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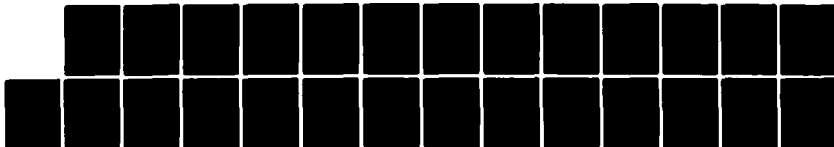
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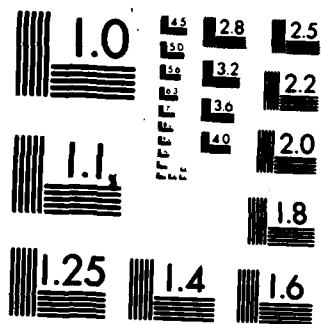
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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
	AD-A133146	
4. TITLE (and Subtitle)	5. TYPE OF REPORT & PERIOD COVERED	
Detection and Quantitation of T-2 Mycotoxin Using a Simplified Protein Synthesis Inhibition Assay	Interim	
7. AUTHOR(s)	6. PERFORMING ORG. REPORT NUMBER	
W. L. Thompson and R. W. Wannemacher, Jr.		
9. PERFORMING ORGANIZATION NAME AND ADDRESS	8. CONTRACT OR GRANT NUMBER(s)	
US Army Medical Research Institute of Infectious Diseases, SGRD-UIS Fort Detrick, Frederick, MD 21701		
11. CONTROLLING OFFICE NAME AND ADDRESS	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS	
US Army Medical Research & Development Command Office of the Surgeon General Department of the Army, Washington, D.C. 20314	S10-AQ-013	
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	12. REPORT DATE	
	18 July 1983	
	13. NUMBER OF PAGES	
	17 + 7 Figures	
	15. SECURITY CLASS. (of this report)	
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE	
16. DISTRIBUTION STATEMENT (of this Report)		
Distribution unlimited - Approved for public release		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
Reprints bearing assigned AD number will be forwarded upon receipt. To be published in Toxicology and Applied Pharmacology		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number)		
T-2 mycotoxin, protein synthesis inhibition, tissue culture cells, lymphocytes		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number)		
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Detection and Quantitation of T-2 Mycotoxin
Using a Simplified Protein Synthesis Inhibition Assay

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Running title: T-2 mycotoxin and detection and quantitation

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Abstract

A simple, rapid and sensitive bioassay is described for the detection and quantitation of T-2 mycotoxin using a protein synthesis assay in cultured cells. Increased sensitivity of the cells to the mycotoxin occurs with time up to approximately 60 min. Time and dose response curves demonstrate that an average of 10 to 20 ng/ml T-2 is sufficient to cause 50% inhibition of protein synthesis in tissue culture cells. A wide range of tissue culture cells having varied type, tissue, and species sources, and growth characteristics were tested using this system. All showed approximately the same sensitivity to the mycotoxin. Using a slight modification of the procedure, suspended cultures of mitogen-stimulated lymphocytes also demonstrated an equal degree of sensitivity to the mycotoxin. By simply changing the labeled precursor, the inhibition of RNA, DNA and protein synthesis by T-2 mycotoxin can be compared. Although T-2 has little effect on RNA synthesis, DNA and protein synthesis are equally inhibited. Because of the sensitivity and capacity to quickly assay a large number of samples, this technique has been a valuable tool in screening samples for the presence of active toxin. This assay system has been used to help establish laboratory safety standards for the inactivation of T-2 mycotoxin by chemical agents. It is presently being used in studies of mycotoxin mechanism of action and approaches toward in vivo neutralization of the toxic effects of mycotoxins.

Introduction

Numerous approaches have been used in the past for the detection and quantitation of mycotoxins. Sensitive analytical techniques such as gas chromatography and mass spectroscopy (Pathre et al, 1977) are available for qualitative as well as quantitative detection of the mycotoxins. However, they require expensive instrumentation and a great deal of time and skill in their operation. Thin layer chromatography (Ueno et al, 1973; Gimeno, 1979; Garst-Allman, 1979) can be used for determination and quantitation of mycotoxins present with the appropriate standards and the proper instrumentation such as a scanning spectrophotometer.

Radioimmunoassay (Fontelo et al, 1982) or ELISA (Pestka et al, 1981; Peters et al, 1982) techniques are two of the most sensitive methods for trichothecene detection. However, they require the presence of specific antibodies to the mycotoxin which are presently difficult to acquire in large quantity due either to the poor antigenicity or the immunosuppressive nature of the mycotoxins.

The mouse bioassay (Ueno et al, 1971) and the skin sensitivity test (Ueno et al, 1970; Chung, 1974) are effective bioassay techniques for detection, but require the use of a large number of animals and are not a very accurate means of quantitation.

Extensive studies have been conducted on the mechanism of action of the trichothecenes, some of which might serve as a means of detection. Cell-free protein synthesis using the reticulocyte lysate assay (Wei et al, 1974a; Mizuno, 1975; Cannon et al, 1976), the effects of mycotoxins on isolated ribosomes (Barbacid et al, 1974; Cannon, 1976) and even some approaches toward the use of tissue culture cells (Ueno and Yamakawa, 1970; Ohtsubo et al, 1972; Wei et al, 1974b; Cundliffe et al, 1977, Olkham et al, 1980) are a few

examples. However, these techniques are either difficult, time consuming, or limited in the number of samples which can be tested, and quite often lack sensitivity.

In this paper we describe a simple but functional bioassay technique involving protein synthesis inhibition adapted from studies on diphtheria and pseudomonas exotoxins (Middlebrook et al, 1976a and 1976b) for the detection and quantitation of T-2 mycotoxin. The only requirement is a tissue culture cell line or any other eukaryotic cell source which can be easily isolated. Although, as with the above described techniques, it has its limitations, some of the useful applications, its versatility, and future potential are discussed.

Methods

Cell cultures: Seed stock for all of the tissue culture cell lines tested (see Table I) was obtained from the American Type Culture collection.¹ Cells were transferred at a dose of 1.5×10^5 cells/well to 24 well plates in EMEM + nonessential amino acids medium² containing 10% fetal calf serum³, and allowed to form confluent monolayers for several days before use.

Toxin: T-2 mycotoxin⁴ was dissolved in methanol and stored at 4°C. Just prior to use, dilutions of the toxin to be tested were made in the media to which the cells were exposed. All tests were run on greater than 1/100 dilutions of methanol which in itself had no effect on the cells.

Protein synthesis inhibition assay: Cells in a confluent monolayer were changed over to H199 medium² + 25 mM Hepes buffer² and 5% heat-inactivated (30 mins at 56°C) fetal calf serum, and allowed to equilibrate in a hot box (37°C) for one hour. The cells were then exposed to various concentrations of the toxin for lengths of time from five to 60 minutes. Each toxin dilution was tested in three wells. At the end of the exposure time, the toxin was removed and 2 μ Ci/ml ³H-leucine⁵ in the H199 medium added back to the cells for an additional 30 minutes. The isotope was then removed, the cells washed twice with HBSS media², and 0.1 ml/well of 0.1N NaOH added. The plates were placed on a shaker until the cells released. The cellular protein was absorbed onto 0.5 in. diameter filter paper disks.⁶ One ml of cold 10% TCA was then added to each well to precipitate the protein onto the disk. The disks were then placed in a bottle and washed two times with 5% TCA, once with 1:1 ethanol acetone and once with acetone (5 min per wash, minimum of 1 ml volume per disk). After drying under an IR lamp, the disks were placed in 2 ml toluene⁷ plus liquifluor⁵ and CPM of ³H-leucine incorporated into protein determined on

a scintillation counter. The initial results are expressed as a mean \pm standard error for each dose. Subsequent results are plotted as a percent of the protein synthetic rate in the untreated cells.

RNA and DNA synthesis: The same procedure was used for the determination of inhibition of RNA and DNA synthesis by the toxin except that the ^3H -Leucine was replaced with ^3H -Uridine⁵ for RNA synthesis (0.2 $\mu\text{Ci/ml}$) and ^3H -Thymidine⁵ for DNA synthesis (0.2 $\mu\text{Ci/ml}$).

Inactivation studies: Vero cells were routinely used for studies of T-2 mycotoxin inactivation using the inhibition of protein synthesis assay. A known concentration of mycotoxin was pretreated with various agents or under various conditions for different lengths of time. The sample was then diluted and tested as described above with the Vero cells. Dilutions of the agent alone were also tested as described above with the Vero cells. Dilutions of the agent alone were also tested to rule out any effects on the cells. Determination of the percent inactivation was accomplished by comparison of the treated toxin to an untreated standard.

Inhibition of protein synthesis in rat spleen lymphocytes: The lymphocytes from rat spleens were isolated as previously described (Ford et al, 1973) with several modifications. The cells were teased from the spleen using two flat-tipped spatulas in HBSS media² plus phenol red (no bicarbonate). After dispersing the cells using a pasteur pipette, the debris was allowed to settle out in ice for 8 min. The upper 2/3 of the suspension was removed and the cells centrifuged at 1250 RPM for 5 min in a Sorvall RC2-B centrifuge. The cell pellet was resuspended in RPMI plus heat-inactivated fetal calf serum to a concentration of 1×10^7 cells/ml based on cell counts on a hemacytometer. Microscopic examination of the spleen cell preparations showed less than 10% contamination of red blood cells. One ml aliquots were

placed in each well of a 24 well tissue culture plate. For stimulated lymphocytes, 5 μ g of concanavalin A⁸ was added to each well. Varying concentrations of T-2 mycotoxin were added at various times after the addition of Con A. Inhibition of mitogen stimulation was measured by a one hour pulse of ³H-Leucine or ³H-Thymidine 2 days after the addition of Con A. The cells were then collected and centrifuged at 2500 RPM for 5 minutes. They were then treated with 0.1 ml of 0.1N NaOH, absorbed on filter paper disks and processed as described under the protein synthesis assay.

Results

Effects of T-2 mycotoxin on protein, RNA and DNA synthesis in Vero

cells: A typical dose response curve of Vero cells to T-2 mycotoxin plotted as actual counts per minute and showing the standard error bars is shown in Figure 1. Between experiment variability is due mainly to the rate of protein synthesis of the cells depending on their stage of growth and/or actual cell numbers. However, the degree of protein synthesis inhibition by the T-2 mycotoxin appears to be independent of the growth stage of the cells. Since the standard errors on all triplicate samples are reproducibly small, and in order to compare the results between experiments, all subsequent results are plotted as a percent of the no-toxin control protein synthesis levels by comparing mean values. A progressive decrease in the concentration of T-2 mycotoxin necessary to inhibit protein synthesis is shown in the time and dose response curves and the 50% inhibition levels for each time period (Fig. 2). Exposure times greater than 60 minutes cause little if any shift to the left of the 60 min curve. The dose response curves for the inhibition of DNA synthesis are very similar to the protein synthesis inhibition curves (Fig. 3). Comparison of the 50% inhibition levels at the various response times show that they are practically identical. Therefore, it appears that shutdown

of protein and DNA synthesis occurs almost simultaneously. As previously reported (Liao et al, 1976), very little effect on RNA synthesis is caused by T-2 mycotoxin treatment up to the 60 min. exposure time tested (Fig. 4). The lowest level of RNA synthesis as a result of any dilution or exposure time to T-2 mycotoxin is 75 to 80% of the no-toxin control.

Based on the above results, the standard established for further study of T-2 mycotoxin inhibition of protein synthesis was an intermediate preexposure time to the toxin of 30 min. followed by a 30 min. pulse with the labeled Leucine.

Comparison of the effects of T-2 mycotoxin in a variety of tissue culture cell lines: Table 1 includes a list of 8 different tissue culture cell lines tested for protein synthesis inhibition levels by T-2 mycotoxin. Analysis of variance using Duncan's multiple range test was used to compare 50% protein synthesis inhibition levels of individual cell lines, species sources, tissue sources, cell types (epithelial or fibroblast), and growth rates. Only the L929 cell line showed a significantly increased sensitivity to the toxin when compared to the least sensitive cell line, the CHO cells. This is also reflected in the species and tissue source comparisons since the L929 are the only representatives from mouse and connective tissue, respectively. Generally, however, the range of 50% inhibition levels for the various groups lies well within what would be expected from experiment to experiment variation. Probably the largest source of error is due to slight variations in the actual concentration of the toxin since extensive dilution to get it in a testable range is required. Stocks of these dilutions cannot be used over a period of time since we have found that loss of activity results from storing T-2 mycotoxin in aqueous solutions.

Inactivation studies: Many different agents or conditions such as hydrogen peroxide, sulfuric acid, formaldehyde, ultraviolet light, and heat were tested for their possible use as a decontaminant for T-2 mycotoxin. Most of them either had no effect on the mycotoxin or were in themselves harmful to the tissue culture cells, and could not be tested using this system. However, sodium hypochlorite in combination with sodium hydroxide was found to be a very effective means of inactivation. The results in Table II demonstrate how this assay can be effectively used to establish safety standards for the decontamination of samples exposed to T-2 mycotoxin. The use of as low as 0.25% sodium hypochlorite in the presence of 0.025 M NaOH for four hours will inactivate over 99% of the toxin. If used without the NaOH, a minimum four hour exposure with greater than 1% sodium hypochlorite is required to cause almost complete inactivation.

Effects of T-2 mycotoxin on lymphocyte stimulation: Rat spleen cell preparations were first tested for optimal in vitro stimulation time with Con A. By 48 hrs the incorporation of ^3H -leucine was more than five fold over unstimulated cells while ^3H -thymidine incorporation was over ten fold. Stimulation remained at this level through day three. Therefore, in order to minimize contamination and save time, the mycotoxin was tested during the course of a two-day stimulation of lymphocytes with Con A. Table III shows the compiled results from several lymphocyte preparations. The rate of protein synthesis is still well below the 50% level with 5 ng/ml T-2 mycotoxin. Therefore, it appears that the lymphocytes are even more sensitive to the mycotoxin than tissue culture cells, perhaps due to a variation in the procedure which is necessary since the lymphocytes do not form a monolayer on the plates. Since the cells cannot be washed, they are in constant exposure

to the mycotoxin, even during the pulse with ^3H -leucine. The level of inhibition of the toxin appears to be independent of the time of addition of T-2 after mitogen stimulation.

Discussion

Although numerous approaches are currently available for the study of mycotoxins, none of them provide the versatility and simplicity of the protein synthesis inhibition assay described in this paper. With the availability of tissue culture cells or any other eukaryotic cell that can be easily isolated such as lymphocytes, and most probably macrophages (Gerberick et al, 1981) or hepatocytes (Emeh et al, 1981; Terao et al, 1981), the detection and quantitation of T-2 mycotoxin can be accomplished within one day's work. One 24 well plate is sufficient to test 8 dilutions of toxin in triplicate. A 10 to 20 plate assay is well within the limits of a single one day experiment. Since T-2 is such a potent protein synthesis inhibitor, this assay will detect the mycotoxin at nanogram levels with a narrow range of sensitivity of about one log between no inhibition and complete inhibition of protein synthesis. The sample must be extensively diluted to bring the toxin to an effective range for testing. Therefore, problems with any interfering toxic substances in the assay sample are usually eliminated.

The assay has its limitations in that it lacks specificity in identifying which toxins are present in the sample. In the case of a mixture of mycotoxins, their amounts cannot be quantitated due to a variable toxicity of the various trichothecene derivatives. However, the results described in this paper demonstrate the potential for use of this assay in the study of mycotoxins in several areas of interest. Although differences in response to the mycotoxins in several cell lines have been reported by other techniques (Bamberg et al, 1971), results using this procedure with a wide range of

tissue culture cells and rat spleen cells indicate that the T-2 mycotoxin inhibits protein synthesis to about the same degree in any eukaryotic cell it encounters. The non-preferential effect is supported by the fact that all of the cell lines tested are equally inhibited regardless of the growth stage of the cells. Also, the level of protein synthesis in rat spleen cells is equally affected at any point after mitogenic stimulation of the cells. In addition to T-2, inactivation of a wide range of trichothecene mycotoxins which are all protein synthesis inhibitors can be tested. A similar field of study would be the search for a means of in vivo neutralization of mycotoxin effects using antibodies or chemical agents by first testing for protection from the toxin in the in vitro protein synthesis inhibition assay. The possible use of this procedure for mechanism of action studies was demonstrated by the comparison of RNA, protein and DNA synthesis in Vero cells exposed to T-2 mycotoxin. Whether the simultaneous shut down of protein and DNA synthesis involves a direct effect of the toxin on both processes has not yet been proved or disproved. However, it is possible that inhibition of DNA replication is only a secondary effect of protein synthesis inhibition since it has been shown that several known protein synthesis inhibitors also shut down DNA synthesis within the same time frame as seen with the T-2 mycotoxin (Mizuno, 1975).

Structure-function relationships, the uptake and release of labeled precursors and toxins and viability studies are also currently underway using this basic system in an effort to better understand the mechanism of action of the mycotoxins.

Acknowledgements

The author wishes to thank Mr. Dennis Leatherman for help in developing the protein synthesis inhibition technique, and for the expert technical assistance of Jennie Cejka and Stanley Griffin.

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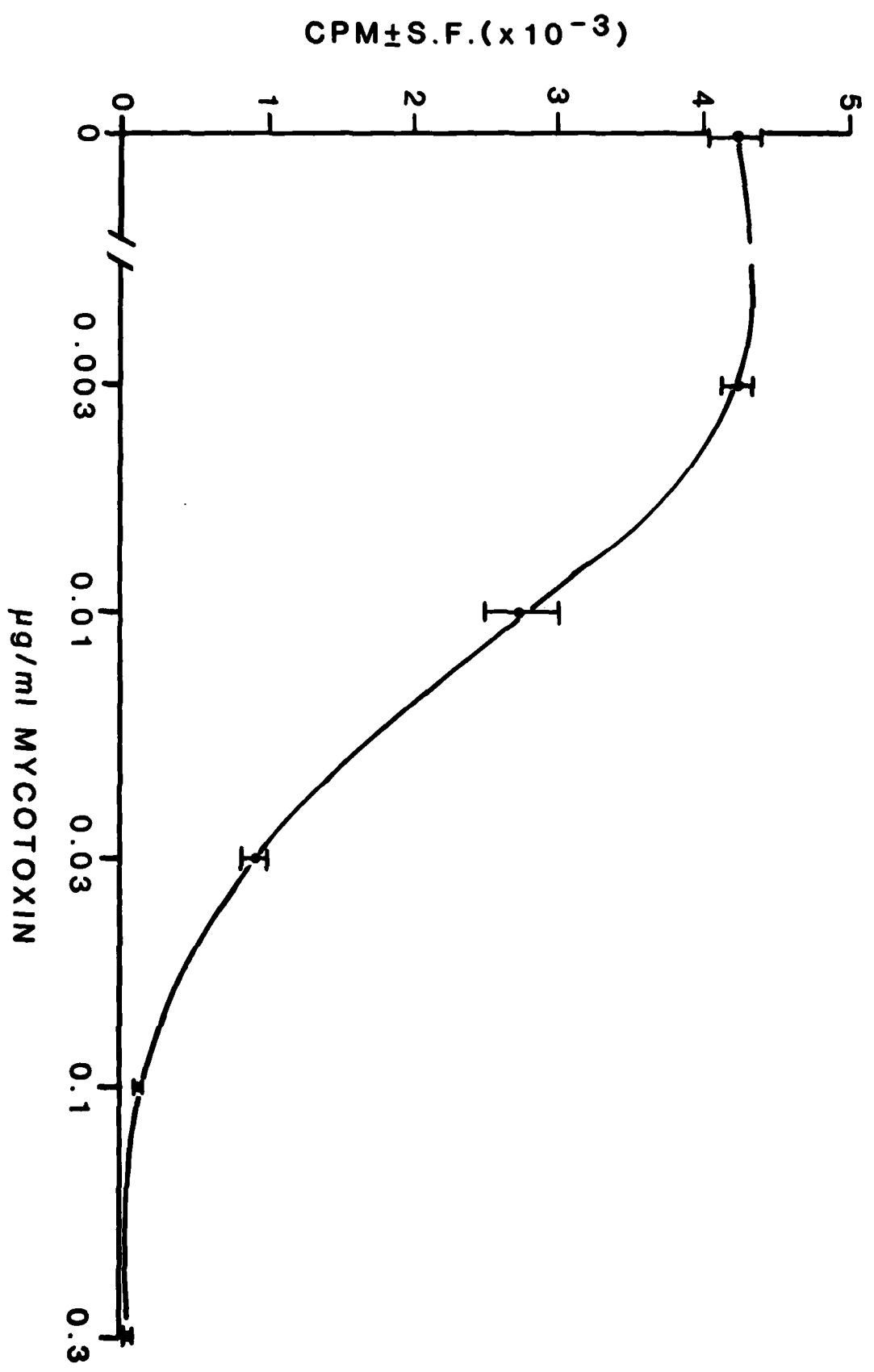
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3. KC Biologicals, Kansas City, KS
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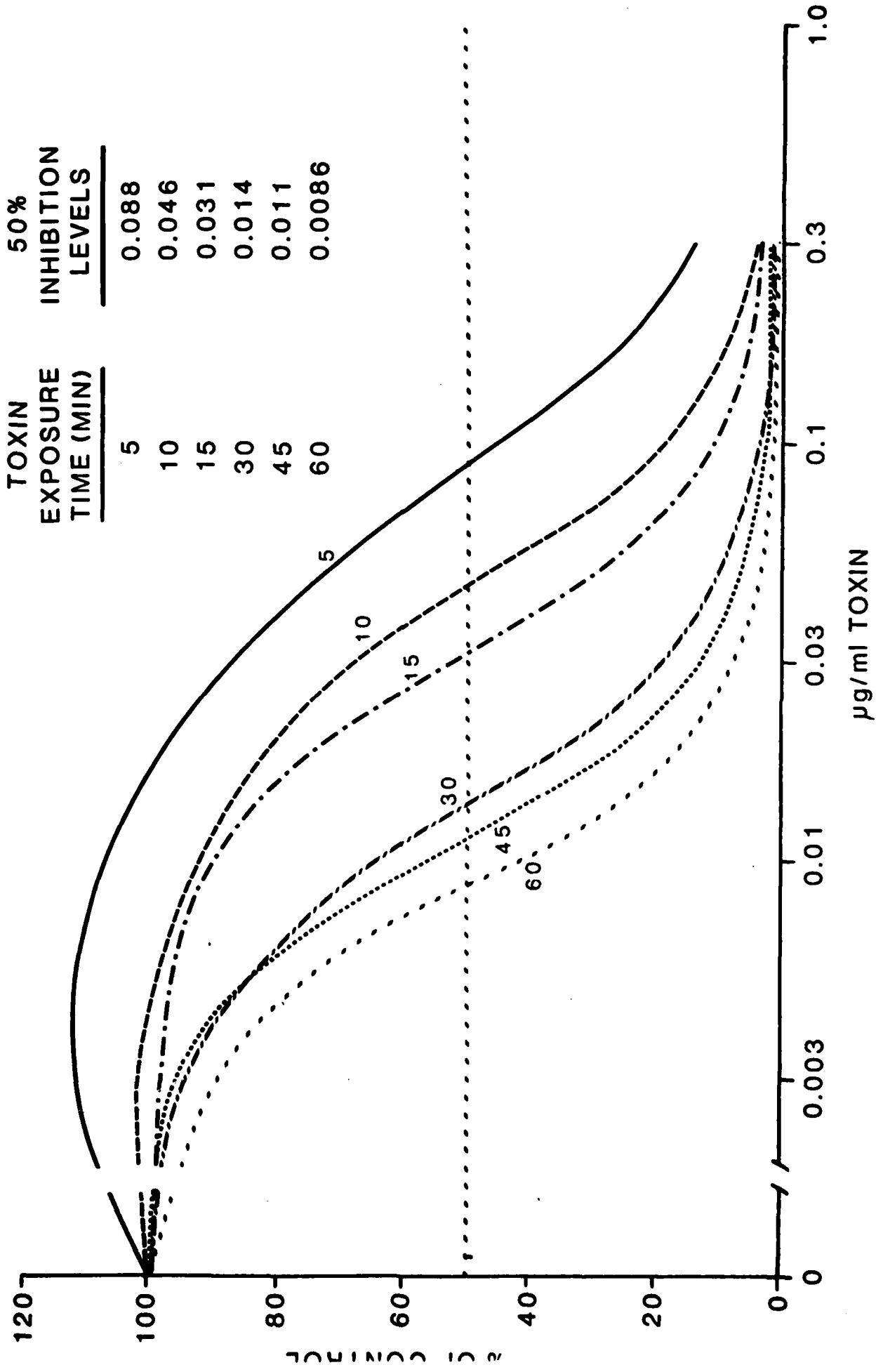
Legends to Figures

- Figure 1.** Effect of T-2 mycotoxin on protein synthesis in Vero cells. Each point represents the mean \pm standard error of three samples.
- Figure 2.** Time and dose response curves of T-2 mycotoxin protein synthesis inhibition in Vero cells plotted as a per cent of untreated cells. 50% inhibition levels taken from the curves are included.
- Figure 3.** Time and dose response curves of T-2 mycotoxin DNA synthesis inhibition in Vero cells plotted as a per cent of untreated cells. 50% inhibition levels taken from the curves are included.
- Figure 4.** Time and dose response curves of T-2 mycotoxin RNA synthesis inhibition in Vero cells plotted as a per cent of untreated cells.

T-2 MYCOTOXIN INHIBITION OF PROTEIN SYNTHESIS IN VERO CELLS

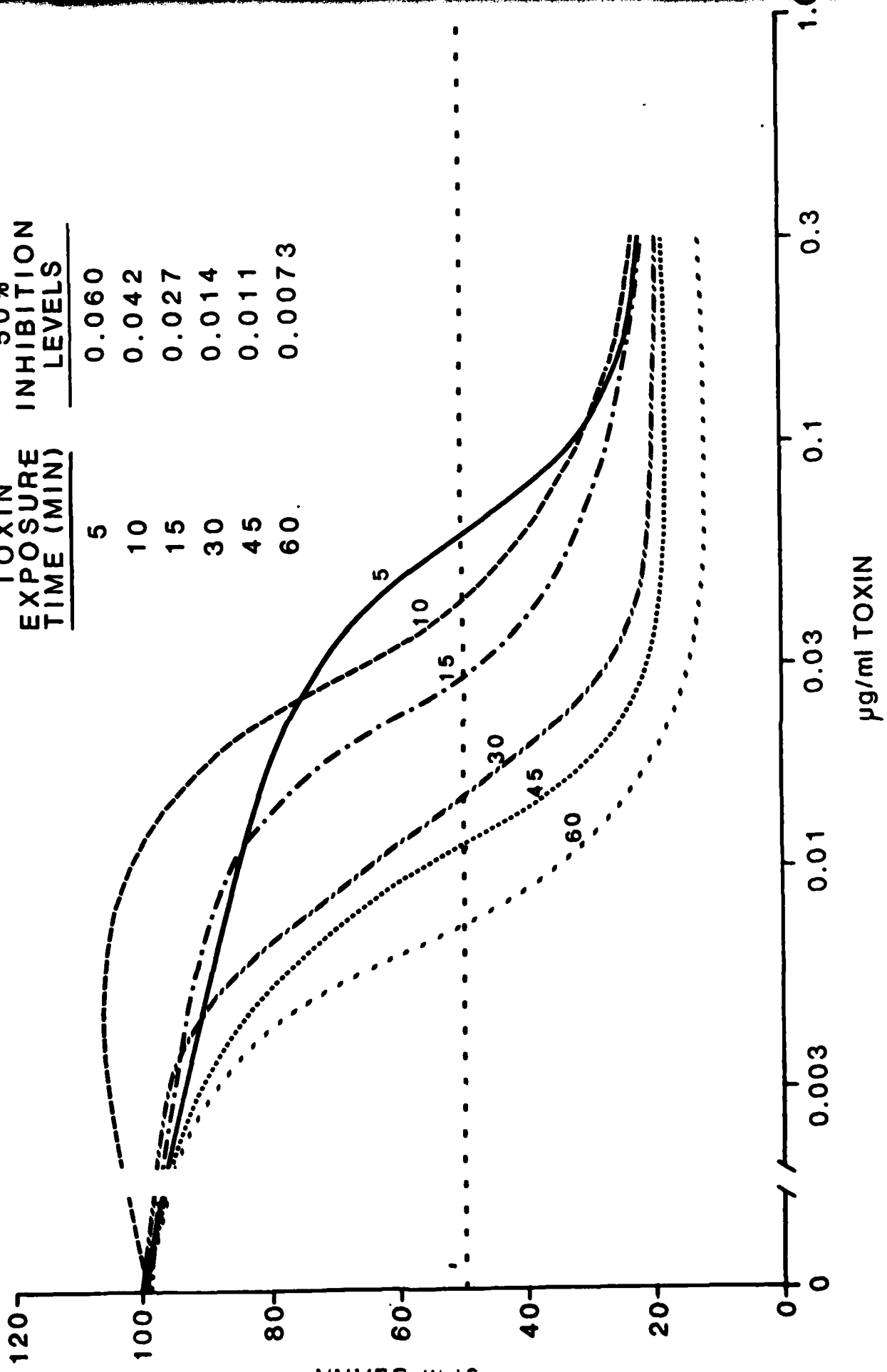


T-2 MYCOTOXIN INHIBITION OF PROTEIN SYNTHESIS IN VERO CELLS



T-2 MYCOTOXIN - DNA SYNTHESIS INHIBITION

TOXIN EXPOSURE TIME (MIN)	50% INHIBITION LEVELS
5	0.060
10	0.042
15	0.027
30	0.014
45	0.011
60	0.0073



T-2 MYCOTOXIN RNA SYNTHESIS INHIBITION IN VERO CELLS

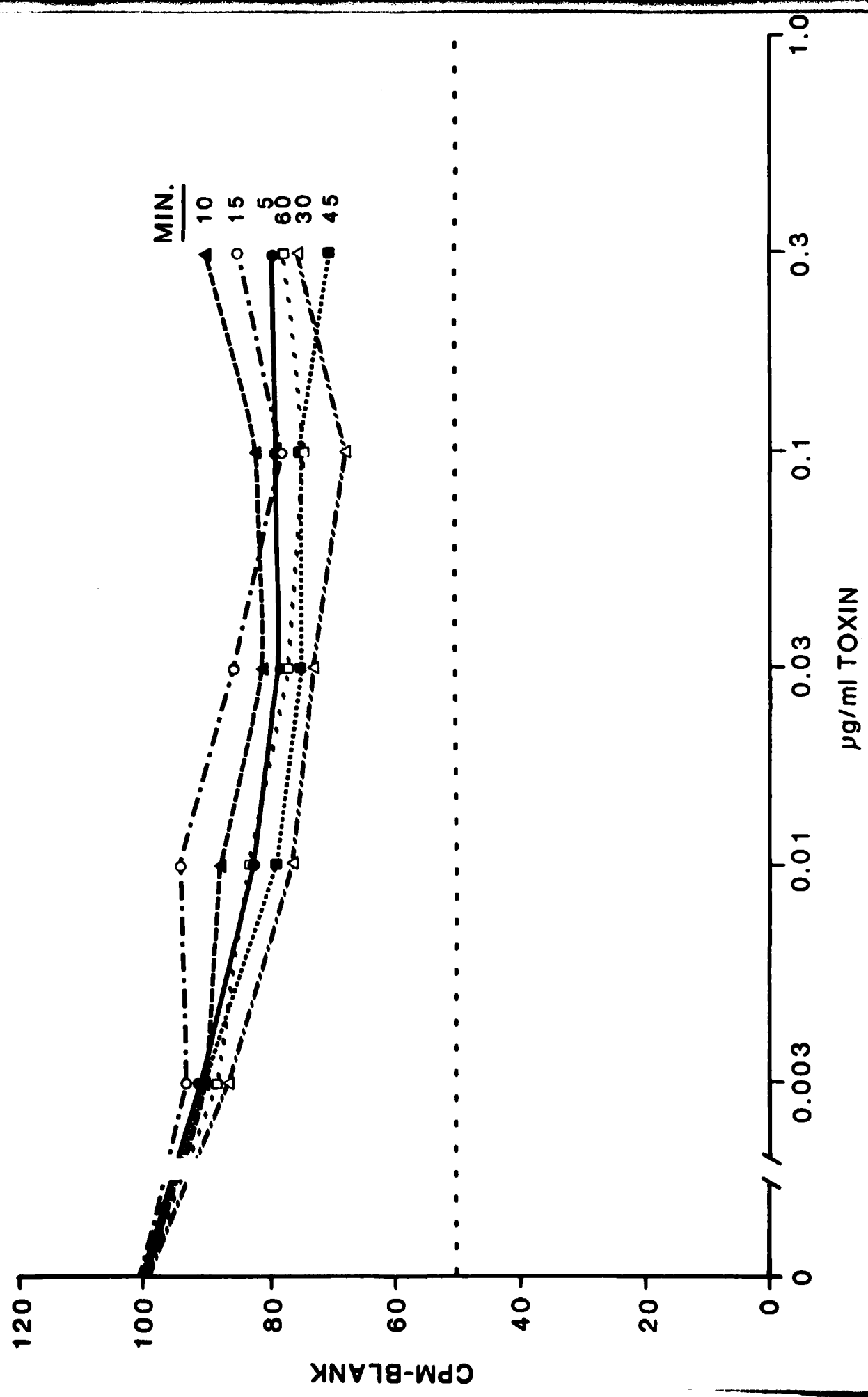


Table I. Tissue Culture Cell Lines Tested for Inhibition of Protein Synthesis by T-2 Mycotoxin

Cell Line (ATCC #)	Species Source	Tissue Source	Growth Char.	N	50% Inhib. Level ^a
<u>Epithelial</u>					
CHO (61)	Hamster	Ovary	15-20X in 7 days	4	17.3 ± 0.61
Hela (2)	Human	Cervix (Carcinoma)	15X in 7 days	2	17.0 ± 1.0
E-6 (^b)	Monkey (African green)	Kidney	30X in 7 days	6	13.1 ± 0.93
A-549 (185)	Human	Lung (Carcinoma)	15X in 7 days	4	12.5 ± 1.76
LLC-MK2 (7)	Monkey (Rhesus)	Kidney	5-8X in 7 days	9	11.1 ± 1.2
<u>Fibroblast</u>					
BHK-21 (10)	Hamster	Kidney	4X in 4 days	8	17.0 ± 2.9
Vero (81)	Monkey (African green)	Kidney	30X in 7 days	28	16.8 ± 1.01
DBS-FRHL (160)	Monkey (Rhesus)	Lung	3X in 7 days	5	14.2 ± 2.54
MRC-5 (171)	Human	Lung	3-5X in 7 days	6	12.6 ± 1.14
MCTC clone 929 (1)	Mouse	Connective tissue	8-9X in 7 days	7	7.65 ± 0.65

^aµg/ml T-2 mycotoxin ± S.E. required to inhibit protein synthesis 50% after 30 min exposure to the cells.

^bE-6 cells (Vero derivative) were supplied by the Center for Disease Control, Atlanta, GA.

Table II. Inactivation of T-2 Mycotoxin with Sodium Hypochlorite + NaOH

Treatment		% Inactivation by Exposure Time			
Na Hypochlorite (%)	NaOH (M)	10 Min	30 Min	4 Hr	16 Hr
2.5	0	95.2	<u>99.6</u>	99.8	
2.5	0.25		<u>100.0</u>	100.00	100.0
1.0	0	71.8	95.15	<u>98.1</u>	
1.0	0.05		<u>98.4</u>	99.8	99.9
0.25	0	47.6	68.8	77.6	
0.25	0.025		89.9	<u>99.7</u>	99.7
0.10	0	15	50.0	33.3	
0.10	0.005		48.0	96.7	<u>98.9</u>

Underlined values denote acceptable level and time for inactivation of T-2 mycotoxin with various treatments (>98%).

Table III. Effects of T-2 Exposure on CON A Stimulated Lymphocytes

T-2 Challenge after CON A addition	% Inhibition of Protein Synthesis by the following Toxin Doses:		
	5 ng/ml	10 ng/ml	100 ng/ml
0 Time	89.9	97.8	98.5
4 hrs	62.0	92.4	98.7
24 hrs	87.2	97.7	98.8
48 hrs	84.8	93.8	98.6

MED
8