THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

A COMPARATIVE STUDY OF TRICHOTHECIN, AMPHOTERICIN B AND 5-FLUOROCYTOSINE AGAINST CRYPTOCOCCUS NEOFORMANS IN VITRO AND IN VIVO

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSPHY

BY

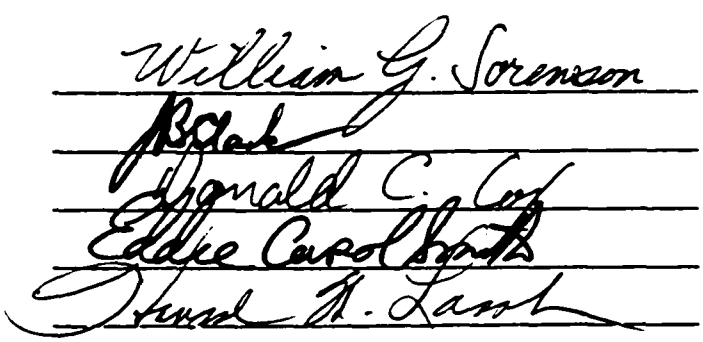
Mark Robert Sneller

Norman, Oklahoma

1976

A COMPARATIVE STUDY OF TRICHOTHECIN, AMPHOTERICIN B AND 5-FLUOROCYTOSINE AGAINST CRYPTOCOCCUS NEOFORMANS IN VITRO AND IN VIVO

APPROVED BY



DISSERTATION COMMITTEE

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank all members of my graduate committee, Drs. Sorenson, Larsh, Cox, Clark, Smith, and Lancaster for contributing to my positive learning experience.

In particular I would like to thank Dr. Howard Larsh and Dr. William Sorenson for their financial support of this study. I would further like to thank Dr. William Sorenson for his assistance in the preparation of this manuscript, and for his guidance, understanding and inspiration.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Trichothecin (T-cin), amphotericin B (AB) and 5-fluorocytosine (FC) were compared singly and in combination against Cryptococcus neoformans in terms of relative fungistasis, inhibition of DNA, RNA and protein synthesis and chemotherapeutically in experimentally infected mice. The minimum inhibitory concentration (MIC) for T-cin, AB and FC was found to be 0.5, 0.2 and 5.0 µg/ml respectively. In vitro viability studies demonstrated the occurrence of synergy with the AB-FC combination and additivity with the AB-T-cin combination for a three day period. Studies on the cellular level were inconclusive in the explanation of these phenomena. In mice the ED_{50} (50% effective dose) value for AB was found to be 0.38 mg/kg ip and for FC it was 100 mg/kg ip for a 30 day time period. T-cin was tested from 0.1 to 50 mg/kg and no ED_{50} value could be ascertained, however 1.0 mg/kg offered the best protection in terms of extending mean life expectancy. The most effective drug combination in vivo was AB-T-cin where drug synergy was demonstrated. High dose T-cin controls survived despite having received a cumulative dosage of more than twice the reported LD_{50} value. Histopathological evidence supported T-cin toxicity in mice when toxin was used in high concentrations.

A COMPARATIVE STUDY OF TRICHOTHECIN, AMPHOTERICIN B AND 5-FLUOROCYTOSINE AGAINST CRYPTOCOCCUS NEOFORMANS IN VITRO AND IN VIVO

CHAPTER I

INTRODUCTION

Trichothecin (T-cin) is produced by the saprophytic mold Trichothecium roseum. It is one of the trichothecene class of mycotoxins and as such is a sesquiterpene and is an inhibitor of protein synthesis in eucaryotic cells (22,23,44). Freeman found the LD_{50} of T-cin in mice to be over 250 mg/kg (21). T-cin was also reported by Freeman to be toxic to a number of pathogenic fungi in vitro, including Cryptococcus neoformans (Torulopsis neoformans) which was inhibited by 16 µg/ml or less of the compound (21). Preliminary in vitro experiments in our laboratory had demonstrated that C. neoformans was sensitive to this drug at less than 1 µg/ml under more defined conditions. The large ratio between the reported LD_{50} in mice and the in vitro effectiveness against C. neoformans suggested that T-cin might be useful as antibiotic.

Antibiotic properties of the trichothecenes have been investigated previously with varying results. Trichodermin, a trichothecene similar in activity to T-cin (25) is active

against Candida albicans, and because of its low mammalian toxicity has been proposed for treating candida infections in man and for use as an antitumor agent (24). In contrast Freeman (21) stated that the antifungal activity of T-cin is of no practical value in treating infections in animals owing to the high toxicity of the antibiotic. Scheiber et al. (26) compared T-cin, nystatin, and crotocin, an isomer of T-cin in mice experimentally infected via stomach tube with C. albicans. Even though the drugs were found to reduce the number of Candida cells in the faeces of mice the excretion of Candida cells in the controls was not consistent and the results were considered unreliable. Glaz et al. (27) studied the activity of antibiotic T which was subsequently referred to as crotocin (40). They found it to be active against C. albicans in vitro but not in vivo and concluded that it does not promise to be suitable for treatment of systemic mycoses. However, Glaz and coworkers had administered only two treatments of crotocin over a 48 hour period. There appeared to be sufficient ambiguity in the literature regarding antibiosis of T-cin and other trichothecenes to warrant further investigation. C. neoformans infected mice was selected as the test system. For positive controls two drugs presently in use for treatment of systemic cryptococcosis were chosen, amphotericin B (AB) (14) and 5-fluorocytosine (FC) (14,15).

AB is a polyene antibiotic produced by Streptomyces nodosus. It affects membrane properties of fungal and

animal cells by binding to sterols in the membrane (10) resulting in leakage of a variety of cellular constituents (11, 12). The LD_{50} of AB in mice is 280 mg/kg ip (41).

The pyrimidine analogue FC has a very low toxicity to mammalian cells with marked and rather selective antifungal activity against yeast cells in vitro and in vivo (16,17,36). The LD_{50} of FC in mice is 1190 mg/kg ip (17).

There have been recent investigations into the concurrent use of AB and FC in vitro and in vivo (3-5, 31,32,45). These investigations have pointed to wide disparities in C. neoformans strain behavior to AB and FC when used in combination. Little information is available comparing in vitro with in vivo results of a single strain exposed to this drug pair (32). However, under identical circumstances different strains may demonstrate synergism, antagonism or indifference to AB and FC when these drugs are used together. When used singly AB does not demonstrate this disparity and yields more consistent results (30,38,39,42). The activity in vitro of FC has been shown to be affected by a number of variables (45,46). In vivo FC causes the formation of resistant mutants (8,9,16,18). It is theorized that use of AB and FC would eliminate the resistant mutants resulting from FC usage and would reduce the nephrotoxicity caused by AB (6,7) by lowering its concentration and still achieve clinically significant results.

T-cin has been used also in combination with other drugs. In their paper cited above, Scheiber et al. (26) had tested the therapeutic efficacy of their antibiotics during the concurrent oral administration of oxytetracycline incorporated in the food of the mice. To date there have been no literature reports regarding the activity of T-cin when combined with AB or FC in vivo or in vitro. To study the problem of combined drug therapy an experimental animal model similar to that of Block and Bennett was chosen (31).

The desire to compare T-cin with the control drugs on as many bases as feasible lead to the development of two other phases of the investigation. The first was an initial in vitro screening of these drugs in terms of their single and combined effects on the viability of C. neoformans, as reported previously for AB and FC (13,29,30,45).

The second was a study of drug interaction on the molecular level. Similar investigations have included at least one member of the drug pair in high or in varying concentrations (13,28,32). The use of low and constant concentrations of each drug studied singly and in combination over time appeared to us to be a necessary link in the correlation of this work with viability counts. This seemed especially true if the same concentrations could be used in both cases.

The investigation of the comparative effects of T-cin with AB and FC was therefore divided into three primary phases: 1) in vitro viability studies to determine the minimum inhibitory concentration (MIC) of T-cin, AB and FC against

C. neoformans for a three day period. This phase also included combinations of these drugs at one-half MIC (MIC/2) and one-quarter MIC (MIC/4) levels to determine the relative fungistatic or fungicidal characteristics of the combination pairs, 2) a study at the molecular level of single drugs and two-drug combinations in an attempt to corroborate viability studies and to investigate the mechanism of action of the drug combinations. This work included uptake of ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-leucine by C. neoformans at MIC/2 concentrations over a four hour time period, 3) in vivo establishment for each drug of an effective dose at the 50% level (ED_{50}) against C. neoformans infected mice for a 30 day treatment period. This phase included an investigation into combined drug therapy in which the drugs were combined at ED_{50} or one-half ED_{50} ($ED_{50}/2$) levels. Limited histopathological data are presented.

CHAPTER II

MATERIALS AND METHODS

Medium. A modified Wickerham's medium designated M9A was employed in all experiments to grow the cells. The medium consisted of Wickerham's concentration of trace elements and salts, 1% (w/v) glucose, 0.5% (w/v) vitamin free casamino acids (Difco) and thiamin at 0.2 µg/ml. Ammonium sulfate was omitted.

Cells. Cryptococcus neoformans strain No. 184 was used in all in vitro and in vivo studies. The strain was originally isolated from a human patient and was provided by Dr. L. Friedman, Charity Hospital, New Orleans, Louisiana.

Growth. For minimum inhibitory concentration (MIC) studies cells were inoculated by the loop method and grown for 24 hours in M9A on a rotating shaker at 37 C, diluted with fresh medium to 10^5 cells/ml and allowed to grow overnight until ca. 10^6 cells/ml was obtained. They were then transferred in 50 ml aliquots to sterile flasks to which drugs were added singly at varying concentrations. After an initial screening, two-fold serial dilutions of each drug were prepared to determine the MIC. In subsequent experiments drugs were added singly or in combination at fractional MIC levels. Ten ml from each flask was transferred to

each of four 125 X 15 mm test tubes and placed on a New Brunswick tube platform (New Brunswick, N.J.) on a 15 degree angle, and shaken for three days at 37 C. Duplicate tubes were removed after 24 and 72 hours. Cell counts were made after dilutions by spread plating in triplicate on Sabouraud Dextrose Agar (SDA) to determine the number of colony forming units (CFU). All experiments were duplicated and the results averaged.

Isotope studies. Cells were grown as above. A 500 ml Erlenmeyer flask containing 250 ml of M9A was used for each cell workup. When ca. 10^6 cells/ml was obtained (hemacytometer count) isotope was added to the stock flask and 30 ml was dispensed into each of seven 125 ml Erlenmeyer flasks. These were replaced on the shaker for 4 hours for adaptation. Drugs were then added singly or in combination at one-half MIC concentrations (MIC/2) to appropriate flasks by addition of 100 µl of each drug. The flasks were placed on the shaker for 5 minutes and the T_o measurements were then taken. Acid precipitable counts were determined by withdrawing one ml from each flask and adding to tubes containing 1 ml ice cold 10% trichloroacetic acid (TCA). Samples were filtered through millipore filters (0.45 µ) and tubes were washed three times with 10 ml each of 5% ice cold TCA. Duplicate data points were taken hourly at T_o through T_4 hours. DNA, RNA and protein experiments were duplicated and the results averaged.

In vivo studies. Cells were grown for 24 hours in M9A, washed 3X in physiological saline (PS) and diluted to 10^6

cells ml in PS.

In all experiments initial viable counts were also determined to confirm hemacytometer counts.

Isotopes. Thymidine-Methyl-^{3}H (specific activity 43.1 Ci/mmole) and Uridine-5,6-^{3}H (specific activity 45 Ci/mmole) were used at 0.5 μCi/ml final concentration and L-leucine-4,5-^{3}H (specific activity 50 Ci/mmole) was used at 1.5 μCi/ml final concentration. Isotope fluor was dioxane base Aquafluor.

Isotope and fluor were purchased from New England Nuclear (Chicago, Ill.).

Drugs. T-cin was obtained as previously described (33). T-cin stock solutions and dilutions were prepared in distilled water (DH_2O). For animal inoculations T-cin was dissolved in light mineral oil (Markol 52, Exxon Petroleum Co.).

Crystalline 5-fluorocytosine was purchased from Hoffman-La Roach (Nutley, N.J.) and prepared in DH_2O for in vitro studies and in PS for in vivo experiments.

Amphotericin B (Fungizone, Squibb) was reconstituted and diluted with 5% dextrose in water.

All drug stock solutions were sterilized by filtration. They were kept in dark containers and refrigerated at 4 C when not in use.

Assessment of drug activity. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of each compound which completely inhibited multiplication of C. neoformans strain 184 over the three day experimental time

period. The exception to this was FC for which a two day period was chosen since overgrowth could not be prevented with this strain after two days even with much higher concentrations of the drug.

An additive effect will be defined as the same effect achieved by the sum of two drugs used in combination at reduced concentration as that effect achieved by the single most effective drug when used alone. Under these circumstances the single drug is used at a dose equivalent to that of the mixture (1,2).

In the viability studies antifungal synergy was defined as a decrease in 10-fold or more in colony counts after three days when drugs were used in combination at reduced concentration as compared to the counts when a single drug was used alone (13).

Mice. Six to eight week old BALB/cj inbred white mice were used. Mouse weights varied between 25-30 grams per experiment. Drug dosages were adjusted accordingly. All groups of mice were weighed at the time of treatment. On day 1 mice were inoculated intravenously via a lateral tail vein with 0.1 ml of a 10^6 cells/ml suspension of C. neoformans strain 184. Drug treatment began on day 4 and 14 alternate day dosages were administered for a 30 day experimental time period. All drugs were administered via the intraperitoneal route. Injection amounts varied between 0.1 to 0.4 ml depending upon the drug and its concentration. Mice were housed in groups of five. Each dosage group contained 10

mice, 5 males and 5 females. They were provided water and food (Purina Lab Chow) ad libitum. Mice were maintained on a 12 hour day-night cycle.

Dead mice in all animal experiments and surviving mice of the combined drug experiment were autopsied. The spleen and brain were ground with a tissue homogenizer and plated on SDA containing 100 units of penicillin and 100 µg streptomycin/ml. Growth of yeast-like colonies at 37 C and examination of random colonies by India ink wet mount constituted positive identification criteria for C. neoformans. Plates containing less than four colonies were classified as negative. All plates classified as positive produced confluent growth.

Experimental. Three mouse experiments were conducted. The first consisted of 1.0, 2.5, 5.0, 7.5 and 10 mg/kg levels of T-cin only. The second experiment consisted of mice receiving treatment by AB, FC or T-cin. AB dosages tested included 0.1, .25, .50, .75, 1.0 and 1.5 mg/kg. FC dosages tested included 10, 25, 50, 100 and 150 mg/kg. Due to the large quantities of T-cin necessary for the study and the desire to maintain uniform numbers of mice for each dosage regimen the second experimental T-cin group contained only five mice per dosage (males). These included the 0.1, 1.0, 10, 25 and 50 mg/kg levels. All other animal groups contained ten mice per dosage regimen. Control groups consisted of uninfected, infected, solvent and high dose controls. The latter group contained five mice each in the case of T-cin. At the

conclusion of the experiment the high dose T-cin controls were inoculated with C. neoformans. In addition ten other mice of the same age were inoculated to serve as positive controls.

Synergy studies. The third mouse experiment was an investigation of drug combinations in infected mice. Combinations were studied at the ED_{50} and $ED_{50}/2$ levels, which were ascertained as a result of experiment two. Controls consisted of T-cin at 1.0 mg/kg and 0.5 mg/kg, FC and AB at ED_{50} levels, uninfected and infected mice.

Histopathological. In addition to the three mouse experiments two mice were injected ip with T-cin at a concentration of ca. 250 mg/kg and one was injected ip with T-cin at a concentration of 50 mg/kg. The toxin was dissolved in warmed corn oil. The mice used in this work were six week old CBA Bar Harbor white mice, with an average weight of 17-18 grams. The mice were sacrificed after 24 hours, autopsied, and the thymus, bone marrow, liver, and kidney were examined histopathologically by Dr. James Price at the University of Oklahoma Health Sciences Center.

CHAPTER III

RESULTS

In vitro. Table I lists the MICs and their respective cell counts in the viability studies. The MIC of T-cin was only about twice the MIC of AB. The MIC of FC was determined to be 5 µg/ml for a two day period or > 150 µg/ml for three days since all concentrations tested up to 150 µg/ml were unable to maintain viable counts at or below 10^6 CFU/ml for the longer time period. The most effective MIC/2 combination (each drug at one-half MIC) after three days (Table II) was AB-FC which demonstrated synergy by the criterion of Medoff et al. (13). The AB-T-cin MIC/2 combination approximated an additive effect based upon the comparative value of its CFU with that of the MIC with the lowest counts after three days (AB). An additive effect would be interpreted for the FC-T-cin MIC/2 combination for the same reason. However due to the apparent inactivity of FC after two days the combination might be expected to equal T-cin/2 controls; CFU for the combination is seven fold below this value. Other combinations tested and their CFU are presented in Table II for comparative purposes.

Uptake of labelled substrates. All combinations in thymidine uptake (Figure 1) fall below single drug points for the greater part of the four hour time period but differences are often insignificant. Significance was determined by comparison of standard errors based upon differences between means. (Time periods at which differences are significant are marked with an arrow.) Only the synthesis of RNA in general appears to be significantly affected by individual drugs and their combinations (Figure 2) as evidenced by negative slopes. The AB-T-cin combination appears to exert its major effect here since all points are significantly below points belonging to single drugs. The other two combinations appear to exert greater than additive effects at T_o and T_2, but the meaning of the occasional synergy in terms of long term cellular inhibition is not clear. Protein synthesis (Figure 3) appears to be the least affected of the three cellular processes studied. For the most part combinations had little or no apparent effect on the uptake of leucine compared with the single drugs except for the AB-T-cin and AB-FC combinations for which one point of significant difference was noted at T_o and T_1 respectively. A direct comparison of drug combinations for the three cellular processes studied is presented in Figure 4. While FC-T-cin shows relative inactivity compared with the other combinations in all uptake measurements, the AB-T-cin group shows the lowest initial counts in protein synthesis and after four hours in RNA

synthesis. Finally, the AB-FC pair shows a significant reduction in DNA synthesis after three hours compared with the other two combinations.

In vivo single drug experiments. The curative effect of various concentrations of AB and FC on mice infected with C. neoformans is graphically represented in Figure 5 (A and B). The ED_{50} of AB was determined as .38 mg/kg on the basis of percent survivors. The ED_{50} of FC was 100 mg/kg. No ED_{50} for T-cin could be obtained at 30 days since all concentrations tested fluctuated around control values (Figure 6C). All five of the 50 mg/kg high dose T-cin controls survived despite having received a total of 700 mg/kg, more than twice the estimated LD_{50} value (25). When challenged on day 33 with C. neoformans all five mice died within the next 30 days (Figure 6D) while only 7 out of 10 controls infected at the same time succumbed. A comparison of T-cin with AB and FC for the single drug experiment is presented in Table III. It should be noted that the concentration of 1.0 mg/kg T-cin offered the best protection to mice of all T-cin concentrations tested in terms of extending mean life expectancy (MLE).

Histopathological. The mice injected with 250 mg/kg T-cin exhibited marked cortical depletion of the thymus and evidence of hemorrhage in the kidney, liver and lung and definitely decreased myeloid elements in the marrow of one with a hyperplastic myeloid marrow in the other. The mouse injected with 50 mg/kg T-cin demonstrated liver parenchymal cells with areas of fatty degeneration or intracellular fatty

infiltration. The kidney showed moderate congestion in the area of medullary rays. The corn oil injected control possessed tissues described as unremarkable.

Combined drug experiment. The various drug combinations used in the combined drug experiment are compared in Figure 7. Results are expressed as percent survivors at 30 days. It can be seen that the AB-FC ED_{50} group (each drug at ED_{50} level) allowed greater percent survivors than either control but since each drug in the combination was at ED_{50} concentration twice the drug dosage was given. At one-half the ED_{50} concentration of each drug ($ED_{50}/2$) the same results were achieved as the AB ED_{50}. This is considered an additive effect. The value of 1.0 mg/kg T-cin was chosen for use in the combined drug experiment for the purpose of conservation of toxin and since this concentration had offered the greatest protection to the mice in terms of extending mean life expectancy (MLE). Both AB-T-cin combinations demonstrated synergy since a greater percentage of survivors over controls was noted even though T-cin is inactive by itself. The FC-T-cin pair shows effects approximating those of FC alone, suggesting that T-cin does not act in combination with this drug.

In Figure 8 the MLE of the various groups is depicted. The relative positions of the drug pairs are similar to those described for Figure 7. In general, drug combinations extended the MLE over single drug controls. The MLE of the untreated controls is 21.0 days in this experiment in contrast to 15.7 days in the single drug experiments.

In Figure 9 the cumulative total weight gain per mouse is shown over the 30 day experimental time period. It can be seen that the most effective combinations were the high dose groups in which little weight loss was observed. Compared with other low dose combinations the AB-T-cin $ED_{50}/2$ pair was the most effective.

Results of the combined drug experiment are summarized in Table IV. Positive organ cultures are shown as a fraction of total mice autopsied. Spleen cultures considered negative were observed for all high dose drug combinations and brain cultures considered negative were observed only for the FC ED_{50} and AB-FC $ED_{50}/2$ groups. All of the high dose T-cin control mice (uninfected) suffered severe weight loss of up to 11 grams per mouse and their coats were unkempt and ruffled after having received a total of 250-350 mg/kg T-cin. Thereafter recovery and weight gain were rapid. At the conclusion of the experiment the mice weighed only 4.6 grams less than their initial weight at the start of the experiment.

Table I. Minimum inhibitory concentrations (MIC) for T-cin and AB were based on a three day time period. The MIC for FC was determined after two days since all concentrations tested were ineffective after this time. Results in viable counts are expressed as colony forming units (CFU) per ml. Combined drug values will be compared with MIC values.

$T_o = 10^6$ CFU/ml

	MIC µg/ml	CFU[a]/ml Day 1	Day 2	Day 3
T-cin	0.5	9.8×10^5	b	9.0×10^5
AB	0.2	5.9×10^5	b	5.8×10^5
FC	5.0	4.7×10^5	5.7×10^5	4.4×10^7
Controls	---	1.8×10^7	b	9.4×10^7

[a]Colony forming units

[b]Not determined

Table II. Results of in vitro drug combination studies. The most effective combination was AB-FC one-half MIC, i.e. each drug at MIC/2 concentration, in which CFU were reduced more than ten fold below the MIC for the most effective single drug. Single drugs and drug combinations other than MIC/2 pairs are shown for comparative purposes only.

$T_o = 10^6$ CFU/ml

	µg/ml	CFU[a]/ml Day 1	Day 3
Untreated controls		2.6×10^7	9.0×10^7
Single drugs:			
T-cin/2	.25	5.1×10^6	6.7×10^6
AB/2	.1	1.9×10^5	9.2×10^7
FC/2	2.5	8.9×10^5	3.5×10^7
T-cin/4	.125	1.6×10^7	5.6×10^7
AB/4	.05	7.0×10^6	8.0×10^7
FC/4	1.25	1.2×10^6	5.6×10^7
Combinations:			
AB/2 - FC/2		5.8×10^3	6.3×10^3
AB/2 - T-cin/2		6.3×10^4	4.3×10^5
FC/2 - T-cin/2		9.4×10^5	9.5×10^5
FC/4 - T-cin/4		8.8×10^5	9.9×10^5
AB/4 - FC/4		2.3×10^5	4.0×10^6
AB/4 - T-cin/4		1.1×10^6	1.2×10^7
AB/2 - FC/4		8.7×10^3	6.3×10^4
AB/4 - T-cin/2		2.5×10^5	7.0×10^5
FC/2 - T-cin/4		8.8×10^5	1.0×10^6
FC/4 - T-cin/2		9.4×10^5	1.0×10^6
AB/4 - FC/2		1.0×10^5	1.5×10^6
AB/2 - T-cin/4		1.3×10^5	6.7×10^6

[a]Colony forming units

Figure 1. Inhibition of DNA synthesis by single drugs each at one-half MIC (MIC/2) level and by two drug combinations. Points where differences are significant are marked by an arrow. Although drug combinations were usually below single drug counts, differences were often insignificant and continuous inhibition by the combinations was not observed.

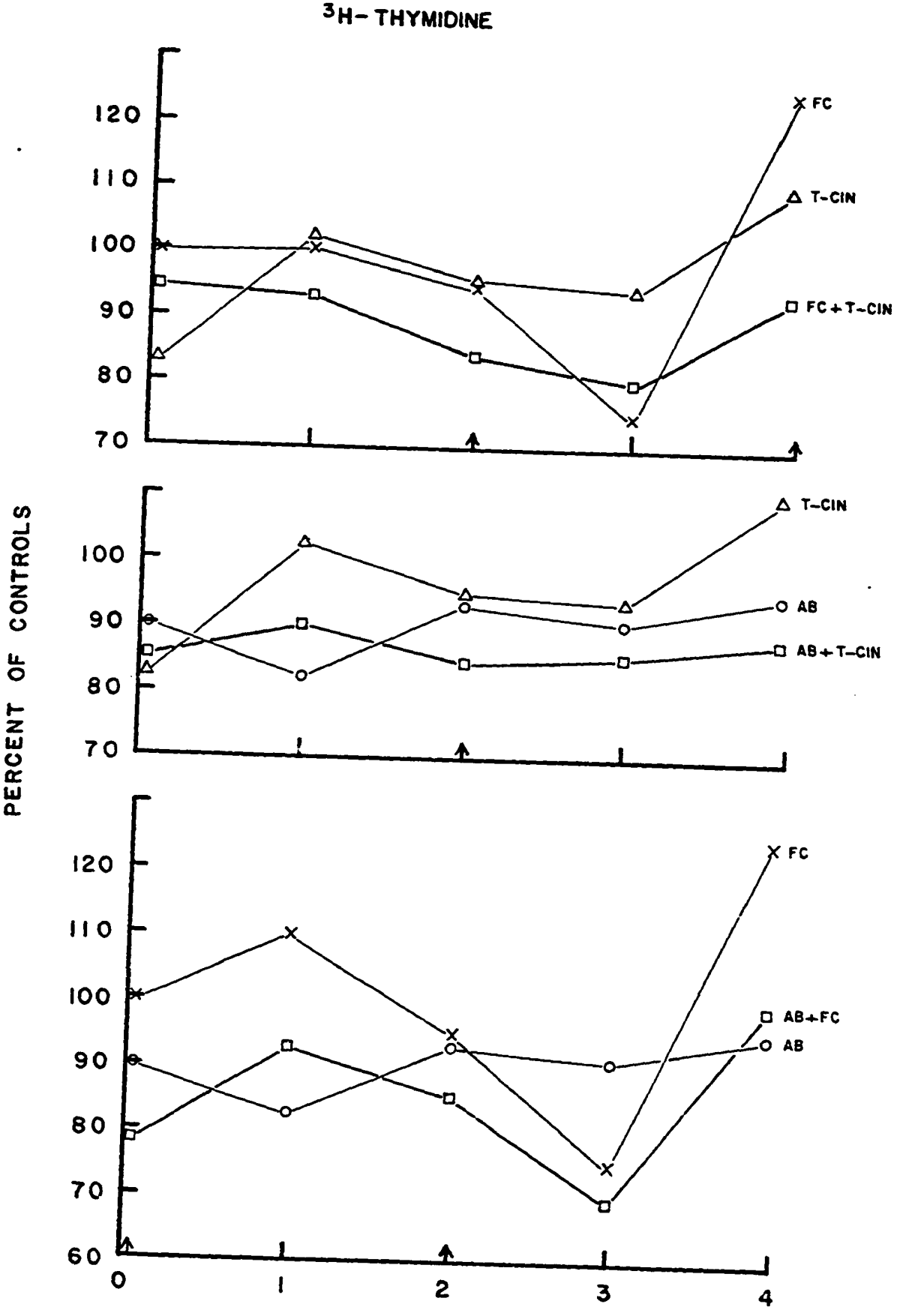

^{3}H- THYMIDINE
PERCENT OF CONTROLS
120
110
100
90
80
70
FC
T-CIN
FC+T-CIN
100
90
80
70
T-CIN
AB
AB+T-CIN
120
110
100
90
80
70
60
FC
AB+FC
AB
0
1
2
3
4

Figure 2. Inhibition of RNA synthesis by single drugs each at one-half MIC (MIC/2) level and by two drug combinations. Points where differences are significant are denoted by an arrow. Marked inhibition by all drugs and combinations is observed. The most effective drug pair appears to be AB-Tcin where percentage inhibition is well below single drug effects for the entire four hour period.

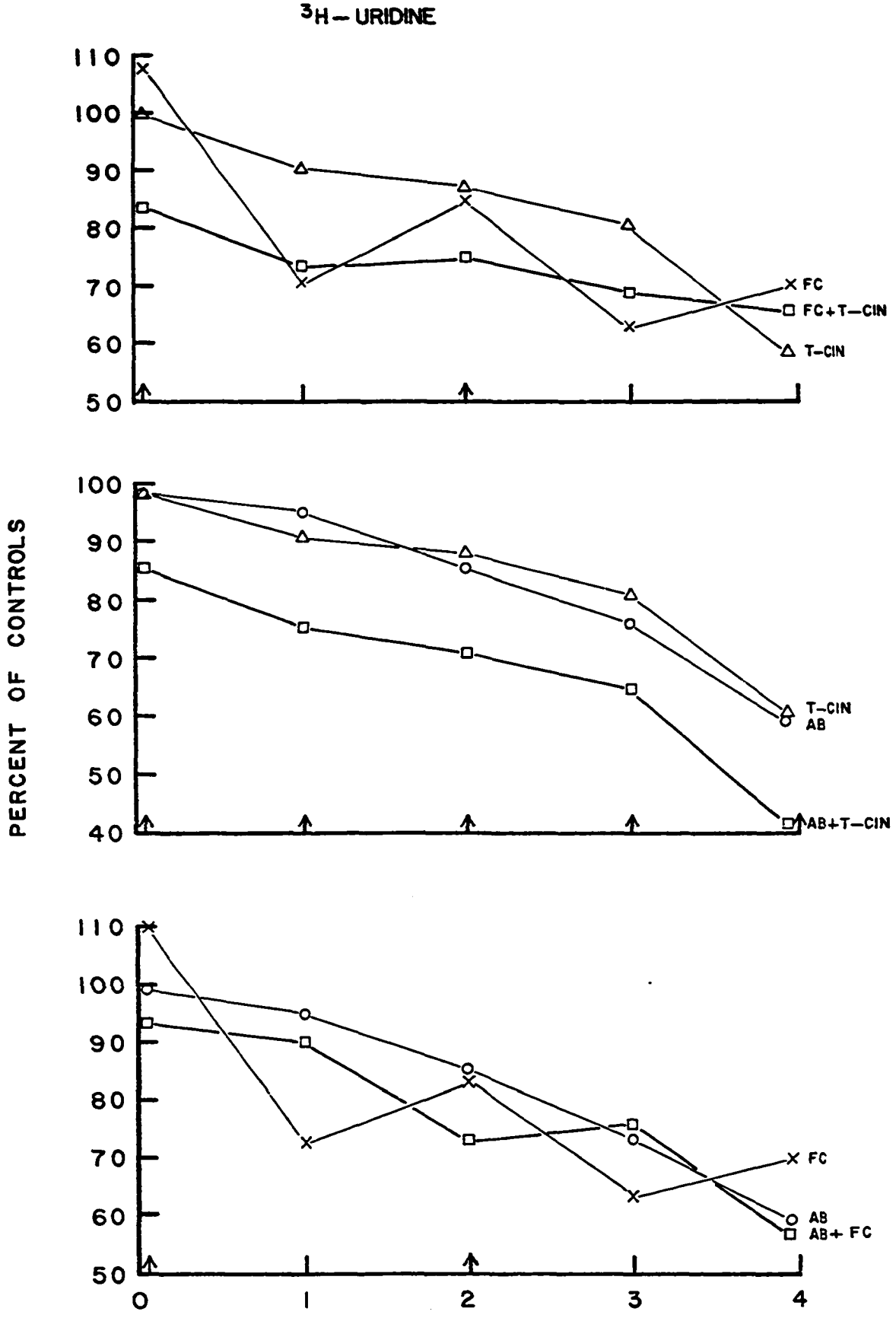

^{3}H – URIDINE
PERCENT OF CONTROLS
110
100
90
80
70
60
50
FC
FC+T–CIN
T–CIN
100
90
80
70
60
50
40
T–CIN
AB
AB+T–CIN
110
100
90
80
70
60
50
FC
AB
AB+ FC
0
1
2
3
4

Figure 3. Inhibition of protein synthesis by single drugs each at one-half MIC (MIC/2) level and by two drug combinations. Points where differences are significant are marked by an arrow. Drug combinations appeared to have very little effect on protein synthesis.

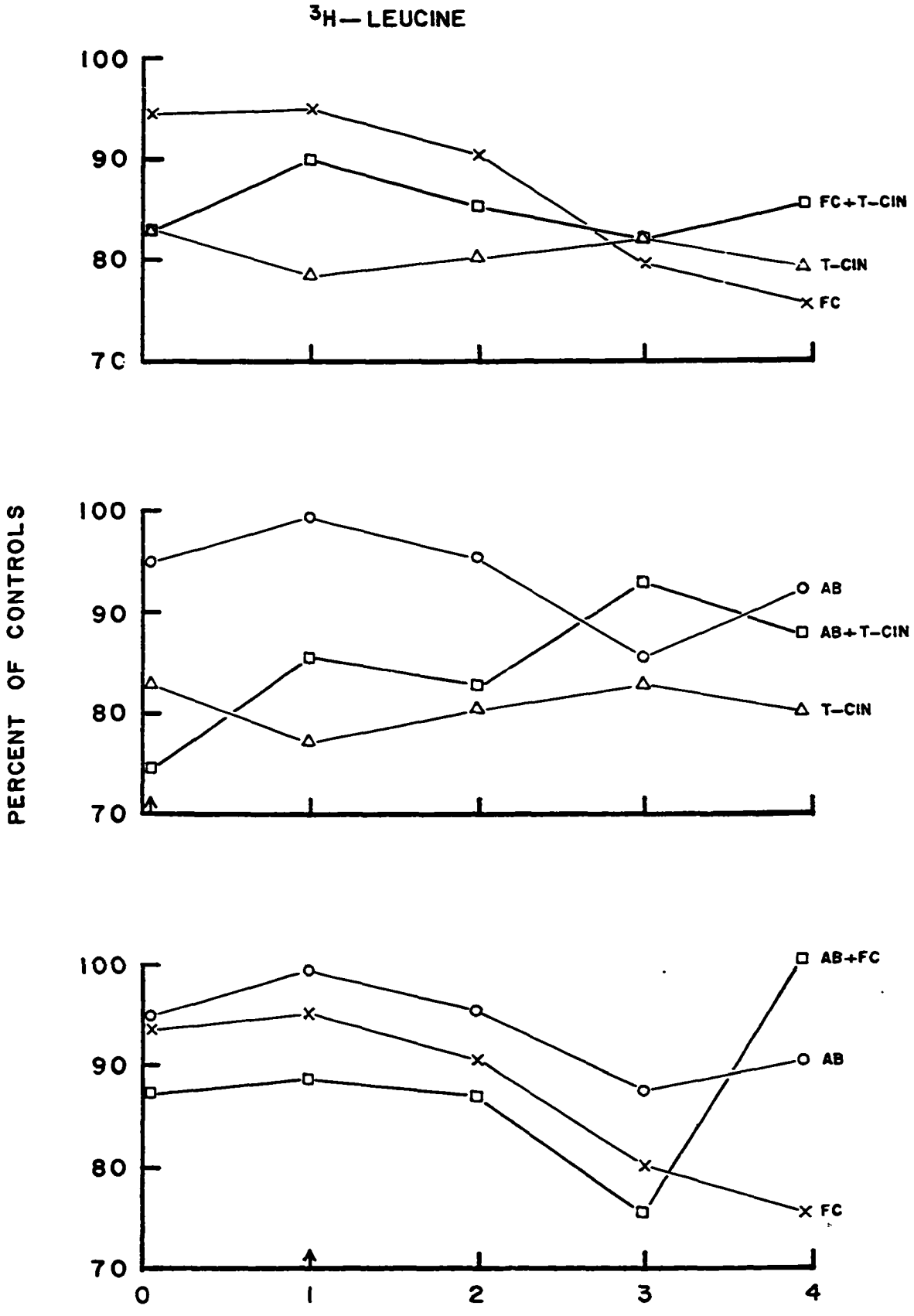

^{3}H—LEUCINE
PERCENT OF CONTROLS
100
90
80
70
FC+T-CIN
T-CIN
FC
AB
AB+T-CIN
T-CIN
AB+FC
AB
FC
0
1
2
3
4

Figure 4. A comparison of the three two-drug combinations is shown for each of the cellular processes studied. Points where differences are significant are denoted by an arrow. O = AB-FC, X = AB-T-cin and □ = FC-T-cin. The AB-T-cin combination was the most effective of the three in terms of inhibition of RNA and protein synthesis. The AB-FC combination was the most effective in terms of DNA inhibition. The FC-T-cin pair was relatively ineffective compared with the other two combinations.

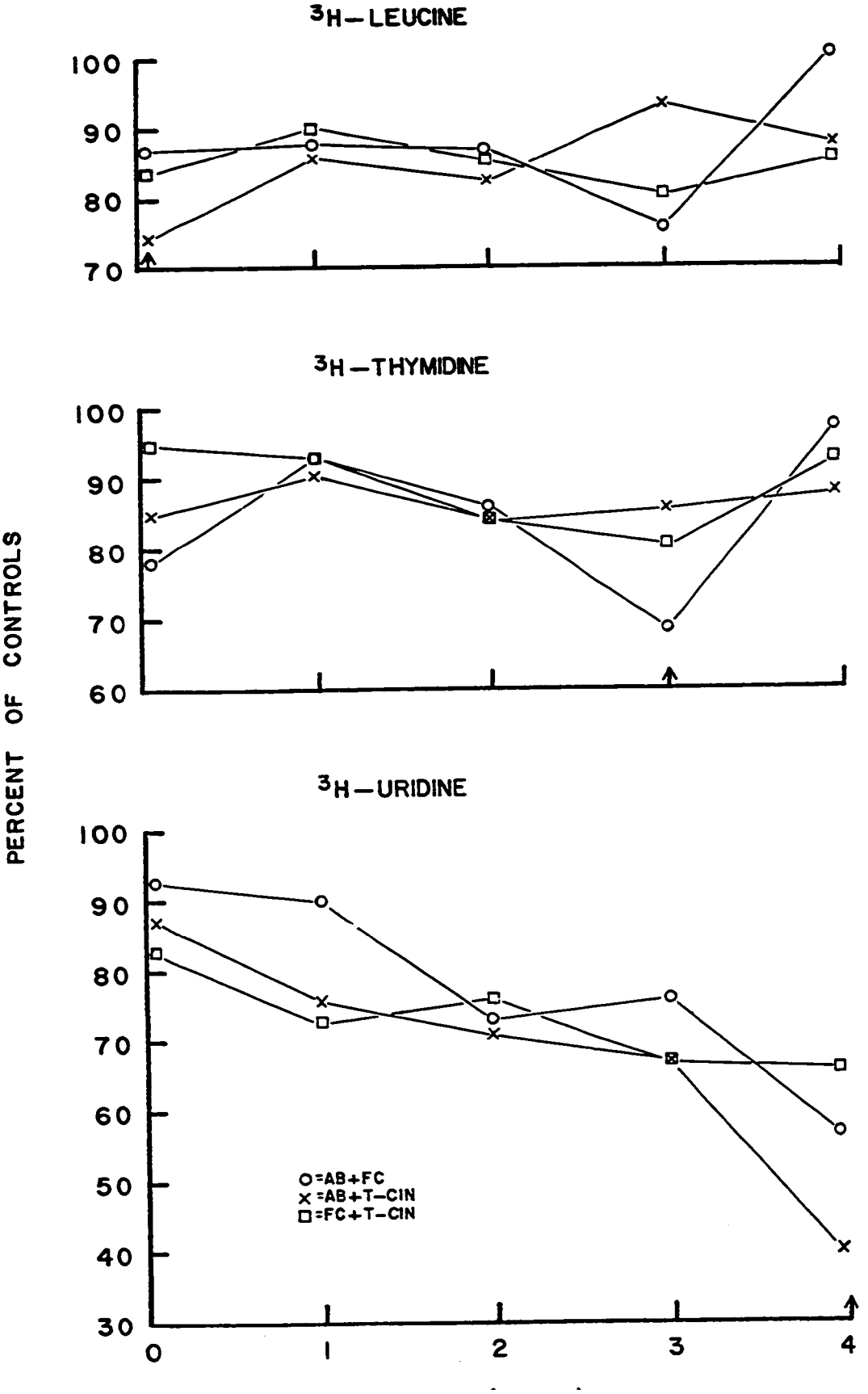

^{3}H—LEUCINE
100
90
80
70
^{3}H—THYMIDINE
100
90
80
70
60
PERCENT OF CONTROLS
^{3}H—URIDINE
100
90
80
70
60
50
40
30
0
1
2
3
4
○=AB+FC
×=AB+T-CIN
□=FC+T-CIN

Figure 5. Mortality curves for amphotericin B (graph A) and 5-fluorocytosine (graph B) compared with untreated controls. The ED_{50} for amphotericin B was established at .38 mg/kg and 100 mg/kg for 5 fluorocytosine.

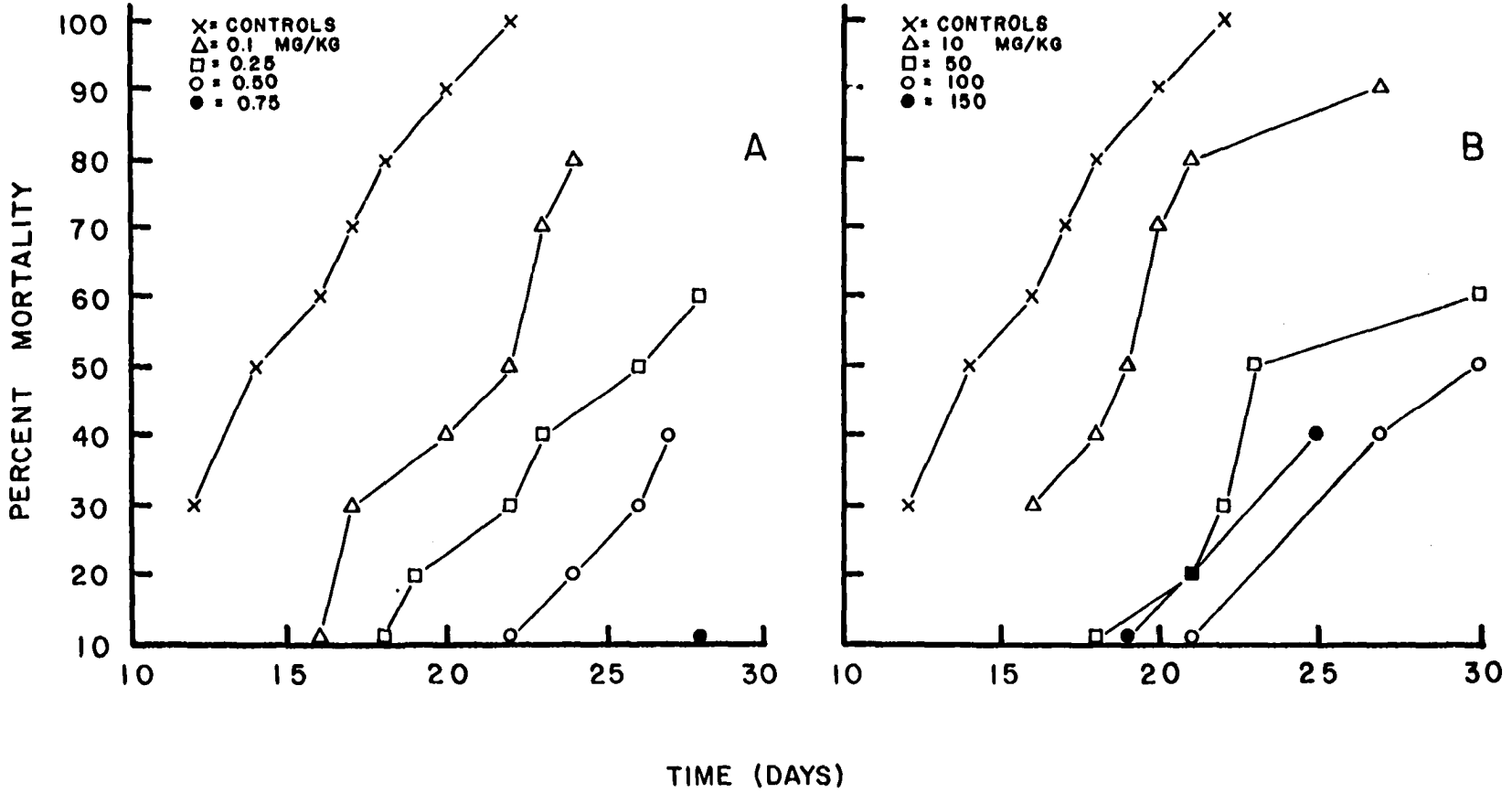

X= CONTROLS
△= 0.1 MG/KG
□= 0.25
○= 0.50
● = 0.75
A
X= CONTROLS
△= 10 MG/KG
□= 50
○= 100
● = 150
B
PERCENT MORTALITY
100
90
80
70
60
50
40
30
20
10
10
15
20
25
30
TIME (DAYS)

Figure 6. Graph C: Mortality curves for trichothecin (T-cin) treated mice. All groups contained five animals except controls which had ten mice. The longest survival time of the dosages tested was offered by the 1.0 mg/kg regimen. No ED_{50} could be obtained since 80-100% death occurred in all cases. Graph D: Mortality curves for infected high dose T-cin treated mice compared with untreated controls. The five high dose T-cin controls from experiment 2 (50 mg/kg) were infected with C. neoformans on day 33. In contrast to T-cin treated mice in experiments 1 and 2 these mice demonstrated a higher mortality rate than controls. Mean life expectancy (MLE) and its standard error ($S_{\bar{D}}$) are shown. MLE was not significantly affected.

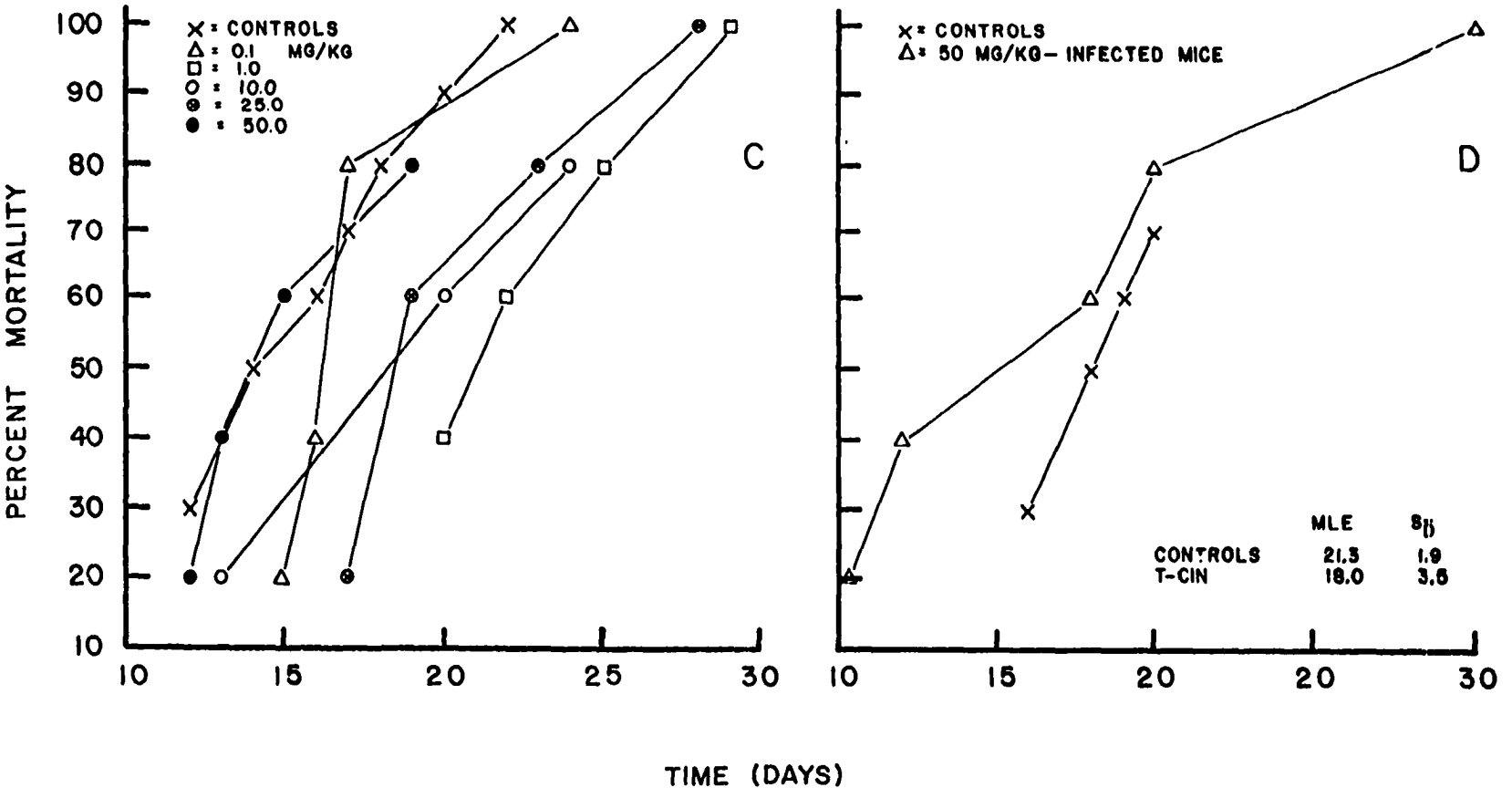

PERCENT MORTALITY
100
90
80
70
60
50
40
30
20
10
× = CONTROLS
△ = 0.1 MG/KG
□ = 1.0
○ = 10.0
⊛ = 25.0
● = 50.0
C
10
15
20
25
30
× = CONTROLS
△ = 50 MG/KG – INFECTED MICE
D
MLE
$S_{(i)}$
CONTROLS 21.5 1.9
T-CIN 18.0 3.5
10
15
20
20
30
TIME (DAYS)

Table III. Summary of single drug animal experiments 1 and 2. Experiment 1 consisted of C. neoformans infected mice treated with T-cin only. In experiment 2 infected mice were treated with T-cin, AB or FC.

Drug (mg/kg)	No. of mice tested	% survivors at 30 days	mean life expectancy (days)	standard error of mean
Experiment 1				
Controls	20	5	17.0	0.8
T-cin 1.0	10	10	18.9	1.6
2.5	10	0	14.7	1.4
5.0	10	10	16.9	1.8
7.5	10	20	17.5	2.3
10.0	10	0	17.7	1.8
Experiment 2				
Controls	10	0	15.7	1.1
T-cin .1	5	0	17.8	1.6
1.0	5	0	23.4	1.9
10.0	5	20	19.3	2.3
25.0	5	0	21.2	2.0
50.0	5	20	14.8	1.5
AB[a] .10	10	20	20.3	1.1
.25	10	40	22.7	1.6
.50	10	60	24.8	1.1
.75	10	90	29.8	0.2
1.0	10	90	29.8	0.2
1.5	10	100	30.0	0
FC[b] 10	10	10	19.2	1.2
50	10	40	22.8	1.6
100	10	50	26.4	1.5
150	10	60	22.5	1.5

[a] ED_{50} = .38 mg/kg

[b] ED_{50} = 100 mg/kg

Figure 7. Results of the combined drug experiment expressed in terms of percent survivors at 30 days. Drug combinations consist of each drug of the pair at the ED_{50} or $ED_{50}/2$ (one-half ED_{50}) concentration; T-cin in these respective combinations is at 1.0 and 0.5 mg/kg. The 1.0 mg/kg T-cin controls are shown. Both AB-FC combinations showed additive effects while synergy was demonstrated with the AB-T-cin combinations since T-cin is inactive when used alone.

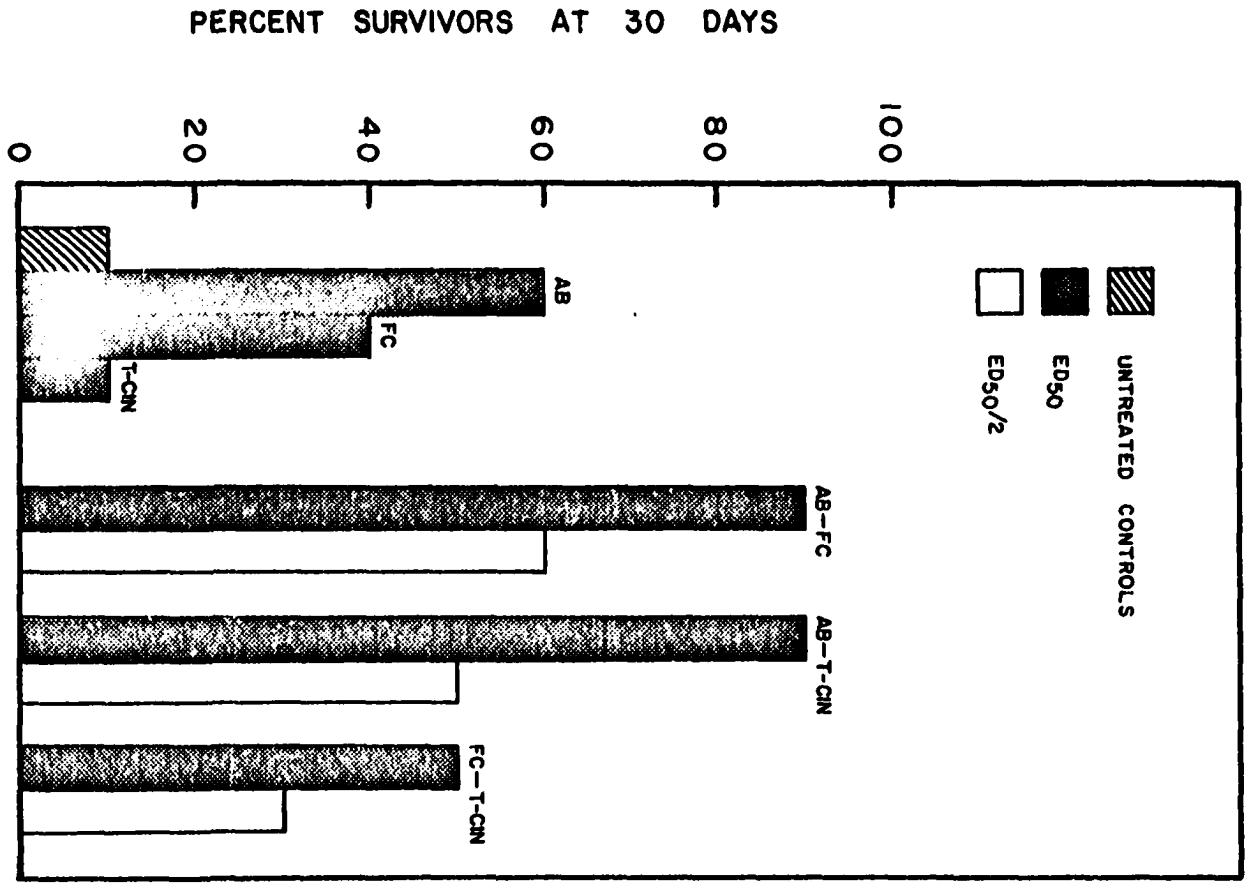

PERCENT SURVIVORS AT 30 DAYS
0
20
40
60
80
100
UNTREATED CONTROLS
ED50
ED50/2
AB
FC
T-CIN
AB-FC
AB-T-CIN
FC-T-CIN

Figure 8. Results of the combined drug experiment expressed in terms of mean life expectancy (MLE). Drug combinations consist of each drug at ED_{50} or $ED_{50}/2$ (one-half ED_{50}) concentration; T-cin in these respective combinations is at 1.0 and 0.5 mg/kg. The 1.0 mg/kg T-cin control group is shown. The relative positions of all combinations approximates that of Figure 7. The FC-T-cin $ED_{50}/2$ group showed a significantly lower MLE than the controls.

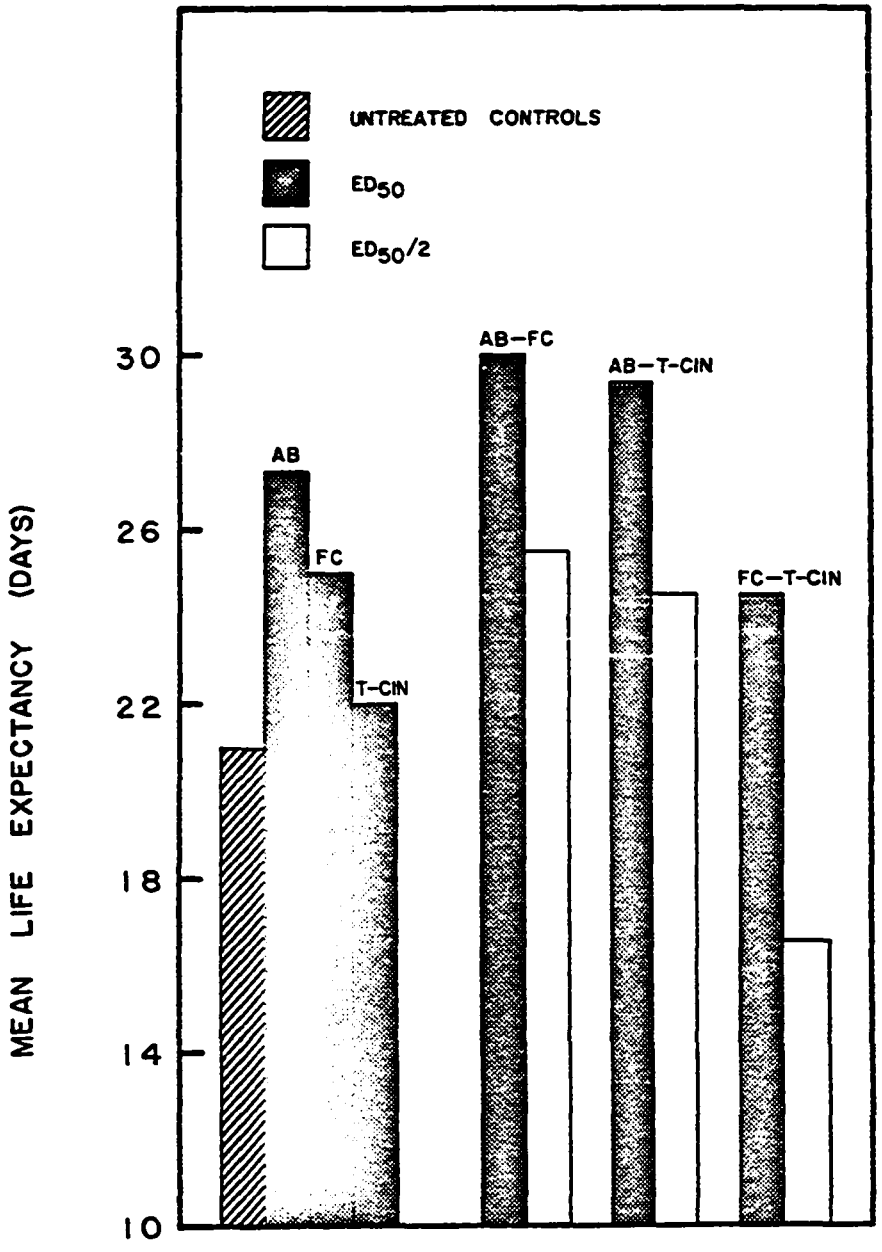

UNTREATED CONTROLS
ED50
ED50/2
MEAN LIFE EXPECTANCY (DAYS)
30
26
22
18
14
10
AB
FC
T-CIN
AB—FC
AB—T-CIN
FC—T-CIN

Figure 9. Results of the combined drug experiment expressed in terms of cumulative total weight gain per mouse after 30 days. Drug combinations consist of each drug at ED_{50} and $ED_{50}/2$ (one-half ED_{50}) concentrations; T-cin in these respective combinations is at 1.0 and 0.5 mg/kg. The 1.0 mg/kg T-cin control group is shown. All high dose drug combinations were effective in preventing significant weight loss and the FC-T-cin low dose group was the least effective. Low dose combinations are shown for comparative purposes only.

CUMULATIVE TOTAL WEIGHT GAIN
PER MOUSE (GMS) AFTER 30 DAYS

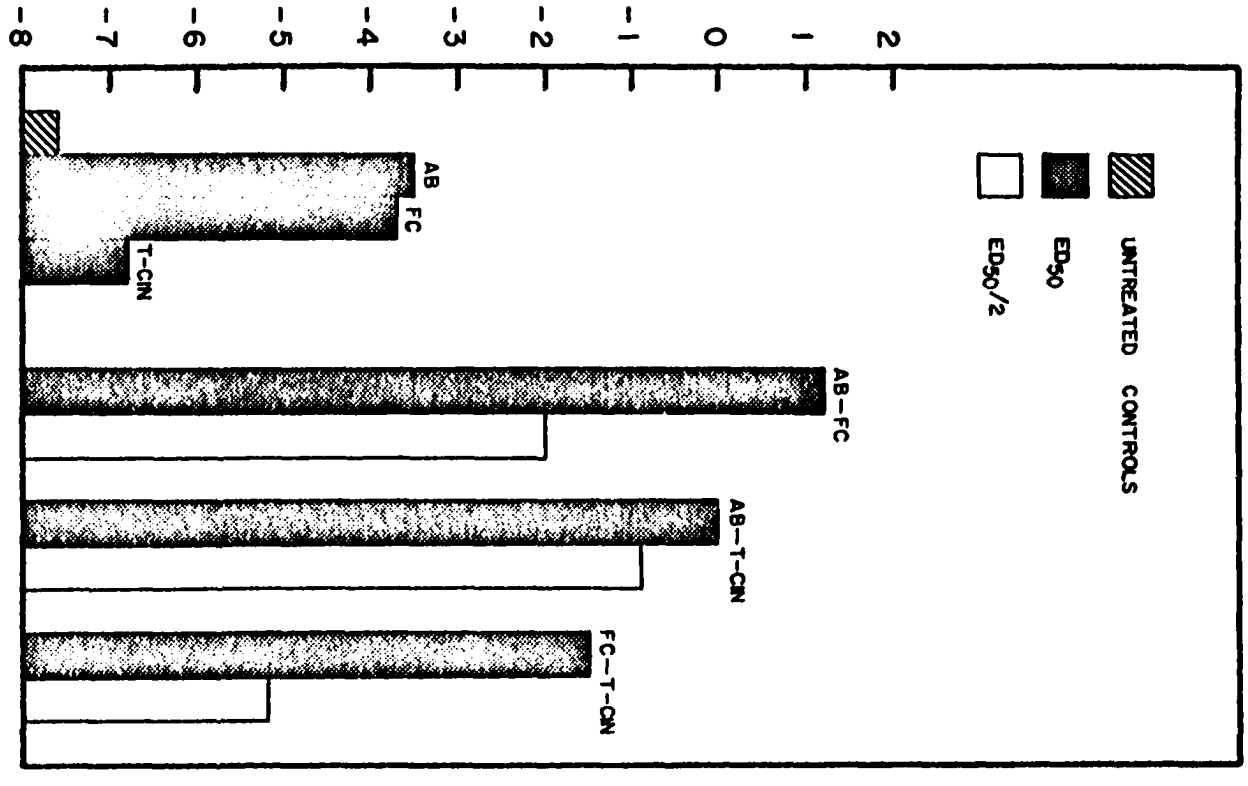


Table IV. Summary of combined drug experiment (experiment 3). All experimental groups contained 10 mice. Drug pairs at ED_{50} level contained AB or FC at the ED_{50} dosage with T-cin at 1.0 mg/kg. Drug pairs at $ED_{50}/2$ level (each drug at one-half ED_{50} dosage) contained AB or FC at $ED_{50}/2$ with T-cin at 0.5 mg/kg.

Group	% survivors at 30 days	MLE[c] (days)	Std. Error (mean)	Fraction of positive cultures		Total wt. gain per mouse (gms) cumulative
				Spleen	Brain	
Experiment 3						
Controls	10	21.0	1.5	1/1	1/1	-7.6
AB ED_{50}	60	27.3	1.3	6/6	6/6	-3.5
FC ED_{50}	40	25.3	2.3	4/4	3/4	-3.7
T-cin 1.0 mg/kg	10	22.1	1.6	1/1	1/1	-6.8
T-cin 0.5 mg/kg	0	18.9	1.9	0	0	-7.3
AB-FC ED_{50}	90	30.0	0	7/9	7/9	+1.2
AB-FC $ED_{50}/2$	60	25.5	0.5	6/6	6/6	-2.0
AB-T-cin ED_{50}[a]	90	29.3	0.7	7/9	9/9	0
AB-T-cin $ED_{50}/2$[b]	50	24.4	2.7	5/5	5/5	-1.1
FC-T-cin ED_{50}[a]	50	24.4	0.9	3/5	5/5	-1.5
FC-T-cin $ED_{50}/2$[b]	30	16.4	1.5	3/3	3/3	-5.2
T-cin high dose	100	30.0	0	5/5	5/5	-4.6

[a] T-cin at 1.0 mg/kg

[b] T-cin at 0.5 mg/kg

[c] MLE = Mean life expectancy

CHAPTER IV

DISCUSSION

The MIC of AB against C. neoformans strain 184 was established at 0.2 µg/ml. This is supported by Shadomy et al. (38) who report 80% of 77 strains of this yeast to have an MIC of 0.2 µg/ml or less for AB. Nearly 100% of the FC pretreatment isolates of C. neoformans studied by Shadomy (46) and all 55 of those of Block et al. (35) were shown to be sensitive to 10 µg/ml or less of the drug for a 48 hour period, in agreement with our results of 5 µg/ml. After this period of time nine percent of the strains of Block et al. (35) developed drug resistance. This might help explain the high concentrations of FC necessary to suppress growth for longer periods of time in these experiments. The MIC of T-cin against C. neoformans has not been established, although Freeman (21) reports that this yeast is sensitive to 16 µg/ml or less of T-cin. I am reporting the MIC of T-cin to be 0.5 µg/ml for the strain of C. neoformans used in this study.

An examination of cell counts for one day only shows that of the MIC/2 combinations, AB-FC reduced CFU ca. 11-fold

below the AB-T-cin pair which in turn gave counts ca. 15-fold below the FC-T-cin pair. The difference between the first and last of these three pairs is over 160-fold. No clearcut quantitative differences could be observed in the radioisotope experiments even though the same drug concentrations and medium were employed. Therefore these experiments could not clearly define the mechanisms by which this synergism was occurring. The statement by Medoff et al. seems particularly appropos. "The effects of the drugs on shortterm growth or RNA synthesis are much less marked than the reduction in colony counts at 7 days but they are consistent with a cumulative inhibition that finally kills the cells" (13).

Fluctuations between single drugs and their combinations is often a time dependent occurrence (1) and in our case is exemplified by the changing ratios of the combinations to one another or to their controls in viability counts at one and three days. Assuming this fluctuation may also occur on the molecular level different combinations appear effective at different times. Of the three combinations tested AB-FC significantly demonstrates the lowest relative counts in DNA synthesis at three hours and the AB-T-cin pair the lowest relative counts in protein and RNA synthesis at one and four hours respectively.

In their study regarding AB and FC synergy on the cellular level Medoff et al. (13) were able to show that less than one-half MIC of AB was capable of potentiating the effects of FC and rifampicin in C. albicans. On the other hand,

these concentrations of AB alone resulted in no marked inhibition in RNA synthesis. Kwan et al. (28) reported similar results in Saccharomyces cerevisiae. Marked inhibition of RNA synthesis caused by T-cin is difficult to justify and is in conflict with published reports on other trichothecenes (43,48,49). This difference is as yet unexplained. The effects of FC of RNA synthesis in yeast have been described (19,20). It is also difficult to explain the apparent cytocidal effect of the AB-FC and AB-T-cin combinations in viability studies (Table II) on the basis of four hour isotope experiments. Viability platings made at the same time that isotope data points are taken might reveal a closer correlation between the two.

In the study by Block and Bennett (31) in which AB and FC were used to treat mice experimentally infected with C. neoformans, they ascertained the ED_{50} of FC to be 50 mg/kg when ip injections were given daily. In this experiment 100 mg/kg was administered on an alternate day basis, for equivalent dosage. These authors have also reported a ED_{50} of .86 mg/kg for AB when injected intravenously. In this work the value was .38 mg/kg ip. The difference could be due to the strain of mouse used and route of inoculation as well as to other variables (37).

The AB-FC ED_{50} group permitted 90% survival in mice, however total drug concentrations equalled twice as much as individual ED_{50} controls. Since doubling the ED_{50} concentration of AB also gives improved survival rate and MLE, it

seems likely that the same holds true for FC.

Although the AB-FC MIC/2 group was the most effective in vitro, the AB-FC $ED_{50}/2$ group demonstrated only additive effects in vivo, similar to the results obtained by Block and Bennett (31). These authors did not state whether their strain of C. neoformans had demonstrated synergism with these two drugs in vitro.

At the concentrations tested the in vitro experiments suggest only an additive effect when AB and T-cin are used in combination. Yet synergy appears to be occurring with both AB-T-cin ED_{50} and $ED_{50}/2$ combinations in vivo. Increased effect due to the addition of an inactive agent is clearly synergistic (47). One possible explanation for this difference between in vitro and in vivo results is that T-cin may be biologically modified in the mouse into a compound that is also active against C. neoformans. This compound might be less permeable to the yeast cell than is T-cin, unless it is assisted by AB, but not FC. This may explain why an effect approximating that of FC when used alone is achieved in the FC-T-cin combinations in vivo. The data presented in Figure 6C and the fact that high dose T-cin controls survive despite the large cumulative amount of toxin received suggests that T-cin is inactivated in the mouse. Supportive evidence for the degradation of T-cin and crotocin is offered by Horvath and Varga (50) who showed that these compounds were able to induce the synthesis of enzymes in Penicillium which lead to

their inactivation by ester cleavage. In addition, Glaz et al. (27) found that intravenous injection of crotocin at two to four mg per mouse resulted in a serum level ten times lower than expected. These authors point out that although the antifungal effect of crotocin and T-cin is inactivated in human or mouse serum the alcohol component of crotocin is not inactivated and that it is easily soluble in water. The close structure-activity relationship between crotocin and T-cin suggests that similar physical and biological properties can be expected of T-cin. Structure-activity relationships and the relative solubility of trichothecene parent alcohols is reviewed by Bamburg and Strong (25)and Wei and McLaughlin (44).

High dose T-cin control mice demonstrated clinical signs only when mice were given near lethal doses, results similar to those of Kosuri et al. (34) who studied T-2 toxin in rats. There was a gradual loss of body weight in our mice not seen in solvent treated controls which reached a minimum after mice had received 500 mg/kg T-cin. Thereafter recovery was rapid. The gradual loss of weight suggests a lack of tolerance to the concentrations of drug used. The survival and apparent recovery of these mice by the conclusion of the experimental period did not rule out possible adverse effects of T-cin on the mammalian system. Subsequent infection of this group with C. neoformans resulted in 100% mortality compared with 70% mortality in the control group. The survival of 30% of the controls may be due to the physical and

immunological maturity of these mice which were now 10 to 12 weeks old as compared to the six to eight week old mouse, upon which initial dosages were based. A greater resistence to the pathogen would be expected in the mature mouse. Therefore the enhanced death rate observed in T-cin treated mice suggests toxic effects of high concentrations of T-cin in these animals. This is supported by our histopathological findings on mice injected with a single high dosage of T-cin.

In conclusion, although T-cin is active in vitro against C. neoformans it may be rapidly degraded in the mouse and is ineffective when used alone in the treatment of systemic cryptococcosis. In vitro the AB-T-cin combination demonstrated an additive effect while marked synergism was noted for this drug pair in vivo. A more comprehensive study of this drug combination using low concentrations of T-cin and AB coupled with histopathological studies might reveal more concerning the relative toxicity of this drug pair compared with AB alone and hence better describe the chemotherapeutic efficacy of T-cin.

LITERATURE CITED

1. Jawetz, E., and J.B. Gunnison. 1953. Antibiotic synergism and antagonism: an assessment of the problem. Pharm. Rev. 5:175-192.

2. Jawetz, E. 1967. Combined antibiotic action: some definitions and correlations between laboratory and clinical results. Antimicrob. Ag. Chemother. 203-209. 1968.

3. Hoeprich, P.D., and P.D. Finn. Activity of combinations of antifungal agents against Candida albicans and Cryptococcus neoformans (abstract). In Proceedings of the 72nd Meeting of the American Society for Microbiology, Washington, D.C. 1972. p. 135.

4. Shadomy, S., and B.A. Davis. In vitro and in vivo studies with combinations of amphotericin B and 5-fluorocytosine. In Proceedings of the 13th Interscience Conference on Antimicrobial Agents and Chemotherapy. 1973.

5. Garriques, I.L., M.A. Sande, J.P. Utz, G.L. Mandell, J.F. Warner, R.F. McGehee, and S. Shadomy. Combined amphotericin B-flucytosine chemotherapy in human cryptococcosis (abstract). In Proceedings of the 13th Interscience Conference on Antimicrobial Agents and Chemotherapy. 1973.

6. Utz, J.P., J.E. Bennett, M.W. Brandriss, W.T. Butler, and G.J. Hill. 1964. Amphotericin B toxicity. Ann. Intern. Med. 61:334-354.

7. Doughas, J.B., and J.K. Healy. 1969. Nephrotoxic effects of amphotericin B, including renal tubular acidosis. Am. J. Med. 46:154-162.

8. Fass, R.J., and R.L. Perkins. 1971. 5-fluorocytosine in the treatment of cryptococcal and candida mycoses. Ann. Intern. Med. 74:535-539.

9. Block, E.R., A.E. Jennings, and J.E. Bennett. 1973. 5-fluorocytosine resistance in Cryptococcus neoformans. Antimicrob. Ag. Chemother. 3:649-656.

10. Kinsky, S.C. 1963. Membrane sterols and the selective toxicity of polyene antifungal antibiotics. Antimicrob. Ag. Chemother. 387-394. 1964.

11. Kinsky, S.C. 1961a. The effect of polyene antibiotics on permeability in Neurospora crassa. Biochem. Biophys. Res. Commun. 4:353-357.

12. Kinsky, S.C. 1961b. Alterations in the permeability of Neurospora crassa due to polyene antibiotics. J. Bacteriol. 82:889-897.

13. Medoff, G., G.S. Kobayashi, C.N. Kwan, D. Schlessinger, and P. Venkov. 1972. Potentiation of rifampicin and 5-fluorocytosine as antifungal antibiotics by amphotericin B. Proc. Nat. Acad. Sci. U.S.A. 69:196-199.

14. Pankey, G.A., W.R. Lockwood, and J.M. Montalvo. 1972. 5-Fluorocytosine: a replacement for amphotericin B in the treatment of candida and cryptococcus infections. J. La. State Med. Soc. 122:365-369.

15. Utz, J.P., B.S. Tynes, H.J. Shadomy, R.J. Duma, M.M. Kannan, and K.N. Mason. 1968. 5-fluorocytosine in human cryptococcosis. Antimicrob. Ag. Chemother. 344-346. 1969.

16. Malbica, J., L. Sello, B. Tabenkin, J. Berger, E. Grunberg, J.H. Burchenal, J.J. Fox, I. Wempen, T. Gabriel, and R. Duchinsky. 1962. Some biological properties of 5-fluorocytosine and its derivatives. Fed. Proc. 21: 384.

17. Grunberg, E., E. Titsworth, and M. Bennett. 1964. Chemotherapeutic activity of 5-fluorocytosine. Antimicrob. Ag. Chemother. 566-568. 1963.

18. Block, E.R., and J.E. Bennett. 1972. Clinical and pharmacological studies with 5-fluorocytosine (5-FC). Clin. Res. 20:525.

19. Kempner, E.S., and J.H. Miller, 1963. The mechanism of action of purine and pyrimidine analogs in microorganisms. Biochem. Biophys. Acta. 76:341-346.

20. Polak, A., and H.J. Scholer. 1973. Fungistatic activity, uptake and incorporation of 5-fluorocytosine in Candida albicans as influenced by pyrimidines and purines. II. Studies on distribution and incorporation. Path. Microbiol. 39:334-347.

21. Freeman, G.G. 1955. Further biological properties of trichothecin, an antifungal substance from Trichothecium roseum Link and its derivatives. J. Gen. Microbiol. 12:213-221.

22. Cundliffe, E., M. Cannon, and J. Davies. 1974. Mechanism of inhibition of eukaryotic protein synthesis by trichothecene fungal toxins. Proc. Nat. Acad. Sci. U.S.A. 71:30-34.

23. Schindler, D. 1974. Two classes of inhibitors of peptidyl transferase activity in eukaryotes. Nature (London). 249:38-41.

24. Loevens Kemiske Fabrik Produktionsaktieselskab. 1964. Netherlands Patent 320,527. WG 696 A new antibiotic. Chem. Abst. 1965. 1050 f.

25. Bamburg, J.R., and F.M. Strong. 1971. 12,13-Epoxytrichothecenes. In Microbial Toxins, Vol. VIII. Academic Press, N.Y. pp 207-292.

26. Scheiber, E., T. Kakosy, and E.T. Glaz. 1961. Effect of antifungal agents on the faecal yeast flora of mice. Acta. Microbiol. Acad. Sci. Hung. 8:173-179.

27. Glaz, E.T., E. Scheiber, and K. Jarfas. 1960. Studies on a new antifungal antibiotic. Acta Physiologica Acad. Scient. Hung. 18:225-232.

28. Kwan, C.N., G. Medoff, G.S. Kobayashi, D. Schlessinger, and H.J. Raskas. 1972. Potentiation of the antifungal effects of antibiotics by amphotericin B. Antimicrob. Ag. Chemother. 2:61-65.

29. Medoff, G., M. Comfort, and G.S. Kobayashi. 1971. Synergistic action of amphotericin B and 5-fluorocytosine against yeast-like organisms. Proc. Soc. Exp. Biol. Med. 138:571-574.

30. Shadomy, S., G. Wagner, A. Espinel-Ingroff, and B.A. Davis. 1975. In vitro studies with combinations of 5-fluorocytosine and amphotericin B. Antimicrob. Ag. Chemother. 8:117-121.

31. Block, E.R., and J.E. Bennett. 1973. The combined effect of 5-fluorocytosine and amphotericin B in the therapy of murine cryptococcosis. Proc. Soc. Exp. Biol. Med. 142:476-480.

32. Hamilton, J.D., and D.M. Elliott. 1975. Combined activity of 5-fluorocytosine and amphotericin B against Cryptococcus neoformans in vitro and in vivo in mice. J. Inf. Dis. 131:129-137.

33. Sorenson, W.G., M.R. Sneller, and H.W. Larsh. 1975. Qualitative and quantitative assay of trichothecin: a mycotoxin produced by Trichothecium roseum. Appl. Micro. 29:653-657.

34. Kosuri, N.R., E.B. Smalley, and R.E. Nichols. 1971. Toxicologic studies of Fusarium tricinctum (Corda) Synder et Hansen from Moldy Corn. Am. J. Vet. Res. 32:1843-1850.

35. Block, E.R., A.E. Jennings, and J.E. Bennett. 1973. Variables influencing susceptibility testing of Cryptococcus neoformans to 5-fluorocytosine. Antimicrob. Ag. Chemother. 4:392-395.

36. Titsworth, E., and E. Grunberg. 1973. Chemotherapeutic activity of 5-fluorocytosine and amphotericin B against Candida albicans in mice. Antimicrob. Ag. Chemother. 4:306-308.

37. Barnes, C.D., and L.G. Eltherington. Drug dosages in laboratory animals. 2nd ed. Univ. of California Press, Berkeley. 1973.

38. Shadomy, S., H.J. Shadomy, J. McCoy, and J. Utz. 1969. In vitro susceptibility of Cryptococcus neoformans to amphotericin B, hamycin, and 5-fluorocytosine. Antimicrob. Ag. Chemother. 452-460. 1968.

39. Rhoades, E.R., F.G. Felton, J. Wilkus, and H.G. Muchmore. 1968. Susceptibility of human and environmental isolates of Cryptococcus neoformans to amphotericin B. Antimicrob. Ag. Chemother. 736-738. 1967.

40. Glaz, E.T., E. Csanyi, and J. Gyimesi. 1966. Supplementary data on crotocin--an antifungal antibiotic. Nature 212:617-618.

41. Merck Index 8th Ed. Merck and Co., Inc. Pahway, N.J. 1968.

42. Goodman, L.S., and A. Gilman (eds.). The pharmacological basis of therapeutics. 5th ed. The MacMillan Co., N.Y. 1975.

43. Ueno, Y., and K. Fukushima. 1968. Inhibition of protein and DNA synthesis in Ehrlich ascites tumour by nibalenol, a toxic principle of Fusarium nivale-growing rice. Experientia 24:1032-1033.

44. Wei, Cha-Mer, and C.S. McLaughlin. 1974. Structure-function relationship in the 12,13-epoxytrichothecenes. Biochem. Biophys. Res. Commun. 57:838-844.

45. Polak, A. 1974. Determination de la synergie entre la 5-fluorocytosine et l'amphotericine B au moyen de differents modeles in vitro et in vivo. Bull. Soc. Fr. Mycol. Med. III (2) 175-178.

46. Shadomy, S. 1969. In vitro studies with 5-fluorocytosine. Appl. Microbiol. 17:871-877.

47. Lacey, B.W. 1958. Mechanisms of chemotherapeutic synergy. The strategy of chemotherapy, 8th Symposium of the Society for General Microbiology. pp.247-287.

48. Ohtsubo, K., Y. Masa-atsu, and S. Mamoru. 1968. Inhibitory effect of nivalenol, a toxic metabolite of Fusarium nivale, on the growth cycle and biopolymer synthesis of HeLa cells. Jap. J. Med. Sci. Biol. 21:185-194.

49. Nakano, N. 1968. Inhibitory effects of fusarenon on multiplication of Tetrahymena pyriformis. Jap. J. Med. Sci. Biol. 21:351-357.

50. Horvath, I., and J.M. Varga. 1961. Enzymatic inactivation of trichothecin and crotocin. Nature 192:88.

APPENDIX I

FILTRATE AND MYCELIAL QUANTITATION OF TRICHOTHECIN BY MULTIPLE ASSAY TECHNIQUES

In a previous paper Sorenson et al. (1) described the development of three assay techniques for the quantitation of trichothecin, a mycotoxin produced by the saprophytic mold, Trichothecium roseum. These techniques included a colorimetric assay (Poltorak), a bioassay using Candida albicans as the test organism, and quantitative thin layer chromatography (TLC). These methods were shown to be in close agreement with one another when applied toward the quantitation of T-cin in filtrate fractions under the growth conditions described. In subsequent work with the bioassay and Poltorak method, discrepancies were occasionally noted. This appeared to be especially true in attempts to quantitate the amount of toxin present in the mycelium. In addition, the relative values of these two assay methods occasionally varied with different strains (1).

Both the bioassay and Poltorak methods of assay are semiquantitative. Since many carbon tetrachloride (CCl_4) soluble compounds are capable of inhibiting C. albicans in addition to T-cin their presence could cause an increase in zone size in the bioassay. Although intended as a specific assay for T-cin, the Poltorak method is also subject to

variation in that the final blue color can be masked by the presence of reactive metabolic biproducts. These metabolites may react in the sulfuric acid step of the reaction but not the methanol step. The darkened solution therefore tends to obscure the colorimetric reaction.

It appeared that a more thorough comparative study of these assay techniques might provide evidence for the presence of other toxins as well as information regarding the metabolism of T-cin by T. roseum. In order to determine conditions of maximum toxin production as measured by two or more of the techniques described above, an active toxigenic strain of T. roseum (NRRL 1665) was grown under varying culture conditions using either glucose or sucrose as a carbon source.

MATERIALS AND METHODS

All growth experiments were carried out using 2.8 liter Fernback flasks containing 750 ml of the medium of Freeman and Morrison (2). Each flask was inoculated with a 10-day old slant of T. roseum grown on malt extract agar. All flasks were incubated in the dark.

Two experiments were conducted. In the first experiment glucose and sucrose were used separately as carbon sources at varying concentrations and at ambient room temperature for a three-week time period. Each carbon source was tested in duplicate flasks at concentrations of 50, 60, 70, 80, and 90 grams/liter. After three weeks the flasks were autoclaved. The culture was then filtered through a double thickness of cheese cloth, extracted, and quantitated by bioassay and Poltorak method, as previously described (1). The remaining mycelium was washed with three 100 ml portions of distilled water, removed from the flask, allowed to drain through cheese cloth for a few minutes and then dried out for three hours at 110 C. Each mycelial sample was then powdered in a mortar and weighed. This experiment was conducted twice and the results averaged.

In the second experiment sucrose was used at a concentration of 80 grams/liter since experiment one had shown

maximum T-cin production at this level. Both filtrate and mycelium were assayed at two, three and four weeks and at 20 C, ambient room temperature and 28 C. The methods of quantitation were bioassay, Poltorak method and TLC. The latter was applied to the filtrate but not to the mycelial fraction because of the viscous nature of the mycelial extract and because of the presence of a high concentration of metabolic biproducts which tended to obscure the trichothecin spot. The TLC solvent system used was benzene-ethyl acetate (95:5) double development (1).

The filtrate and mycelial fractions were prepared as in the first experiment. Each mycelial sample was then extracted in a Soxhlet apparatus for 24 hours with CCl_4, evaporated in vacuo and reconstituted to 10 ml in CCl_4 for quantitation.

RESULTS

Figure 1 shows the toxin production of T. roseum when glucose and sucrose were used as carbon sources at varying concentrations. The dry weight of mycelium is also shown. Results are expressed as µg T-cin/ml filtrate. With strain 1665, glucose proved to be a better carbon source for toxin production at all sugar concentrations tested. The dry weight of the mycelium was also higher with glucose as a carbon source with maximum weight yield at 80 g/l, which correlated with peak toxin yield. T-cin was assayed at ca. 300 µg/ml filtrate with the use of glucose in contrast to sucrose which allowed the production of ca. 150 µg T-cin/ml filtrate. In all analyses the bioassay gave higher values than the colorimetric assay. There appeared to be less discrepancy between the two methods of analysis when sucrose was the carbon source.

Figure 2 shows the results of filtrate and mycelial analysis of sucrose cultures over time. At all temperatures toxin production was maximal in the filtrate after four weeks as measured by either assay method. In analyses of filtrates, bioassay values were consistently higher than Poltorak values at all temperatures studied. On the other hand, Poltorak

values tended to be higher in mycelial analyses. This difference appears to be consistent with T. roseum strain 1665. In the mycelium the greatest toxin yields were at room temperature (ca. 20 mg T-cin/g mycelium). Both assay methods were in agreement after four weeks at this temperature. In the mycelium the greatest discrepancy between the two assay methods was at 28 C.

Figure 1. Trichothecin production by T. roseum using varying concentrations of glucose or sucrose as the carbon source. Filtrate analysis was performed by bioassay and Poltorak method. Mycelium extracted from glucose and sucrose cultures is shown as a solid line and brcken line, respectively. Experimental time period was three weeks.

Filtrate analysis

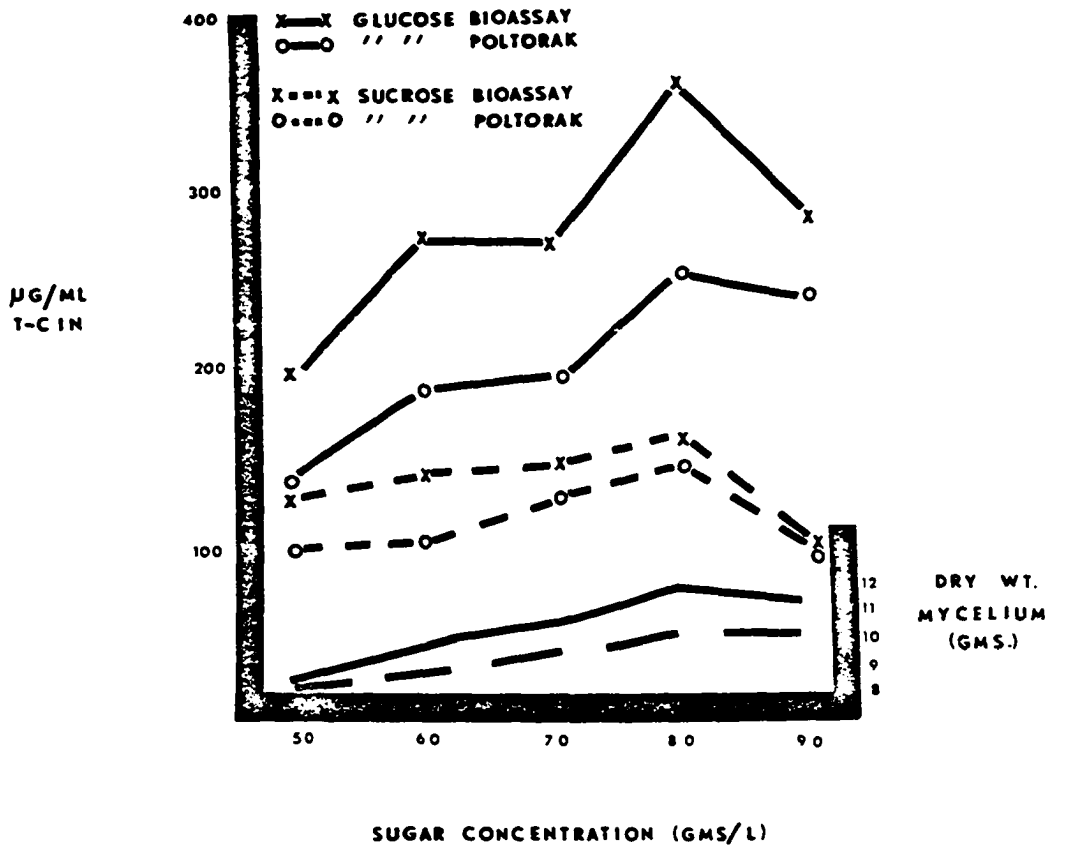
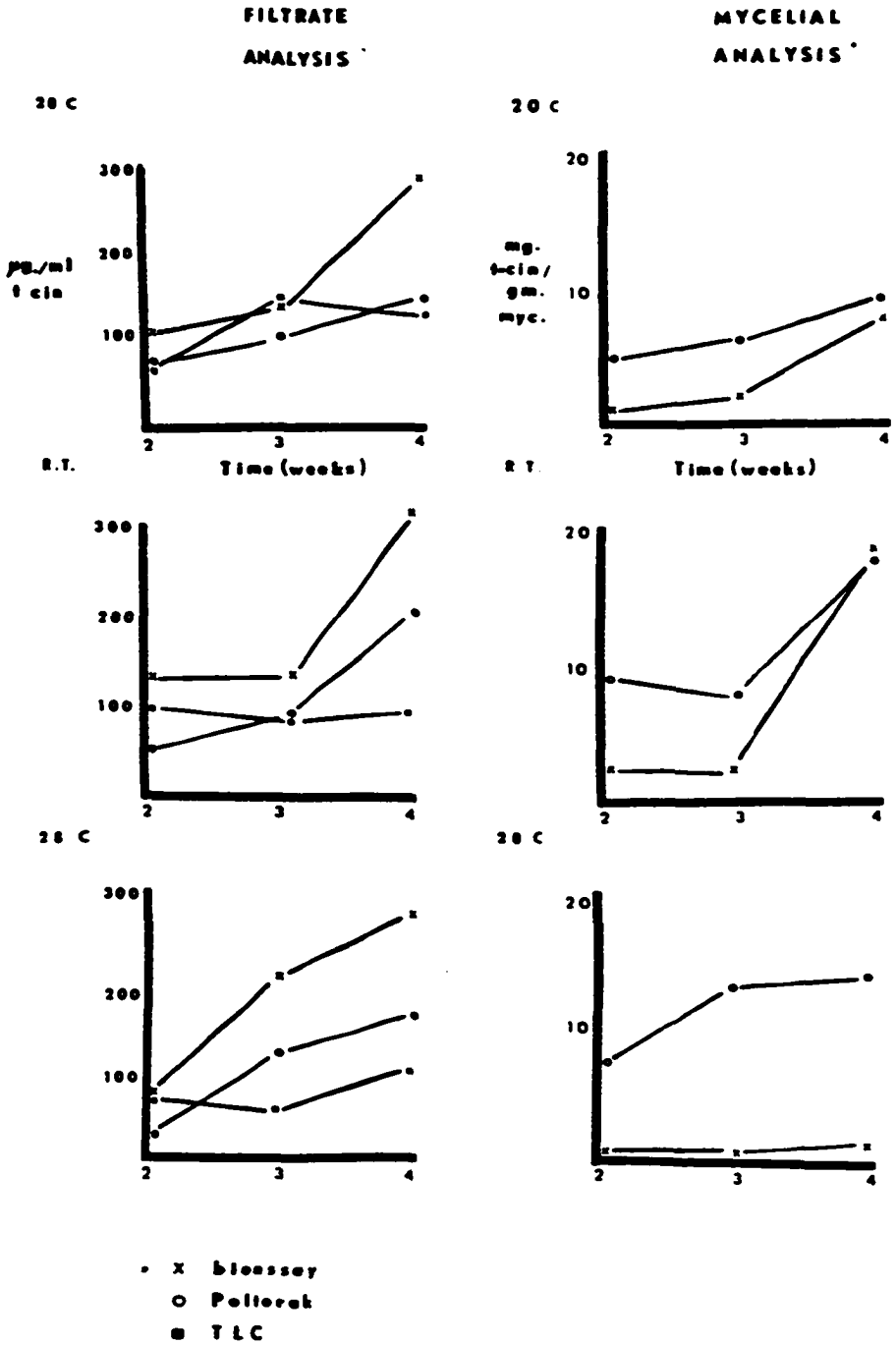


Figure 2. Filtrate and mycelial analysis of trichothecin over time using sucrose as the carbon source. The filtrate was quantitated by bioassay, Poltorak method and TLC. The mycelium was quantitated by bioassay and Poltorak method only.

Sucrose

80 gms /l



DISCUSSION

The high bioassay values in the filtrates suggest the possible presence of an additional biologically active compound. In addition to T-cin, crotocin and trichothecolone have also been recovered from culture filtrates of T. roseum (3). Therefore the lower Poltorak measurements probably more closely reflect the true T-cin concentration. In filtrate analyses assay methods were in closer agreement earlier in the growth period. In mycelial analysis, higher relative Poltorak values suggests that contaminating metabolic by-products are present and therefore bioassay values are probably closer to the true T-cin concentration. At 20 C appearance of T-cin in the mycelium increases almost linearly with time as does appearance of T-cin in the filtrate. At room temperature mycelial release of toxin occurs at an increasing rate, peaking at three weeks. Analysis at 28 C suggests that very little T-cin is retained in the mycelium (although other biologically active substances may be) and release of toxin(s) into the supernatant occurs rapidly. If additional biologically active compounds are present, they would probably be produced at varying times and at different rates. Thus differences between the assay results might be affected by incubation time.

In conclusion, the use of either the bioassay or the Poltorak colorimetric method for the quantitation of T-cin under the conditions described can yield anomalous results. These two assay techniques should ideally be used in conjunction with one another, preferably in conjunction with TLC. However, for initial screening the Poltorak method is recommended for supernatant analysis and the bioassay for mycelial analysis. It appears that these three assay systems may be useful to study the kinetics of toxin production within the mycelium as well as its extra-cellular release.

LITERATURE CITED

1. Sorenson, W.G., M.R. Sneller, and H.W. Larsh. 1975. Qualitative and quantitative assay of trichothecin, a mycotoxin produced by Trichothecium roseum. Appl. Micro. 29:653-657.

2. Freeman, G.G., and R.I. Morrison. 1949. The isolation and chemical properties of trichothecin, an antifungal substance from Trichothecium roseum Link. Biochem. J. 44:1-5.

3. Achilladelis, B., and J.R. Hanson. 1969. Minor terpenoids of Trichothecium roseum. Phytochem. 8:765-767.

APPENDIX II

APPLIED MICROBIOLOGY, May 1975, p. 653-657
Vol. 29, No. 5

Qualitative and Quantitative Assay of Trichothecin: a Mycotoxin Produced by *Trichothecium roseum*

W. G. SORENSON,* M. R. SNELLER, AND H. W. LARSH
Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma 73069

Received for publication 31 January 1975

A method for quantitative determination of trichothecin in crude culture filtrates was presented. The method utilized an agar diffusion bioassay against *Candida albicans*, a colorimetric test involving a halochromatic reaction with sulfuric acid, and subsequent formation of blue color with methanol, and thin-layer chromatography of trichothecin and its dinitrophenylhydrazine derivative. A positive result in all three systems confirmed the presence of trichothecin. Quantitative results were generally in close agreement.

Trichothecin (T-cin) is a member of a family of mycotoxins known as the 12,13-epoxytrichothecenes. Members of this family share the property of strongly inhibiting protein synthesis in eucaryotic cells, although at least two different mechanisms of inhibition exist within the group (3, 10). T-cin is one of the most potent antifungal members of the group and one of the least effective against mammalian cells (2).

Until recently, demonstration of the trichothecenes required relatively pure samples since these compounds are not fluorescent and do not lend themselves easily to the usual methods of assay. For these reasons, assay procedures applicable to crude samples are badly needed (2). Ueno et al. (12) described a procedure for screening cultures and foodstuffs for *Fusarium* trichothecenes based on both biological (animal toxicity and inhibition of reticulocyte protein synthesis) and chemical (thin-layer chromatography) properties. No such procedure is currently available for T-cin.

Ikediobi et al. (6) have developed a gas chromatography system for the detection and quantitation of several trichothecenes including T-cin. Although their system has been used to detect T-2 toxin in crude grain extracts, its use with T-cin has so far been confined to mixtures of pure crystalline samples.

Thin-layer chromatographic methods have been described for all of the trichothecenes (2, 12). We have tried several of these with crude extracts of *Trichothecium roseum* cultures but were unable to achieve adequate separation.

The purpose of this communication is to describe procedures currently being used in our laboratory for the detection of T-cin in crude culture extracts.

MATERIALS AND METHODS

T-cin was prepared by cultivation of *T. roseum* (*Pers.*) Link ex S. F. Gray (NRRL 1665) by the method of Freeman and Morrison (5), except that Fernbach flasks were used, each containing 750 ml of medium. After 21 days of incubation, the mycelium was removed by filtration, and the filtrate was extracted three times with carbon tetrachloride (200 ml/liter of filtrate). The combined extracts were dried by filtration through anhydrous Na_2SO_4, concentrated in vacuo, and diluted to a final volume of 10.0 ml in a volumetric flask. These crude extracts were used for qualitative and quantitative assays and for preparation of crystalline toxin.

Standard samples of crystalline T-cin were prepared from crude CCl_4 extracts by column chromatography on Al_2O_3 (II) with the benzene-ether stepwise elution method of Achilladelis and Hanson (1). Column fractions were screened by bioassay, and active fractions were pooled and concentrated in vacuo. T-cin was crystallized from CH_3OH-H_2O mixtures and recrystallized from petroleum ether. Its identity was confirmed by nuclear magnetic resonance analysis.

Bioassay. Qualitative and semiquantitative assay was carried out by the agar diffusion method against *Candida albicans* strain MI-023 obtained from the Center for Disease Control in Atlanta, Ga. Inoculum was prepared in a modified Wickerham medium (13) consisting of Wickerham's trace elements and salts (ammonium sulfate omitted), 1% glucose, 0.5% vitamin-free Casamino Acids (Difco), and 0.01% yeast extract. A 10-ml basal agar layer was covered by a 6-ml seed agar layer containing 24-h cells at a density of 2×10^5 organisms/ml. Nutrient agar (Difco) was used in both layers. A standard volume (0.1 ml) of the extract or dilution to be tested was added to Schleicher and Schuell antibiotic disks (12.7 mm), and the disks were permitted to stand a minimum of 30 min at ambient room temperature to allow evaporation of the solvent. The disks were applied to the plates 30 min after the seed agar layer was poured,

and the plates were incubated at 37 C for 24 h.

Colorimetric assay. Colorimetric assay of these extracts was accomplished by a modification of the procedure developed by Poltorak (7). Concentrated H_2SO_4 (1.5 ml) was added to 0.5 ml of extract (in CCl_4 or benzene) to be tested. The tube was then agitated immediately on a Vortex mixer and placed in a boiling-water bath for 4 min. The presence of T-cin or other α,β-unsaturated ketones is indicated by the development of a red-brown color (7). After cooling for 1 to 2 min, this mixture was added cautiously to 7.5 ml of absolute methanol. Under these conditions, T-cin produces a blue color within a few minutes which is stable for several hours (7). The final solutions were read at 600 nm in a Beckman DB-G spectrophotometer after a minimum of 30 min.

Thin-layer chromatography. Silica Gel (Brinkman) at a thickness of about 375 μm was applied to plates (20 by 20 cm) with a Desaga spreader. Mallinckrodt Silicar 7G (250 μm) produced essentially similar results. The plates were activated at 110 C overnight and stored in a Lab Con Co vacuum desiccator (atmospheric pressure) prior to use. Separation of substances by chromatography can often be enhanced by repeated development in the same solvent system (11). The plates were spotted for either single or double development (12 cm) and were developed by the following solvent systems: (i) benzene-ethyl acetate (95:5), double development; (ii) benzene-acetone (95:5), double development; and (iii) chloroform-ethyl acetate (9:1), single development.

After development, the plates were sprayed with a 1:1 mixture of concentrated H_2SO_4 and absolute methanol and developed at 110 C for 15 min. T-cin appeared as a brown nonfluorescent spot (long-wave ultraviolet) which eventually began to fade and became surrounded by a white halo.

To enhance the value of thin-layer chromatography in identifying T-cin, 2,4-dinitrophenylhydrazine derivatives were prepared from crude extracts, crystalline T-cin, and acetone. The 2,4-dinitrophenylhydrazine reagent was prepared by the method of Roberts et al. (9). The reagent (1 ml) was mixed with 0.5 ml of extract on a watch glass and stirred occasionally until 2,4-dinitrophenylhydrazones were formed. These were collected by filtration through glass wool in a capillary pipette and washed repeatedly with ethanol. The product was then eluted into a test tube with about 1 ml of chloroform. Dinitrophenylhydrazine derivatives were used as a confirmatory test and were not used for quantitative purposes. The acetone-dinitrophenylhydrazone was used for relative R_f determinations (not shown).

Semiquantitative estimation of T-cin concentrations in crude extracts was done by visual comparison with known concentrations of crystalline T-cin on the same plate.

To compare these procedures for the quantitative analysis of individual samples, a series of eight culture filtrates was prepared and analyzed as described above. In each case, dilutions were prepared with CCl_4 whenever required. Analysis of variance was done by standard methods for two factors and one observation per cell (4).

Several strains of *T. roseum* were used to compare these methods of analysis and to determine whether different strains might present unique problems in extraction or analysis. These were obtained from C. W. Hesseltine of the Northern Regional Research Laboratory (NRRL 1665 and 2307), from J. L. Richard of the National Animal Disease Laboratory, Ames, Iowa (MC-156 and MC-176), and from the American Type Culture Collection (ATCC 13411 and 13422). Two of the strains were isolated from natural sources in Oklahoma (FL-1 from soil in Norman and RH-1 from a squash from Elk City). Each strain was cultured and extracted as described above.

RESULTS

Although the bioassay was intended initially only for qualitative purposes, it can be used semiquantitatively as well; a linear relationship holds between the log of concentration and the diameter of the zone of inhibition over the concentration range of 50 μg/ml to 2 mg/ml (Fig. 1). Since only 0.1 ml of extract is used per disk, a definite zone of inhibition is produced if about 2 to 5 μg of T-cin is present on the disk.

The Poltorak colorimetric procedure yields a linear relationship between 0.2 to 3.0 mg/ml (Fig. 2), although certain unknown substances may cause interference in some cases. The presence of interfering α,β-unsaturated ketones is indicated by a pink or purple color resulting from residual red-colored products of the first reaction.

R_f values for T-cin, T-cin–dinitrophenylhydrazone, and acetone-dinitrophenylhydrazone are presented in Table 1. The minimum amount of T-cin or T-cin–dinitrophenylhydrazone which could be easily detected was 6 to 10 μg/spot. These solvent systems afford good separation of T-cin and an unknown contaminating sub-

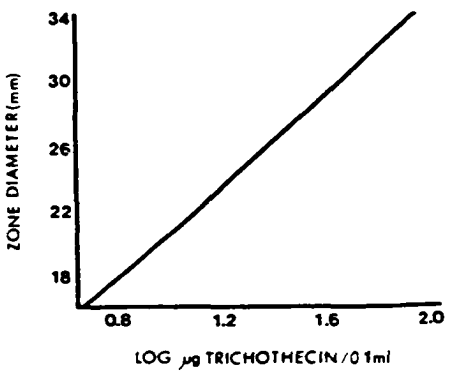


FIG. 1. *Linear relationship between the diameter of the zone of inhibition of C. albicans and the log of the T-cin concentration* ($y = 14.0x + 7.0$; $r = 0.990$).

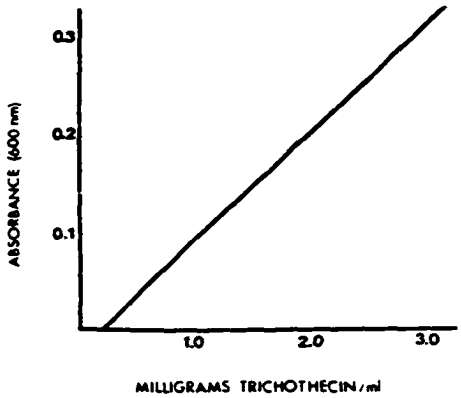


FIG. 2. *Linear relationship between absorbance of reaction products and T-cin concentration* ($y = 0.4x - 0.039$; $r = 0.991$).

TABLE 1. *Characteristic R_f values*

Solvent system	Substance tested		
	T-cin	T-cin-DNP[a]	Acetone-DNP
Benzene-etac[b] (95:5), double development	0.14	0.22	0.67
Benzene acetone (95:5), double development	0.35	0.48	0.76
Chloroform-etac (9:1), single development	0.44	0.58	0.74

[a] DNP, Dinitrophenylhydrazone.
[b] Etac, Ethyl acetate.

stance which overlapped T-cin in several other solvent systems previously reported (Fig. 3).

Table 2 presents the results of a quantitative comparison of a series of eight culture filtrates. Although agreement was not complete (e.g., RH-1 and MC-156), the results of the three assay methods were in general agreement. Analysis of variance of these data (Table 3) shows that the different strains used were significantly different in their production of T-cin, but that there was no statistically significant difference in results obtained by the three assay methods.

DISCUSSION

The minimum detectable levels in the bioassay, Poltorak colorimetric reaction, and thin-layer chromatography are 50 μg/ml, 200 μg/ml, and about 6 μg, respectively. Under the conditions employed, the bioassay is the most sensitive of the three methods. Because of the limited loading capacity inherent in analytical thin-layer chromatography, working samples must contain about 0.5 to 1.0 mg/ml. Therefore thin-layer chromatography is the least sensitive of the methods. A major disadvantage of the bioassay is its lack of specificity, since many naturally occurring substances inhibit *C. albicans*. Its convenience, simplicity, and economy make it a useful screening technique.

The specificity of the colorimetric test is currently not known. According to Poltorak (7), it is dependent upon a halochromatic reaction between concentrated H_2SO_4 and the α,β-unsaturated keto group of T-cin. While we have not studied this point, we have tried quinhydrone (a mixture of quinone and hydroquinone) with H_2SO_4 under the conditions of the Poltorak test. Only a faint yellow color developed at a concentration of 1 mg/ml. Therefore, the red-brown color resulting from halochromation of T-cin is not a general phenomenon of α,β-unsaturated ketones.

It would be helpful to know the structural requirements for this reaction and the subsequent reaction, i.e., the formation of the blue color with methanol. The Poltorak reaction is somewhat limited by its uncertain specificity and by the possibility of interference resulting from the occasional occurrence of turbid solu-

TABLE 2. *Comparative analysis of crude extracts*[a]

Strain	Assay method		
	Bioassay[b]	Colorimetric[b]	TLC[b]
1665	16.2	14.2	13.3
2307	16.6	16.1	16.7
FL-1	5.5	5.8	5.0
RH-1	11.6	6.8	11.4
MC-156	0.3	1.5	0.3
MC-176	1.9	2.3	2.0
13411	1.3	1.5	1.2
13422	2.3	2.9	2.0

[a] Expressed as milligrams of T-cin per milliliter of extract.
[b] Average values: bioassay, 7.0; colorimetric, 6.4; and thin-layer chromatography (TLC), 6.5

TABLE 3. *Analysis of variance*[a]

Source of variation	DF[b]	SS[c]	MS[d]
Strain	7	822.08	117.44[e]
Method of assay	2	1.51	0.76[f]
Error	14	19.69	1.41

[a] Analysis of data in Table 2.
[b] DF, Degrees of freedom.
[c] SS, Sum of squares.
[d] MS, mean square.
[e] Significant at the 5% level of probability.
[f] Insignificant.

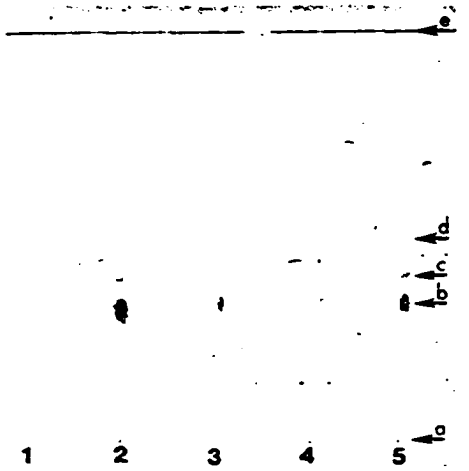


FIG. 3. *Thin-layer chromatographic separation of two culture filtrate extracts in benzene-acetone (95:5, double development). Components were visualized by spraying with CH_3OH-H_2SO_4 (1:1) followed by heating at 110 C for 15 min. Separations: (1) Extract no. 1 (strain MC-176), 2 μl; (2) extract no. 1, 2 μl + 6 μg of T-cin; (3) T-cin standard, 6 μg; (4) extract no. 2 (strain FL-1), 1 μl; and (5) extract no. 2, 1 μl + 6 μg of T-cin. Arrows: (a) origin, (b) T-cin, (c,d) major contaminating substances, and (e) solvent front.*

tions and/or residual red-brown color from the H_2SO_4 step. It is a useful screening tool, however, and offers greater specificity than the bioassay. Interfering substances can be removed by column chromatography if desired, but we believe that this is rarely necessary. The Poltorak colorimetric assay can be scaled down for qualitative purposes. This is helpful in testing column fractions or other samples when conservation of material is desired. In this case, the volumes of samples, H_2SO_4, and methanol are reduced fivefold. Because the sample is in a volatile organic solvent which is readily driven off in the water bath, the sample volume may be increased five- to 10-fold for increased sensitivity. If desired, the final mixture can be poured onto a spot plate for easier visual examination.

The uncertainty of R_f values in identifying organic compounds is well known (11), but by the use of multiple solvent systems and chromatography of derivatives (i.e., 2,4-dinitrophenylhydrazone-T-cin) the problem of specificity can be minimized. Although the spray used resembles the Poltorak reaction system, T-cin does not form a blue spot on thin-layer chromatography plates. However, the spray is useful because it produces fluorescent spots with certain other compounds on the plates, which aids in the interpretation of chromatograms. Also it allows charring of organic compounds present and gives a recognizable reaction with T-cin.

Richard et al. (8) previously reported that *T. roseum* NRRL 2307 does not produce T-cin. However, our results indicate yields comparable to those of NRRL 1665. Perhaps the subculture used in their work had lost its ability to produce the compound. We were able to detect T-cin in all of the extracts reported herein, although the yields were often quite low. It is interesting that we have tried only two fresh isolates from Oklahoma, and that both produced the toxin under these conditions in yields comparable to those originally reported by Freeman and Morrison (5).

Although each of the assay procedures used has certain inherent disadvantages, their use in combination allows qualitative determination of T-cin without purification, and quantitation by these methods yields results which are generally in close agreement.

ACKNOWLEDGMENTS

This investigation was supported by a U. S. Public Health Service training grant (#5 TOI AIOO 123-AID) from the National Institute of Allergy and Infections Diseases, and by the Faculty Research Committee of the University of Oklahoma.

We also thank F. M. Strong of the University of Wisconsin, who kindly supplied a small sample of crystalline trichothecin, and Francis J. Schmitz of the University of Oklahoma for the nuclear magnetic resonance analysis. We also gratefully acknowledge receipt of *T. roseum* cultures from C. W. Hesseltine (ARS Culture Collection Investigations, U.S. Department of Agriculture) and J. L. Richard of the National Animal Disease Laboratory, Ames, Iowa.

LITERATURE CITED

1. Achilladelis, B., and J. R. Hanson. 1969. Minor terpenoids of *Trichothecium roseum*. Phytochemistry 8:765–767.
2. Bamburg, J. R., and F. M. Strong. 1971. 12,13-epoxytrichothecenes. *In* S. Kadis, A. Ciegler, and S. J. Ajl (ed.), Microbial toxins, vol. 7. Academic Press Inc., New York.
3. Cundliffe, E., M. Cannon, and J. Davies. 1974. Mechanism of inhibition of eukaryotic protein synthesis by trichothecene fungal toxins. Proc. Natl. Acad. Sci. U.S.A. 71:30–34.
4. Dixon, W. J., and F. J. Massey, Jr. 1951. Introduction to statistical analysis. McGraw-Hill Book Co., Inc., New York.
5. Freeman, G. G., and R. I. Morrison. 1949. The isolation and chemical properties of trichothecin, an antifungal substance from *Trichothecium roseum* Link. Biochem. J. 44:1–5.
6. Ikediobi, C. O., I. C. Hsu, J. R. Bamburg, and F. M. Strong. 1971. Gas-liquid chromatography of mycotoxins of the trichothecene group. Anal. Chem. 43:327–340.
7. Poltorak, V. A. 1963. Colorimetric determination of trichothecin. (In Russian) Antibiotiki (Moscow) 8:638–640.
8. Richard, J. L., G. W. Engstrom, A. C. Pier, and L. H. Tiffany. 1969. Toxigenicity of *Trichothecium roseum* Link: isolation and partial characterization of a toxic

metabolite. Mycopathol. Mycol. Appl. 39:231-240.
9. Roberts, R. M., J. C. Gilbert, L. B. Rodewald, and A. S. Wingrove. 1969. An introduction to modern experimental organic chemistry. Holt, Rinehart, ar.d Winston, Inc., New York.
10. Schindler, D. 1974. Two classes of inhibitors of peptidyl transferase activity in eukaryotes. Nature (London) 249:38-41.
11. Stahl, E. 1969. Thin-layer chromatography, 2nd ed., p. 86, 100, 127, 217, 219, 900. Springer-Verlag, Inc., New York.
12. Ueno, Y., N. Sato, K. Ishii, K. Sakai, H. Tsunoda, and M. Enomoto. 1973. Biological and chemical detection of trichothecene mycotoxins of *Fusarium* species. Appl. Microbiol. 25:699-704.
13. Wickerham, L. J. 1946. A critical evaluation of the nitrogen assimilation tests as commonly used in classification of yeasts. J. Bacteriol. 52:293-301.