some coenzymes are more sensitive to destruction or that they are more important for cell metabolism.

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Occurrence of thiaminase II in *Saccharomyces cerevisiae*[1]

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Summary. It was found that cell-free extracts of *Saccharomyces cerevisiae* contain thiaminase II which hydrolyzes thiamine and thiamine analogs. The possible involvement of this enzyme and thiamine-synthesizing enzymes in thiamine production from thiamine antagonists is discussed.
Key words. Thiaminase; thiamine; thiamine antagonist; *Saccharomyces cerevisiae.*

Saccharomyces cerevisiae can grow in the presence of two thiamine antagonists, pyrithiamine and oxythiamine, although its growth is inhibited by each antagonist alone[2]. This phenomenon was explained as being due to thiamine production from pyrithiamine and oxythiamine, which was demonstrated using cell suspensions and a crude extract of *S. cerevisiae*[2]. However, the precise enzymatic mechanism of thiamine synthesis from these two thiamine antagonists in yeast cells remained to be established, although 2-methyl-4-amino-5-hydroxymethylpyrimidine(hydroxymethylpyrimidine), the pyrimidine moiety of pyrithiamine, and 4-methyl-5-β-hydroxyethylthiazole (hydroxyethylthiazole), the thiazole moiety of oxythiamine, are supposed to be precursors for thiamine synthesis.

We report in this communication that cell-free extracts of *S. cerevisiae* contain an enzyme hydrolyzing thiamine antagonists as well as thiamine, which gives the first evidence for the existence of thiaminase II (EC 3.5.99.2) in *S. cerevisiae*. Although thiaminase II has been reported from various bacteria[3], its physiological function is unknown. We also describe here that a recently isolated yeast mutant[4] deficient in hydroxyethylthiazole kinase (EC 2.7.1.50) cannot grow with pyrithiamine and oxythiamine, which suggests that, in addition to thiaminase II, other enzymes are involved in the reversal of pyrithiamine-induced growth inhibition of *S. cerevisiae* by oxythiamine.

S. cerevisiae was grown in 10 l of thiamine-free Wickerham's synthetic medium[2] for 15 h at 30 °C with shaking, harvested, and washed once with distilled water. The washed yeast cells were suspended in 20 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM 2-mercaptoethanol and 1 mM EDTA, sonicated for 20 min at 10 kc, and then centrifuged at 15,000 × g for 20 min. The supernatant solution was treated with $^1/_{10}$ volume of 0.8% protamine sulfate solution, and the suspension was centrifuged at 15,000 × g for 20 min. The supernatant solution was then brought to 40% saturation with solid ammonium sulfate. The precipitate formed was removed by centrifugation at 15,000 × g for 20 min, and the supernatant solution was brought to 80% saturation by the addition of ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 10 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM 2-mercaptoethanol and 1mM EDTA. The solution was dialyzed against the same buffer and it was used as enzyme

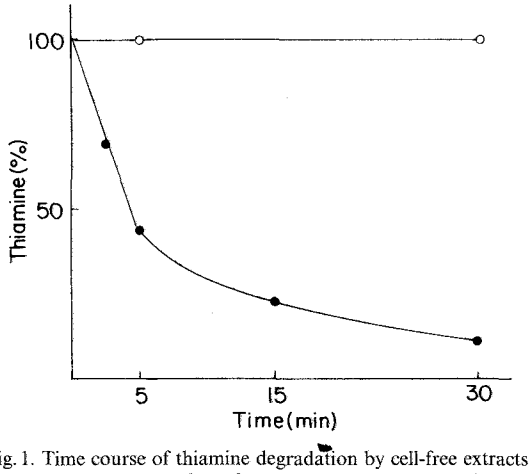

Fig. 1. Time course of thiamine degradation by cell-free extracts of *Saccharomyces cerevisiae:* ●—●, complete; ○—○ complete but boiled enzyme.