Antiprotozoal Activity in Citrinin

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Metabolites of molds growing on foodstuff have caused diseases in man and animals throughout recorded history; but an understanding of the relationship between the mold, the food and the disease has begun to develop only recently (NEWBERNE 1974). Citrinin is such a toxic secondary fungal metabolite; it is produced by a variety of species of Aspergillia and Penicillia (SAITO et al 1971). Yellowish colored rice imported into Japan in 1951 was found to be contaminated with a citrinin producing mold, Penicillium citrinum. Subsequently citrinin-producing organisms have been isolated from foodstuffs, particularly rice, throughout the world (TSUNODA 1970).

Studies in mice and rats have indicated that citrinin causes remarkable histological changes in the kidney which included degeneration and dilation of the lower nephrons beneath Henle's loop (SAKAI et al 1955). Increased mitoses of tubular epithelium or deposits of hematoxylin-stained amorphous material in tubules also were observed after three months of feeding.

Although SMITH (1949) reported that citrinin was antibacterial for gram-positive bacteria but not for gram-negative organisms, in general, information on the effect of citrinin on microorganisms is limited. Since Tetrahymena pyriformis earlier was shown to be a good test organism for mycotoxins (HAYES et al 1974) and in general, an ideal organism for drug action studies (CONKLIN et al 1970), this study was undertaken to investigate the effect of citrinin on growth response, nucleic acids and protein content of T. pyriformis. In addition, effects on cellular respiration and a bioassay for citrinin employing this ciliated protozoan are reported.

MATERIALS AND METHODS

The purity of citrinin, obtained from Dr. N. D. Davis, Auburn University, Auburn, AL, was established by melting point and infrared spectrum.

Tetrahymena pyriformis HSM was grown in 500-ml Nephelometer flasks (Bellco, Vineland, N.J.) containing 50 or 150 ml of 1.5% proteose-peptone (Difco) and 0.1%

Bulletin of Environmental Contamination & Toxicology,
Vol. 15, No. 4 © 1976 by Springer-Verlag New York Inc.

liver fraction L (Nutritional Biochemicals Corp., Cleveland, OH) in distilled H_2O. The medium, prepared fresh for each experiment, was autoclaved 15 min at 121°C, after which the mycotoxin was added aseptically. Working cultures were prepared by inoculating from 48-hr stationary cultures to give a 3% cell suspension containing approximately 10^4 cells/ml. Growth of stationary cultures incubated in the dark at 30 ± 1°C was estimated by turbidimetry at 660 nm in a Coleman Jr. spectrophotometer or by electronic cell count at predetermined intervals over a 60-hr period. Cell number (cells/ml) was estimated in a Coulter Counter Model Z_{BI} (Coulter Electronics, Hialeah, FL) using a 200 micron aperture tube with a 2000 γ (2 ml) manometer. The instrument was calibrated before each run to give an average threshold value of 84 μ^3 per dial division. The lower threshold was set to exclude outside electronic interference. Other control settings were: 1/aperture current = ½; 1/amplification = 4; upper threshold = off. The growth medium was diluted with Isoton prior to counting to give 10^4 cell/ml or less. Cell size determination were estimated in a Coulter Channelyzer attached to the Model Z_{BI}. The channelyzer was calibrated before each run; instrument controls were: count range = 400; base channel threshold = 5; window width = 100.

Effect of citrinin on growth. Different concentrations of citrinin followed by a 3% suspension of T. pyriformis cells were added to Nephelometer flasks after the medium (50 ml) cooled to room temperature. The following citrinin concentrations were tested: 5, 10, 15, 25, 50 and 100 µg/ml of growth medium. The toxin concentration added, unless otherwise stated, was the final amount (µg/ml) in the growth medium. The following protocol was observed: (1) uninoculated controls; (2) inoculated controls; (3) inoculated-solvent controls; and (4) inoculated solvent system plus toxin. The volume of solvent added to 50 ml of culture medium did not exceed 0.5 ml and in most cases was 0.25 ml or less. Experimental variables were in triplicate and each experiment was repeated three times.

Effect of citrinin on respiration. Changes in respiration after exposure of T. pyriformis to citrinin 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 150 ml of culture medium for 48 hr at 30 ± 1°C in the dark. A cell count was

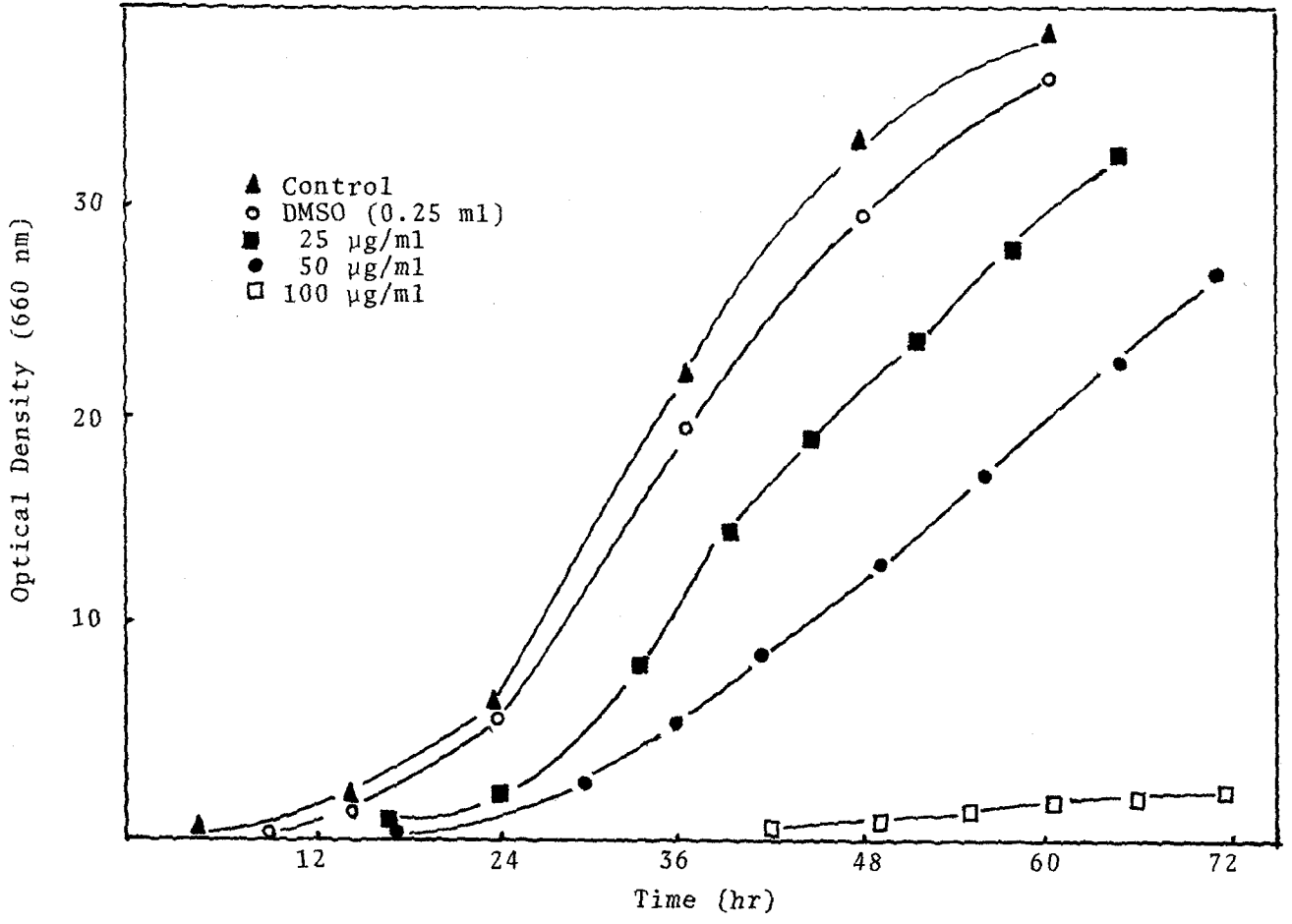
made on a 1-ml aliquot before cells were harvested by centrifugation. Cells were resuspended in 10-ml broth to give approximately 1.5 x 10^4 cell/ml. The main chamber of each respirometer flask contained 0.3 ml of cell suspension in phosphate buffer, pH 7.2, and 1.3 ml co-factor solution (0.001 M NAD^+ and 0.01 M $MgCl_2$ in phosphate buffer, pH 7.2). Toxin, 50 and 100 µg/ml, in 0.1 ml solvent or solvent alone was pipetted into the side arm of the reaction flask. 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 wall 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 transferred rapidly into the main chamber and oxygen consumption was determined at 10 min intervals for 3 hr.

Effect of citrinin on nucleic acids and protein. The effect of citrinin (5, 15 and 25 µg/ml of growth medium) on nucleic acids and protein content of T. pyriformis cells was investigated. Cells were harvested at 48 hr by centrifugation at 15,000 rpm for 15 min at 4°C. Packed cells were washed twice with phosphate buffer (pH 7.0) and recentrifuged after each wash. Cells were transferred to pretared tubes, and the wet weight of cells determined. Deoxyribonucleic acid (DNA) was estimated by the BURTON method (1956) and ribonucleic acid (RNA) was estimated by the orcinal procedure (OGUR and ROSEN 1950). Total protein after lipid extraction was determined by the procedure of LOWRY (1951).

RESULTS AND DISCUSSION

The effects of citrinin in DMSO and DMSO alone on T. pyriformis growth are shown in Fig 1. Growth was slightly inhibited by DMSO throughout the 60-hr observation period. Greater inhibition of growth, as shown by differences in optical density, however, was seen in the flasks containing citrinin. At 36 hr, 50 µg/ml of citrinin had reduced growth by 66%. At 48 hr, 100 µg/ml of citrinin had decreased growth by 97%. Inhibition of growth as shown by optical density was less for 25 µg/ml of citrinin (57% at 36 hr). Differences in optical density compared to DMSO treated cultures were negligible for lower toxin concentrations (data not shown).

Fig 1. Effect of Citrinin in Dimethylsulfoxide on T. pyriformis Growth



A similar effect on growth was observed using cell number as the index of growth. As shown in Fig 2, cell number, at any particular time in the 60-hr period, decreased with increased toxin concentration. At 36 hr, the time of greatest inhibition, growth as measured by cell number was inhibited as follows: 14.5%, 5 µg/ml; 18.6%, 10 µg/ml; 30.8%, 15 µg/ml; 37.1%, 25 µg/ml; 81.8%, 50 µg/ml; and 94.4%, 100 µg/ml.

The size distribution of cells exposed to the higher concentrations of citrinin (25, 50 and 100 µg/ml) was altered markedly. The size distributions of cells in both treated and untreated cultures remained similar through the early stages of growth (up to 24 hr). By 36 hr, however, the largest percentage of the total population of exposed cells was in the lower size ranges between 1052 m^3 and 2567 m^3. A similar distribution of cells was observed at 48 hr. Normal cells had the largest percentage of their total population between 2567 m^3 and 5052 m^3. These size differences became less pronounced by the end of the 60-hr observation period (data not shown).

Citrinin had little effect on the respiration of T. pyriformis. Maximum inhibition on cell respiration in 48-hr cultures was less than 10% even at 100 µg/ml of citrinin (data not shown).

The effect at 48 hr of citrinin on nucleic acids and protein content of T. pyriformis cells is presented in Table 1. There was little change in DNA, RNA or protein at 5 and 15 µg/ml. However, at 25 µg/ml there was a 36% decrease in DNA, a dramatic decrease of RNA (84%) and a 63% decrease in total protein.

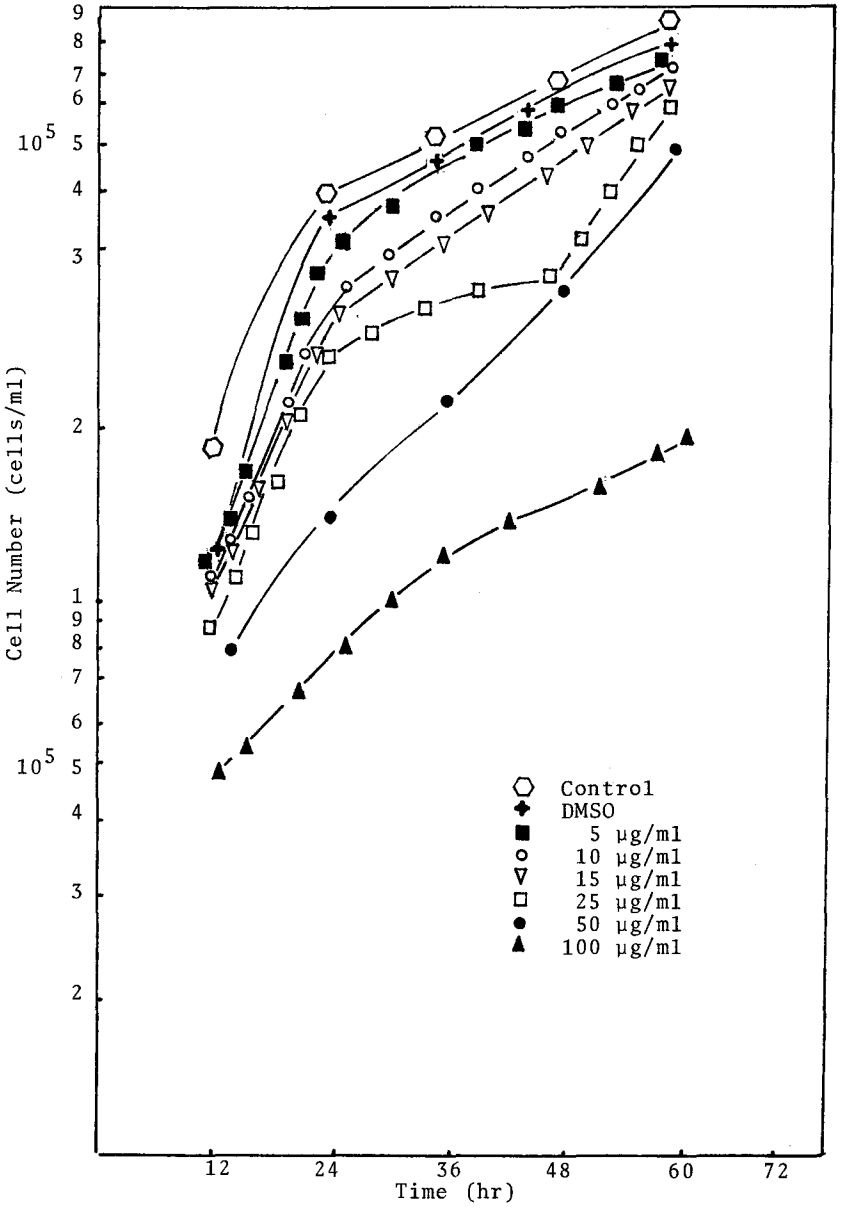
TABLE 1

Effect of Citrinin on Nucleic Acids and Protein Content of T. pyriformis cells at 48 hr[a]

Citrinin (µg/ml of growth medium)	Percent Increase (decrease)		
	DNA	RNA	PROTEIN
5	0.2	4.2	(-) 0.5
15	0.5	12.6	1.5
25	(-)36.6	(-)84.0	(-)63.9

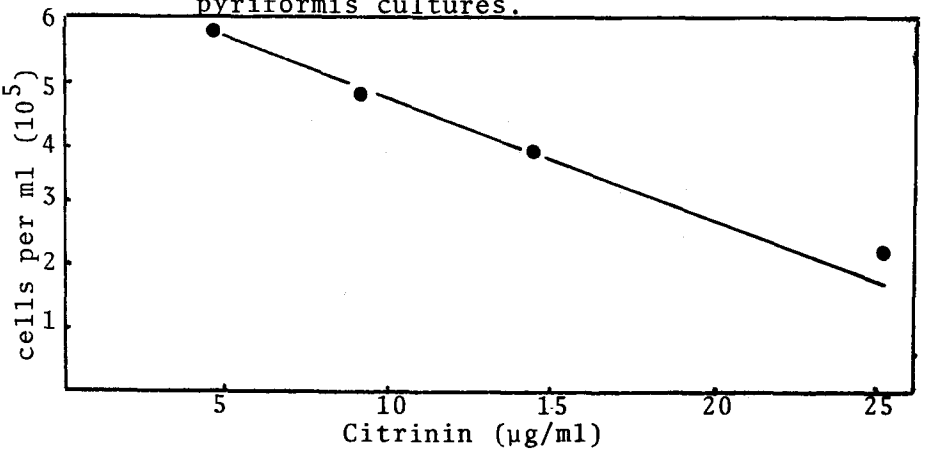
[a]A minimum of three separate experiments.

Fig 2. Effect of Citrinin on T. pyriformis Growth



A biological assay for citrinin employing T. pyriformis is shown in Fig 3. A linear relationship between toxin concentration and cell number was obtained when cells were counted at 36 hr. The lower limit of citrinin detection by this bioassay is in the range of 1 to 5 µg/ml of citrinin.

Fig 3. Relationship of Cell Number to the Amount of Citrinin. Symbols indicate average of three separate samples obtained at 36 hr from T. pyriformis cultures.



SUMMARY

Citrinin has an inhibitory effect on growth of T. pyriformis. Citrinin also caused a shift to smaller cell size, particularly at the higher concentrations (25, 50 and 100 µg/ml). The mycotoxin exerted only a marginal effect on the respiration of T. pyriformis; but citrinin (25 µg/ml) induced an inhibitory effect on DNA, RNA and protein content. The greatest decrease was in RNA, while smaller decreases in protein and DNA were observed. A bioassay employing T. pyriformis was determined; the lower limit of citrinin detection was between 1 and 5 µg/ml.

ACKNOWLEDGEMENTS

Research support was provided by U. S. Public Health Grants ES00464 and ES00674 from the National Institute of Environmental Health Sciences. Dr. Hayes is recipient of Research Career Development Award 1KO4ES 34113, U. S. Public Health Services, National Institutes of Health.

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