# Effect of Aflatoxin $B_1$, Ochratoxin and Rubratoxin B on a Protozoan, *Tetrahymena pyriformis* HSM

by

A. WALLACE HAYES, RENA MELTON, and SHELLEY J. SMITH

*The Department of Microbiology*
*The University of Alabama*
*University, Ala. 35486*

Mycotoxins, which are secondary fungal metabolites, are toxic to a number of animal species (WILSON and HAYES 1973). Although several mycotoxins are elaborated by species of Aspergilli, none outranks the aflatoxins in importance. The aflatoxins are a group of acutely toxic and highly carcinogenic mold metabolites produced by Aspergillus flavus and related Aspergilli which grow on foodstuffs when moisture is sufficient (GOLDBLATT 1969). Aflatoxin is a generic term designating some twelve brightly fluorescing furanocoumarin compounds of which aflatoxin $B_1$ is the prototype. South African workers first isolated ochratoxin A, a greenish-blue fluorescing dicoumarin-phenylalanine complex which produces both liver and kidney damage, as a metabolite of A. ochraceus grown artificially on maize (MERWE et al 1965). It has been identified as a metabolite of Penicillium viridicatum (SCOTT 1965) and has been reported as a natural contaminant of foods (SCOTT 1967, WALBEEK et al 1969). Rubratoxin B, also an hepatotoxin, has been isolated from P. rubrum (HAYES and WILSON 1968) and P. purpurogenum (NATORI et al 1970).

Some information is available on the effect of the aflatoxins on microorganisms, but few data concerning either ochratoxin or rubratoxin have been reported. TEUNISSON and ROBERTSON (1967) reported that aflatoxin $B_1$ and $G_1$ were degraded by Tetrahymena pyriformis but neither compound inhibited growth. HAYES and WYATT (1970) reported that T. pyriformis was the most sensitive to rubratoxin B of the more than 300 microorganisms tested.

The aim of the present study was to investigate the effect of these mycotoxins on growth and cell respiration of T. pyriformis. In addition, a bioassay for rubratoxin B employing this ciliated protozoan is reported.

## MATERIALS AND METHODS

Rubratoxin B was isolated from P. rubrum (HAYES and WILSON 1968). Aflatoxin $B_1$ was purchased from Calbiochem, Los Angeles, Calif. Ochratoxin was supplied by Dr. Alex Ceigler, Northern Regional Laboratory, Peoria, Ill. and contained 93% ochratoxin A and 7% ochratoxin B. Mycotoxins were stored at 4° C in the dark.

Stock cultures of Tetrahymena pyriformis HSM were maintained at 30±1 ° C in the dark and were transferred every three weeks. The culture medium consisted of 1.5% proteose-peptone (Difco) and 0.1%



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liver fraction L (Nutritional Biochemicals Corp., Cleveland, Ohio) dissolved in distilled water. Growth of T. pyriformis was estimated at 3-hr intervals from 0 to 60 hr turbidimetrically at 660 NM in a Coleman Jr. spectrophotometer or by direct cell count. Cell counts were determined in a Bright Line Hemacytometer (36 $mm^2$ area, 100 X magnification). Cell movement was reduced by adding an aliquot of methanol (1:8 v/v) to the cell suspension immediately before counting. Rubratoxin B was dissolved in phosphate buffer, pH 7.0; aflatoxin $B_1$ and ochratoxin, in dimethylsulfoxide (DMSO).

Effect of mycotoxins on growth. Nephelometer flasks (500 ml) containing 50-ml broth were autoclaved for 15 min at 121° C. Different concentrations of the three mycotoxins followed by a 3% suspension of T. pyriformis cells were added after the medium cooled to room temperature. The following toxin concentrations were tested: aflatoxin $B_1$ and ochratoxin, 25, 50 and 100 µg/ml; and rubratoxin B, 5, 10, 25, 50 and 100 µg/ml. Experimental variables were in triplicate and the following protocol was observed: (1) uninoculated controls; (2) inoculated controls; (3) inoculated-solvent controls; and (4) inoculated solvent system plus toxin.

Effect of mycotoxins on respiration. Changes in respiration after exposure of T. pyriformis to each mycotoxin were measured by oxygen consumption in a Gilson differential respirometer (UMBREIT et al 1972). The rate of oxygen uptake was measured as µl/hr/mg dry weight at 30° C. Cells were incubated in a 150-ml culture medium for 48 hr at 30±1° C in the dark. A cell count was made on a 1-ml aliquot before cells were harvested by centrifugation. Cells were resuspended in a 10-ml broth to give approximately 1.5 X $10^4$ cell/ml. The main chamber of each respirometer flask contained 0.3 ml of cell suspension in phosphate buffer, pH 7.2, and 1.3 co-factor solution (0.001 M $NAD^+$ and 0.01 M $MgCl_2$ in phosphate buffer, pH 7.2). Toxin in 0.1 ml solvent or solvent alone was pipetted into the side arm of the reaction flask. The toxin concentrations were: aflatoxin $B_1$ and rubratoxin B, 25, 50, 100, 200 and 400 µg/ml and ochratoxin, 100, 200 and 400 µg/ml. The final volume in the main chamber of each respirometer flask was 3.0 ml. The initial and final pH values of the reaction mixture were 7.2 and 6.8, respectively. The center well contained 0.2 ml of 15% KOH and a small piece of filter paper to increase the surface area for $CO_2$ absorption. Flasks were attached and the reaction mixture was allowed to equilibrate at 30±1° C for 15 min before the respirometer system was closed. The content of each side arm was determined at 10-min intervals for 3 hr.

## RESULTS AND DISCUSSION

The effect of varying the concentrations of the three mycotoxins on growth of T. pyriformis is presented in Table 1. Inhibition was greatest in the presence of rubratoxin B. There was 63% inhibition of growth with 50 µg/ml rubratoxin B whereas less than 6% inhibition was observed with the same concentration of the other two myco-

toxins. Even at the highest ochratoxin concentration (100 µg/ml) tested, inhibition of T. pyriformis growth was less than 1%. An earlier report (TEUNISSON and ROBERTSON 1967) indicated that aflatoxin $B_1$ was degradated by T. pyriformis; however, our thin-layer chromotography studies revealed substantial amounts of aflatoxin $B_1$ after 60-hr incubation with T. pyriformis.

TABLE 1

Effect of Mycotoxins on Tetrahymena pyriformis Growth [a]

| Concentration µg/ml | % Inhibition [b] | | |
|---|---|---|---|
| | Aflatoxin $B_1$ | Ochratoxin | Rubratoxin B |
| 5 | - [c] | - | 3.7 |
| 10 | - | - | 6.1 |
| 25 | 0.7 | 0.5 | 31.8 |
| 50 | 5.5 | 0.2 | 63.6 |
| 100 | 14.7 | 0.5 | 97.9 |

[a] Mycotoxin was added at 0 time; based on cell counts at 48 hr.

[b] Average of three separate experiments.

[c] Experiment not done.

The chelating potential of rubratoxin B as related to toxicity was investigated by determining if divalent metal ions altered the inhibitory effect of rubratoxin B on T. pyriformis growth. Salts of three divalent ions ($Mg^{++}$, $Ca^{++}$ and $Fe^{++}$) sufficient to complex with 50 µg/ml rubratoxin B were dissolved singly or in combination in the culture medium. No alteration in toxicity of rubratoxin B was noted in the presence of any combination of these divalent ions (data not presented).

Data which summarize the effect of the three mycotoxins on cell respiration in 48-hr cultures of T. pyriformis are shown in Table 2. Maximum decreases in respiration of 12.6 and 6.5% were observed at 2.5 hr in 400 µg/ml aflatoxin $B_1$ and ochratoxin, respectively. A 20% decrease in respiration was manifested by 100 µg/ml rubratoxin B. In addition, cultures of T. pyriformis treated with 50 µg/ml of each

toxin at time zero were examined after 20- and 48-hr growth for changes in respiration. No effect was noted. The data indicate that these mycotoxins induced little effect on cell respiration in this ciliated protozoan at toxin concentrations below the 50% growth inhibition level.

TABLE 2

Effect of Mycotoxins on Cell Respiration

| Concentration µg/ml | % Inhibition (At 2.5 hr) | | |
|---|---|---|---|
| | Aflatoxin $B_1$ | Ochratoxin | Rubratoxin B |
| 25 | 4.4 | -[a] | N I[b] |
| 50 | 5.1 | - | N I |
| 100 | 5.7 | - | 20.0 |
| 200 | 9.8 | 4.2 | 22.2 |
| 400 | 12.6 | 6.5 | 19.4 |

[a] Experiment not done

[b] No inhibition (< 1.0%)

A biological assay for rubratoxin B employing T. pyriformis is presented in Fig. 1. A linear relationship between concentration and cell number was obtained when cells were counted at 12 hr. The lower limit of rubratoxin B detection by this bioassay is in the range of 1 to 5 µg/ml.

## SUMMARY

Rubratoxin B is more inhibitory to the growth of T. pyriformis than is either aflatoxin $B_1$ or ochratoxin. Only a marginal effect on cell respiration was exerted by these mycotoxins. The presence of the divalent ions, $Mg^{++}$, $Ca^{++}$ and $Fe^{++}$, did not reduce the toxicity of rubratoxin B. A bioassay sensitive to 1 to 5 µg/ml rubratoxin B has been developed employing T. pyriformis.

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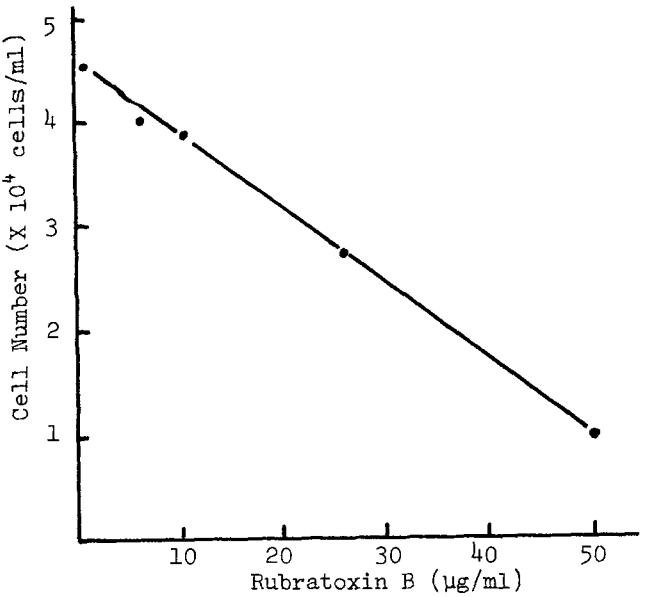


Fig. 1 Relation of cell number to the amount of rubratoxin B. Symbols indicate average of three separate samples obtained at 12 hr from T. pyriformis cultures.

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