CHEMICAL AND BIOLOGICAL EVALUATION OF DETOXIFIED AFLATOXIN

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ABSTRACT

Experiments were undertaken to evaluate testing procedures concerned with detoxification of aflatoxin in foods and feedstuffs. Specific experiments were undertaken to ascertain: (1) the percent decrease in the fluorescence of aflatoxin B_1 when chemically treated with increasing amounts of NaOH, NaOCl, and NH_4OH, (2) the toxicity of native and detoxified aflatoxin B_1, (3) any influence the detoxified molecule may have on the toxicity of the native molecule, (4) changes in the mutagenicity of detoxified aflatoxin B_1 in an "in vitro" assay system, and (5) if the mutagenicity of aflatoxin is destroyed by base treatment.

Although base treatment reduced aflatoxin B_1 fluorescence by 90-95%, it would appear difficult to achieve complete detoxification of the aflatoxin molecule. Toxicity studies demonstrated an effect in which the detoxified aflatoxin molecule increased toxicity of the native molecule. "In vitro" studies showed that although the mutagenicity and potential carcinogenicity of aflatoxin was greatly reduced by base treatment, it was not completely destroyed.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION AND LITERATURE REVIEW	1
	General History and Significance of Aflatoxin	1
	Detoxification of Aflatoxin in Foods and Feeds . . .	2
	Chemical Inactivation	2
	Reaction of aflatoxin with NH_3 and NaOH	2
	Treatment with methylamine, ozone, and hydrogen peroxide	4
	Treatment of aflatoxin with acid	4
	Treatment of aflatoxin with chlorines	7
	Microbial Inactivation	7
	Inactivation by Heat and Radiation	8
	Biological Testing of Aflatoxins	8
	Assay in Mammalian Systems	10
	Assay in Chick Embryo and Day Old Duckling	10
	Assay in Rainbow Trout and Brine Shrimp	12
	Bioassay of Detoxified Aflatoxin B_1	13
	Short Term Assay for Carcinogenesis of Aflatoxin .	13
	Liver Homogenates and Metabolism of Aflatoxin B_1 "in vitro"	16
	Removal of Aflatoxin from Foods and Feeds	17
II.	MATERIALS AND METHODS	19
	Experimental Goals	19

CHAPTER PAGE

Chemical Treatment of Aflatoxin B_1 19

Brine Shrimp Incubation and Testing 20

Salmonella System for Detecting Carcinogens as Mutagens 24

The Use and Storage of Bacterial Tester Strains TA98 and TA100 24

Preparation and Use of the Liver Homogenate . . . 24

Source of liver 24

Preparation and use of the liver homogenate . . 25

Preparation of the S-9 mixture 25

Standard Plate Assay 25

III. EXPERIMENTAL RESULTS 29

Effect of Chemical Treatment of Aflatoxin B_1 29

NaOH Treatment of Aflatoxin B_1 29

NaOCl Treatment of Aflatoxin B_1 29

NH_4OH Treatment of Aflatoxin B_1 29

Toxicity of Native and Base Treated Aflatoxin B_1 to Brine Shrimp 33

"In Vitro" Testing of Native and Base Treated Aflatoxin B_1 in the Salmonella Tester Strains TA98 and TA100 42

Toxicity of Aflatoxin B_1 to S. typhimurium strains TA98 and TA100 42

Dose Response for S. typhimurium TA98 and TA100 when Exposed to Aflatoxin 42

CHAPTER PAGE

Mutagenicity of Native and Base Treated Aflatoxin B_1 45

IV. DISCUSSION 50

V. SUMMARY 54

BIBLIOGRAPHY 55

VITA 62

LIST OF TABLES

TABLE		PAGE
1.	Literature Review of Percent Reduction of Aflatoxin B_1 in Peanut and Cottonseed Meals when Treated with Ammonia as a Function of Time Processed, Percent Moisture of the Meal, and Temperature During Treatment.	3
2.	Literature Review of Percent Reduction of Aflatoxin B_1 in Peanut Meal when Treated with Methylamine, Hydrogen Peroxide, and Ozone as a Function of Time Processed, Percent Moisture of the Meal and Temperature During Treatment	66
3.	Literature Review of the Hepatotoxic Effects Observed in Primary Target Animals Consuming Aflatoxin B_1	9
4.	Literature Review of Bioassay of Aflatoxin B_1 Measuring Response as a Function of Test Animal, Minimal Effective Concentration and Time	11
5.	Literature Review of Bioassay of Aflatoxins Detoxified with NaOH, NH_3, and Chlorine. Effectiveness of Treatment was Evaluated as a Function of Symptoms in the Test Organism	14
6.	Literature Review of Bioassay of Aflatoxin Detoxified with Methylamine, Ozone, H_2O_2, Radiation, and Acid. Effectiveness of Treatment was Evaluated as a Function of Symptoms in the Test Organism	15
7.	Experimental Design for Determination of Aflatoxin B_1 Toxicity to Brine Shrimp Measuring Percent Death of Brine Shrimp as a Function of µg Active Aflatoxin Added. Amount of Aflatoxin B_1 was measured as a Function of Percent Fluorescence.	22
8.	Experimental Design for Determination of Native + Detoxified Aflatoxin B_1 Toxicity to Brine Shrimp Measuring Percent Death of Brine Shrimp as a Function of µg Native + Detoxified Aflatoxin B_1 Remaining After Treatment of Aflatoxin B_1 with NaOH.	23
9.	Experimental Design of One Replicate for Assay of Mutagenicity in <u>S. typhimurium</u> Tester Strains TA98 and TA100 Measuring Number of Revertants Per Plate. Each Petri Plate Equaled One Observation..	28

TABLE PAGE

10. Reaction of NaOH with Aflatoxin B_1 at Room Temperature. Reduction in Fluorescence as a Function of Meq Base Added was Monitored by Spectrofluorometer Measurement at an Excitation Wavelength of 365 nm and an Emission Wavelength of 435 nm. All Data are the Mean of Five Replicates. 30

11. Reaction of NaOCl with Aflatoxin B_1 at Room Temperature. Reduction in Fluorescence as a Function of Meq Base Added was Monitored by Spectrofluorometer Measurement at an Excitation Wavelength of 365 nm and an Emission Wavelength of 435 nm. All Data are the Mean of Five Replicates. 31

12. Reaction of NH_4OH with Aflatoxin B_1 at Room Temperature. Reduction in Fluorescence as a Function of Meq Base Added was Monitored by Spectrofluorometer Measurement at an Excitation Wavelength of 365 nm and an Emission Wavelength of 435 nm. All Data are the Mean of Five Replicates. 32

13. Reaction of NH_4OH with Aflatoxin B_1 at 121° C and 15 psi. Reduction in Fluorescence as a Function of Meq Base Added was Monitored by Spectrofluorometer Measurement at an Excitation Wavelength of 365 nm and an Emission Wavelength of 435 nm. All Data are the Mean of Five Replicates. 34

14. Percent Brine Shrimp Death as a Function of µg Aflatoxin B_1 Added. All Data are the Mean of Five Replicates . . . 37

15. Measurement of Percent Brine Shrimp Death as a Function of µg Active Aflatoxin B_1 and Detoxified Aflatoxin B_1 Remaining after Treatment of Aflatoxin B_1 with NaOH. All Data are the Mean of Five Replicates. 41

16. Evaluation of Toxicity of Aflatoxin B_1 to S. typhimurium Tester Strain TA98 and TA100 as a Function of µg Aflatoxin B_1 Added. Values were Obtained in a Standard Plate Count Using Nutrient Agar Media. All Data are the Mean of Five Replicates 43

17. Dose Response for S. typhimurium TA98 and TA100 When Exposed to Aflatoxin B_1 Measuring the Number of Revertants Per Plate. All Data are the Mean of Ten Observations . . 44

18. Mutagenicity of 4 µg and 2 µg Aflatoxin B_1, 4 µg Aflatoxin B_1 Detoxified by 50 and 90%, and Bacterial Controls in S. typhimurium Tester Strain TA98. Values are the Number of Revertants/Plate. All Data are the Mean of Five Observations. 46

TABLE PAGE

19. These data are the Mean Number of Reversions/Plate of All Experimental Systems tested using Tester Strains S. typhimurium TA98 and TA100. All Data are the Mean of Five Observations. 47

20. Mutagenicity of 4 μg and 2 μg Aflatoxin B_1, 4 μg Aflatoxin B_1 Detoxified by 50 and 90%, and Bacterial Controls in S. typhimurium Tester Strain TA100. Values are the Number of Revertants/Plate. All Data are the Mean of Five Observations. 49

LIST OF FIGURES

FIGURE PAGE

1. Detoxification of aflatoxin B_1. Reaction with Ammonia at 121° C and 15 psi and formation of aflatoxin D_1 (47) . 5

2. Comparison of reaction of NH_4OH with aflatoxin B_1 at room temperature and NH_4OH at 121° C and 15 psi. Reduction in fluorescence as a function of meq base added was monitored by spectrofluorometric measurement at an excitation wavelength of 365 nm and an emission wavelength of 435 nm. All data are the mean of five replicates. 35

3. Comparison of the toxicity of 10 μg aflatoxin B_1 and a combination of 10 μg aflatoxin + 10 μg detoxified aflatoxin B_1 as a function of time. Values are the mean of five replicates. 36

4. Linear regression analysis of the toxicity of aflatoxin B_1 to brine shrimp ($y = 1.64x + 9.16$) and toxicity of a combination of aflatoxin B_1 + detoxified aflatoxin B_1 to brine shrimp ($y = 2.18 + 11.49$). Percent death of brine shrimp (y) was a function of μg aflatoxin B_1 (x). Graph points are calculated from the regression equation. . . . 39

5. Toxicity of aflatoxin B_1 to brine shrimp and toxicity of a combination of aflatoxin B_1 + detoxified aflatoxin B_1 to brine shrimp. Percent death of brine shrimp (y) is a function of μg aflatoxin B_1 (x). These data are the means and their standard deviations of five replicates. . 40

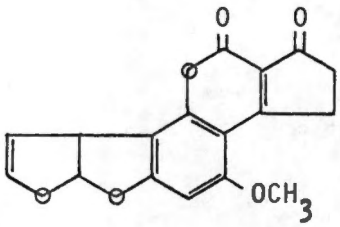
CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

I. GENERAL HISTORY AND SIGNIFICANCE OF AFLATOXIN

Aflatoxin is a metabolite of Aspergillus flavus, although it is known to be produced by other fungi. A. flavus is a ubiquitous mold and may produce aflatoxin in many agricultural products. In 1960 severe mortality occurred in turkey poults aged 3-20 months when fed proprietary and home-compounded rations. In 500 outbreaks of this disease which was termed turkey "X" disease more than 100,000 turkeys died. The toxic meal was found to contain a metabolite of A. flavus which was identified as aflatoxin (64).

Aflatoxins most common to agricultural products are aflatoxin B_1 and G_1, although there are at least eight recognized aflatoxins (40). Aflatoxins have a coumarin lactone structure characteristic of many naturally occuring physiologically active compounds. It produces a highly characteristic lesion of the liver referred to as bile duct hyperplasia (48). Aflatoxin B_1 is acutely toxic and one of the most carcinogenic substances known to man:



AFLATOXIN B_1

Due to the widespread occurrence of A. flavus in foods and feeds and the toxic and carcinogenic nature of aflatoxin, many attempts have been made to reclaim contaminated feeds. In this study we have evaluated the toxicity and biological activity of aflatoxin which has been chemically detoxified in three suggested systems.

II. DETOXIFICATION OF AFLATOXIN IN FOODS AND FEEDS

Chemical Inactivation

Many chemicals have been screened as reagents for the destruction of aflatoxin (7,8,38,32,33,66,67). Reactions of two types have been proposed: (a) additions and oxidations involving the olefinic double bond of the terminal furan ring (20,71) and (b) oxidation of the phenol formed on opening of the lactone ring (29,47,66). Deactivation of the aflatoxin molecule may be closely monitored by monitoring fluorescence of the aflatoxin molecule (17).

a. Reaction of aflatoxin with NH_3 and NaOH. The use of alkali for purpose of detoxification has been the subject of much investigation (27,28,35,36,56,71). The lactone moiety is thought to open readily in the presence of alkalies. Mann et al. (56) have reported that aflatoxin contaminated meal treated with base had only barely detectable levels of the toxin left. Meals containing 519 to 4174 parts per billion (ppb) were reduced to less than 34 ppb by ammoniation (25, 36) (Table 1). Detoxification by NH_4OH required high pressure, moisture, and heat (26,36,56,71). Detoxification with NaOH may be a reversible reaction as a function of pH because the lactone ring may reform (27,56).

TABLE 1

Literature Review of Percent Reduction of Aflatoxin B_1 in Peanut and Cottonseed Meals when Treated with Ammonia as a Function of Time Processed, Percent Moisture of the Meal, and Temperature During Treatment

Aflatoxin (ppb) Before Treatment	After Treatment	% Reduction	Amt. Time Processed	% Moisture	Temp. °C	Ref.
110.0	41.0	63	15 min.	8.6	93	(56)
110.0	5.0	95	15 min.	15.0	93	(56)
110.0	41.0	63	15 min.	15.0	66	(56)
334.0	6.0	99	15 min.	15.0	93	(56)
111.0	5.0	95	15 min.	15.0	68	(27)
10.0[a]	4.0[a]	60	18 days	soln.	25	(71)
2.6[a]	2.4[a]	8	21 days	soln.	25	(71)
121.0	4.0	96	30 min.	15.0	93	(36)
510.0	5.0	99	30 min.	12.0	121	(36)
121.0	5.0	95	30 min.	15.0	38	(36)
187.0	N. D.[b]	100	60 min.	7.0	81	(25)
4174.0	34.0	99	15 min.	15.0	81	(25)
214.0	11.0	95	120 min.	12.5	38	(56)

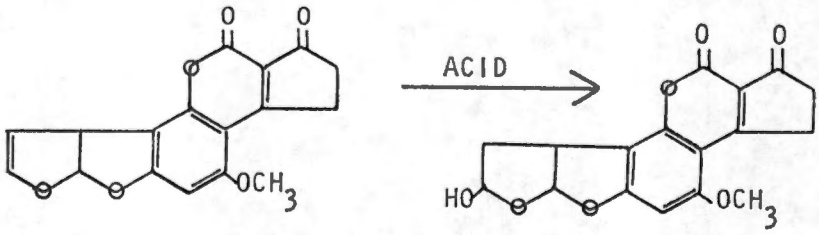
[a] milligrams

[b] N. D. = none detectable

Lee et al. (47) have demonstrated that ammoniation of aflatoxin B_1 with heat and pressure results in a major reaction product referred to as aflatoxin D_1. This reaction is not reversible (Figure 1)

b. Treatment with methylamine, ozone, and hydrogen peroxide. Sreenivasamurthy et al. (66) indicated that 100% detoxification of aflatoxin containing meals is possible using hydrogen peroxide in alkaline media. However, conditions are most critical to achieve 100% destruction (Table 2). Studies with methylamine and ozone (Table 2) suggested that moisture content is a highly critical variable since increasing moisture greatly enhanced the effectiveness of these chemicals (26,30,56). However, increasing moisture had a negative effect on the quality of the meal (25).

c. Treatment of aflatoxin with acid. Ciegler and Peterson (20) have shown that weak acid such as produced by mold or 0.1N citric acid caused the formation of a new compound with the disappearance of aflatoxin B_1. Aflatoxin B_1 and possibly aflatoxin G_1 are hydroxylated in the presence of acids to yield hydroxydihydroaflatoxins with the hydroxyl in the two position:



Aflatoxin B_1 Hydroxydihydroaflatoxin B_1

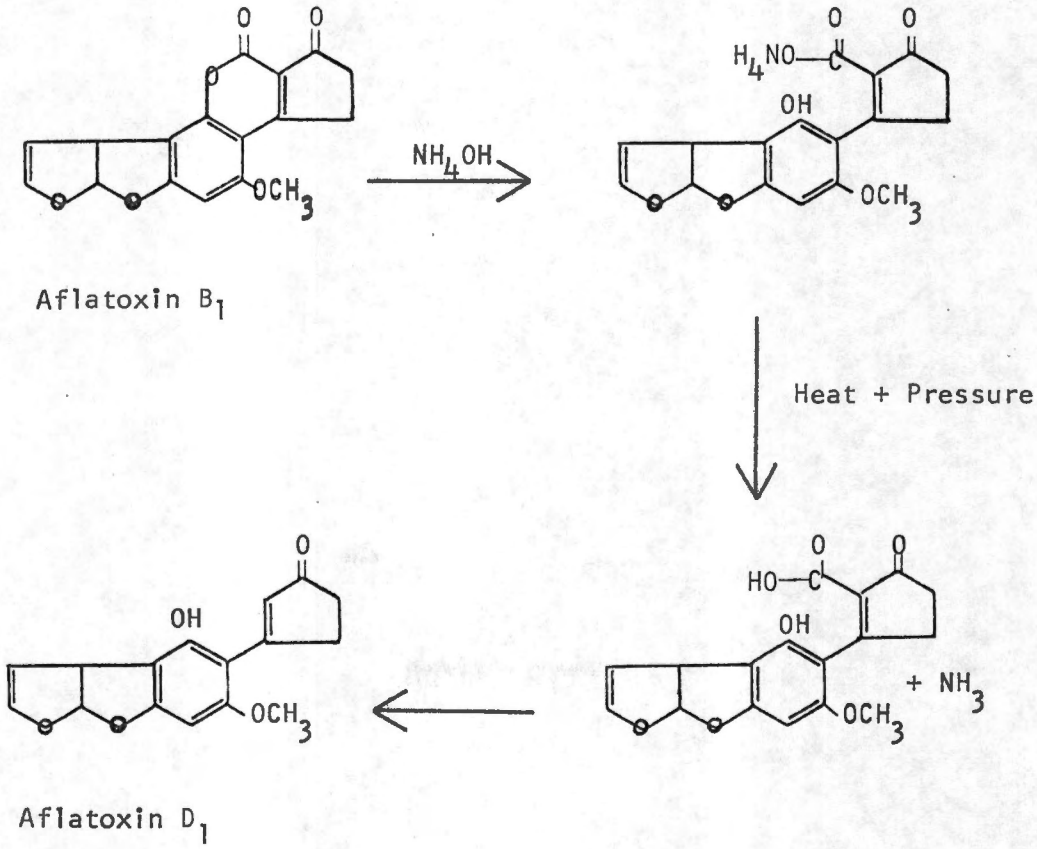


Figure 1. Detoxification of aflatoxin B_1. Reaction with ammonia at 121° C and 15 psi and formation of aflatoxin D_1.

TABLE 2

Literature Review of Percent Reduction of Aflatoxin B_1 in Peanut Meal when Treated with Methylamine, Hydrogen Peroxide, and Ozone as a Function of Time Processed, Percent Moisture of the Meal and Temperature During Treatment

Chemical	% Moisture	pH	°C Temp.	Time Proc.	% Red.	Ref.
METHYLAMINE	15	---	100.0	2 hr.	93	(30)
METHYLAMINE	30	---	100.0	20 min.	100	(56)
H_2O_2	00	9.5	80.0	30 min.	91	(66)
OZONE	22	---	100.0	2 hr.	91	(28)

Preliminary results indicated this to be the same compound as aflatoxin B_{2a} (62). Feuell (32) found that a toxic meal when boiled with 10% HCl and neutralized did not cause liver lesions in ducklings. Since acid brings about the conversion of aflatoxin B_1 to a less toxic metabolite, detoxification in shelled corn by ensiling has been attempted. However, lactic fermentation of corn did not cause adequate detoxification since high concentrations of acid were not formed (52).

d. Treatment of aflatoxin with chlorines. The effectiveness of treatment of contaminated meals with chlorines is questionable at this time. Fischbach and Campbell (33) reported that peanut meal containing 100 μg/kg aflatoxin lost 90% fluorescence and was not toxic to chick embryos after overnight treatment with 10% chlorine gas. Trager and Stoloff (68) reported reaction products of NaOCl treated meals were non-toxic to chick embryo and tissue culture. However, Feuell (32) observed that treatment of peanut meal with chlorine reduced duckling toxicity but not liver lesions. He also warned that chlorinated fats might be toxic. Treatment with chlorine gas also altered the organoleptic quality of peanut meals because it left a residual chlorine odor (66). Natarajan et al. (57) investigated the effect of pH on chlorine treatment of aflatoxin. In alkaline solution the opened lactone was oxidized by NaOCl. In acidic solution chlorination appeared more prevalent. Sodium hypochlorite is thought to be more suitable for detoxification of work areas to protect personnel working with aflatoxin than for detoxification of foods or feeds (24).

Microbial Inactivation

Of 1000 organisms screened no yeast, actinomyces or algae

examined appeared capable of degrading aflatoxin (19). Some molds and mold spores partially transformed B_1 to a different compound. One bacterium, Flavobacterium aurantiacum, NRRL B-184 was capable of destroying aflatoxin. Viable cells (greater than 2×10^{13}) of the F. aurantiacum completely removed 600 µg aflatoxin from milk, 700 µg from vegetable oil, and 700 µg from peanut butter.

Inactivation by Heat and Radiation

Aflatoxins are very stable to heat up to their melting point of approximately 250° C (32). However, Coomes et al. (22) autoclaved groundnut meal containing 10% moisture and reduced its aflatoxin content from 7000 to 370 µg/kg. The quantity of aflatoxin in 20% moisture cottonseed meal could be reduced from 144 to 44 ppb by heating at 100° C for two hours (56). Roasting of peanuts containing 1500 to 100,000 ppb aflatoxin resulted in a 35% loss in fluorescence of aflatoxin (47). These studies suggested that moisture was required for heat destruction of aflatoxin. Even at high moisture levels, cooking did not reduce the aflatoxin levels to those considered acceptable for feed use in the United States.

III. BIOLOGICAL TESTING OF AFLATOXINS

Aflatoxin has been evaluated in a variety of biological systems. The object of bioassay is to establish the relative potency of a compound in a reliable biological system (48). Aflatoxins have been found to be highly toxic (73), carcinogenic (13), teratogenic (31), and mutagenic (3,4,55). Hepatotoxic effects of aflatoxin are reported in Table 3.

TABLE 3

Literature Review of the Hepatotoxic Effects Observed in Primary Target Animals Consuming Aflatoxin B_1

HEPATOTOXIC EFFECT	PRIMARY TARGET ANIMAL
Acute necrosis and hemorrhage	[b] D, TP
Chronic Fibrosis	K, P
Regeneration nodules	K, P, TP
Bile duct hyperplasia	K, P, D, TP, T, R
Veno-occlusive lesions	K
Enlarged cells (Megalocytosis)	K, P, D, TP, R
Enlarged Nuclei	K, P, D, TP, C
Inflammatory cell infiltration	TP, C
Liver tumors	D, T, S, R
Indian Childhood cirrhosis	H

[a] Allcroft and Lancaster (1)

[b] C = Chicken; D = Duckling; H = Human; K = Cattle; P = Pig; R = Rat; S = Sheep; T = Trout; TP = Turkey poult.

Assay in Mammalian Systems

In the rat, maximum sensitivity and toxic response were observed in young male animals, however, tumor induction occurred equally in both sexes. The liver exhibited the initial and most extensive damage (46), with the kidneys and pancreas showing less damage (42). Six months to a year was the minimal time lapse for tumor induction with doses of 0.8 to 4.0 ppm (63).

The mouse is considered one of the more resistant animals to aflatoxin injury with an LD_{50} estimated by Butler as 9 mg/kg (13). Platinow (60) described a three month feeding trial at high levels of aflatoxin (4.5 ppm) (Table 4) that failed to produce any significant changes. The hamster exhibited fair sensitivity with an LD_{50} of 10.2 mg/kg (73) but due to involved techniques is seldom used. However, Elis and DiPaolo (31) studied the teratogenic action of aflatoxin in hamsters and described the development of central nervous system defects when 4 mg/kg aflatoxin B_1 was given intraperitoneally on day eight of pregnancy. Foy et al. (34) reported bile duct proliferation in the rhesus monkey after feeding diets containing 15 mg/kg aflatoxin for three weeks. This study confirmed that primates as well as lower animals were susceptible to hepatotoxic effects of aflatoxin.

Assay in Chick Embryo and Day Old Duckling

The chick embryo is a sensitive bioassay procedure with an LD_{50} of 0.025 μg/egg (70). Verrett et al. (72) reported air cell injection was a more sensitive technique than yolk injection. Sensitivity decreased rapidly with increased embryo age. Platt et al. (61)

TABLE 4

Literature Review of Bioassay of Aflatoxin B_1 Measuring Response as a Function of Test Animal, Minimal Effective Concentration and Time

TEST ANIMAL	MINIMAL EFFECTIVE CONCENTRATION	RESPONSE	TIME PERIOD	REF.
Chick embryo	.30 µg	100% death	2 da.	(59)
Duckling	.36 µg/kg	LD_{50}	72 hr.	(16)
Duckling	.40 mg/kg	LD_{50}	72 hr.	(56)
Duckling	.04 mg/kg	bile duct hyper.	5 da.	(74)
Rainbow trout	1.50 mg/kg	LD_{50}	10 da.	(6)
Rainbow trout	1.00 mg/kg	LD_{50}	10 da.	(74)
Rainbow trout	80.00 ppb	90% hepatomas	12 mon.	(73)
Rainbow trout	20.00 ppb	83% hepatomas	12 mon.	(42)
Male rats	7.20 mg/kg	LD_{50}	4 da.	(14)
Female rats	19.70 mg/kg	LD_{50}	4 da.	(14)
Male weanling rats	50.00 µg	liver lesions	20 da.	(52)
Young rats	4.00 ppm	83% hepatomas	82 wk.	(13)
Mice	4.50 ppm	no change	3 mon.	(58)
Hamster	4.00 mg/kg	teratogenic	3 da.	(29)
Young rhesus monkey	15.00 mg/kg	bile duct hyper.	33 wk.	(67)
Guinea pig	1.0 mg/kg	LD_{50}	3 da.	(15)

noted the chick embryo assay was simple, reproducible, economical and sensitive.

Sensitivity to aflatoxin injury and immediate bile duct proliferation has led to widespread use of the day old duckling test (9). Butler and Barnes (16) reported bile duct proliferation reached a maximum in three days with a single dose of aflatoxin and then regressed with subsequent repair of liver parenchyma. The type of response and difficulties in standardization of feed and environment make the day old duckling test semi-quantitative at best (48).

Assay in Rainbow Trout and Brine Shrimp

Rainbow trout are well suited for qualitative studies of potential aflatoxicosis. Concentrations of 20 ppb produce tumors in six to twelve months (43). Tumors were produced within nine months in trout consuming aflatoxin in commercial trout rations (65). Ashley et al. (7) found that one of five samples of cottonseed meal containing 300 ppb aflatoxin produced typical liver cell neoplasms in five months. Halver (42) in studies with crude aflatoxin estimated that the LD_{50} in 50 gram trout for B_1 and G_1 mixtures was 0.5 to 1.0 mg/kg.

Brine shrimp are convenient test organisms for aflatoxin assay since larvae may be obtained in large numbers and have low natural mortality (44). Brown (12) reported that 2 μg/ml aflatoxin produced 100% death in 24 hours. Curtis et al. (23) warned that extracts of A. flavus may carry fatty acids which are highly toxic to brine shrimp and may interfere with mycotoxin testing. Brown et al. (11) observed that maximum sensitivity of brine shrimp occurred at 37.5° C. The

test could be conducted in 24 hours and highly trained personnel were unneccessary.

Bioassay of Detoxified Aflatoxin B_1

Bioassay of detoxified meals containing aflatoxin has presented difficulties due to formation of toxic by-products (70), anaphalactic responses (32), toxicity of chemicals used for inactivation (56), and the long term testing required for carcinogenesis studies (14,73). Contradictory reports as to the efficiency of various treatments have been reported throughout the literature (Tables 5 and 6).

Deactivation of aflatoxin with ammonia at high temperature and pressure has been the most frequently suggested treatment (35). Mann et al. (56) observed that ammonia treatment of meals did not produce any markedly toxic agents or symptoms of aflatoxicosis in ducklings, although, reductions in PER (Protein Efficiency Ratio) resulted from treatments ranging from 18.1% for ammoniation to 28.0% for methylamine. Hydroxydihydro derivatives of aflatoxin B_1, referred to as aflatoxin B_{2a}, produced no deaths or bile duct hyperplasia. However, fatty metamorphosis of the liver was observed (20). In India, the preferred detoxification of aflatoxin is treatment with hydrogen peroxide. Screenivasamurthy et al. (66) reported 100% detoxification using contaminated peanut meal. He observed, however, that toxicity was destroyed only to a marked degree.

Short Term Assay for Carcinogenesis of Aflatoxin

The assessment of carcinogenic potential of aflatoxin presently requires lengthy and expensive animal studies. Assay of carcinogenicity would benefit by the development of rapid yet reliable tests for

TABLE 5

Literature Review of Bioassay of Aflatoxins Detoxified with NaOH, NH_3 and Chlorine. Effectiveness of Treatment was Evaluated as a Function of Symptoms in the Test Organism

TREATMENT	SYMPTOMS	TEST ORGANISM	REF.
NaOH	none	duckling	(56)
NaOH	liver lesions	duckling	(32)
NH_3	teratogenic	duckling	(10)
NH_3	low PER	rats	(28)
NH_3	no symptoms	duckling	(56)
NH_3	no symptoms	duckling	(28)
Chlorine	no lesions	duckling	(32)
Chlorine	nontoxic	chick embryo	(33)
Chlorine	nontoxic	chick embryo	(68)
Chlorine	none	duckling	(75)
Chlorine gas	liver lesions	duckling	(32)

TABLE 6

Literature Review of Bioassay of Aflatoxin Detoxified with Methylamine, Ozone, H_2O_2, Radiation, and Acid. Effectiveness of Treatment was Evaluated as a Function of Symptoms in the Test Organism

TREATMENT	SYMPTOM	TEST ORGANISM	REF.
Methylamine	none	duckling	(56)
Methylamine	low PER	rat	(28)
H_2O_2	no deaths	duck embryo	(59)
H_2O_2	none	duckling	(66)
Radiation—UV	death with lesions	duckling	(32)
Radiation—Heat	slight toxicity	duckling	(32)
Radiation—Heat	severe lesions	duckling	(22)
Radiation—Gamma	death with lesions	duckling	(32)
Ozone	low PER	rat	(28)
Acid	none	duckling	(32)
Acid	fatty metamorphosis of liver	duckling	(20)

carcinogens. Mutagenesis and DNA repair in bacterial systems have received widespread examination as potential screening systems for carcinogens (67).

Ames et al. (6) described a set of bacterial tester strains for detecting carcinogens and mutagens which are highly sensitive to aflatoxin. Aflatoxin B_1 is activated by a liver homogenate to form a potent frameshift mutagen which has a planar ring system capable of intercalation and covalent bonding to DNA base pairs. It intercalates with the DNA of the bacterial mutant allowing reversion to the wild type (3,4). Metabolites of aflatoxin B_1 have been detected in urine studies (21,29). Aflatoxins M_1, P_1,B_{2a}, and aflatoxicol were studied by Garner et al. (37) using tester strain S. typhimurium TA1538. Hsieh et et. (45) observed that aflatoxin Q_1 was a product of detoxification rather than a product of activation using TA1538. Ames et al. (6) have developed two new tester strains, TA98 and TA100, which have an increased sensitivity to aflatoxin by a magnitude of ten.

Other short term screening tests which are highly sensitive to aflatoxin include: (a) induction of bacteriophage in lysogenic bacteria (49), (b) mitotic inhibition in cell culture, (c) inhibition of DNA synthesis in cell culture (50), and (d) inhibition of DNA polymerase deficient E. coli (53).

Liver Homogenates and Metabolism of Aflatoxin B_1 "in vitro"

Phenobarbital has been used to induce metabolism of aflatoxin B_1 by liver homogenate (4). Phenobarbital is a potent inducer of cytochrome P-450 in rats and enhances metabolism of drugs "in vivo"

as well as "in vitro" (2). Garner et al. (37) observed very high activity of livers from phenobarbital treated rats using S. typhimurium TA1530 "in vitro". He later (38) observed a protective effect of phenobarbital "in vivo". Metabolism of aflatoxin B_1 "in vitro" resulted in a derivative toxic to S. typhimurium TA1530. He observed that the apparent contradiction between the "in vivo" and the "in vitro" system may be that aflatoxin B_1 is in excess in the "in vitro" system but probably limiting in the "in vivo" system.

Removal of Aflatoxin From Foods and Feeds

Many methods have been suggested for detoxification of aflatoxin in foods and feedstuffs (28,32,33,66,68,70). Only one study has been performed detoxifying pure aflatoxin B_1 molecule with base. This reaction has not been precisely defined. Biological testing of detoxified feeds were reported to be preliminary in nature. Closer examination of the literature has shown contradictory information concerning bioassay of detoxified materials. Short term testing for the carcinogenicity of detoxified aflatoxin has not been attempted.

Considering these observations the present study was undertaken to obtain answers to the following questions:

1. What is the percent decrease in fluorescence of aflatoxin B_1 when chemically treated with increasing amounts of NaOH, NH_4OH, and NaOCl?
2. How toxic is aflatoxin B_1 to brine shrimp before and after base treatment?
3. Does the detoxified molecule have any toxicity of its own?

4. Does the presence of detoxified aflatoxin B_1 influence the toxicity of native aflatoxin B_1?
5. Can changes in carcinogenicity of detoxified aflatoxin B_1 be detected in a short term assay for carcinogenesis?
6. Is the carcinogenicity of detoxified aflatoxin less than that of native aflatoxin in an "in vitro" assay?

CHAPTER II

MATERIALS AND METHODS

I. EXPERIMENTAL GOALS

The objective of this study was to elucidate testing procedures which might be applicable to studies concerned with detoxification of aflatoxin in foods. Aflatoxins were used because of the large amount of work in this area and the importance of aflatoxin to the foods industry. All studies were performed with pure aflatoxin B_1 (greater than 95% purity, Calbiochem). Specific experiments were performed to ascertain: (a) the decrease in fluorescence of aflatoxin B_1 molecules when chemically treated with NaOH, NH_4OH, and NaOCl, (b) the toxicity of aflatoxin B_1, (c) the toxicity of aflatoxin B_1 after treatment with NaOH, NH_4OH, and NaOCl, (d) the toxicity of aflatoxin B_1 in the presence of detoxified aflatoxin B_1, (e) the carcinogenicity of aflatoxin B_1 "in vitro", and (f) the carcinogenicity of detoxified aflatoxin B_1 "in vitro".

II. CHEMICAL TREATMENT OF AFLATOXIN B_1

The most reactive functional group of the aflatoxin molecule the lactone ring. The lactone can be readily opened by strong alkalies and the extent of this reaction monitored by the decrease in fluorescence of the aflatoxin molecule (28). Decarboxylation occurs after ring opening in systems utilizing NH_4OH at elevated temperatures (47).

Treatment with NH_4OH; NaOH, and NaOCl was performed to define

their effect on the fluorescence of aflatoxin B_1. Reactions were carried out in aluminum foil covered erlenmyer flasks. The foil was used to protect the aflatoxin from the degradative effects of light. Ten µg of aflatoxin B_1 in benzene were added to 5 ml distilled water at 65° C to evaporate the benzene and disperse aflatoxin in the aqueous solution. Fluorescence readings were taken after five minutes utilizing a model 203 Perkin-Elmer fluorescence spectrophotometer with an excitation wavelength of 365 nm and an emission wavelength of 435 nm.

The NH_4OH treated aflatoxin B_1 was autoclaved for ten minutes at 15 psi and 121° C. Samples were plunged into an ice bath on removal from the autoclave and fluorometry readings taken when samples had equilibrated to room temperature. All other treatments were performed at room temperature according to the above protocol. Five replicates of each experiment were performed.

III. BRINE SHRIMP INCUBATION AND TESTING

The brine shrimp (Artemia salina) assay has several distinct advantages over the duckling or trout test in studies utilizing aflatoxins in solution (11): (a) low cost and no need for extensive laboratory equipment, (b) availability of large numbers of organisms with identical genetic and physiological characteristics (11), (c) ease of handling and observation, and (d) testing requires less than 24 hours.

Brine shrimp testing and hatching was carried out with some modification in the procedure of Brown et al. (11). Metaframe vacuum packed dried brine shrimp eggs were placed in artificial sea water (5% NaCl) at 32° C. Hatching occurred within 24 hours. Once the

container was opened, unused brine shrimp eggs were stored in a refrigerated dessicator at reduced pressure. Brine shrimp of uniform vitality were obtained by selecting larvae that swam from a darkened to an illuminated environment. The brine shrimp eggs were placed on one side of a barrier in the brine shrimp rearing dish. A light source at the front of the dish attracted the swimming larvae under the barrier and also caused larvae to congregate in one location where sufficient numbers were easily drawn into a pipet.

The tests were conducted in ceramic spot plates (12.5 x 10 cm). The desired quantity of aflatoxin disolved in chloroform was drawn up into a syringe and delivered into each spot. The chloroform was then evaporated off in a 60° drying oven. About 15 - 30 brine shrimp were drawn with 0.5 ml artificial sea water into a graduated 1 ml pipet and transferred to each spot. In experiments with detoxified aflatoxin B_1 20 µg aflatoxin B_1 was delivered into each spot. The appropriate amount of base for detoxification was then added and 15 - 30 brine shrimp were transferred to each spot. The volume was brought to 0.5 ml with 5% NaCl. The spot plates were maintained at 37° C up to six hours underneath glass plates. Death was judged to occur if the brine shrimp lost motility and sank to the bottom of the depression. Readings were taken each hour and all data was calculated on a percentage of original number basis. Controls containing NaOH and chloroform (evaporated) were evaluated to determine their toxicity to brine shrimp. A positive control (100% death expected) containing cadmium was evaluated. Regression analysis was performed for the two experimental systems shown in Tables 7 and 8. Regression curves were constructed from these data. A time

TABLE 7

Experimental Design
For Determination of Aflatoxin B_1 Toxicity to Brine Shrimp Measuring Percent Death of Brine Shrimp as a Function of µg Active Aflatoxin Added. Amount of Aflatoxin B_1 was Measured as a Function of Percent Fluorescence.

AMOUNT--µg AFLATOXIN B_1	EXPERIMENTAL SYSTEM	% FLUORESCENCE
0	A	0
2	B	10
4	C	20
6	D	30
8	E	40
10	F	50
12	G	60
14	H	70
16	I	80
18	J	90
20	K	100

TABLE 8

Experimental Design
For Determination of Native + Detoxified Aflatoxin B_1 Toxicity to Brine Shrimp Measuring Percent Death of Brine Shrimp as a Function of µg Native + Detoxified Aflatoxin B_1 Remaining after Treatment of Aflatoxin B_1 with NaOH

EXPERIMENTAL[a] SYSTEM	AFLATOXIN B_1-µg	DETOXIFIED AFLATOXIN B_1-µg	% FLUORESCENCE
A	20	0	100
B	18	2	90
C	16	4	80
D	14	6	70
E	12	8	60
F	10	10	50
G	8	12	40
H	6	14	30
I	4	16	20
J	2	18	10
K	0	20	00

[a] All experimental systems contained 20 µg aflatoxin B_1 initially.

response curve was constructed from data obtained in hourly readings. Five replicates of each experiment were performed.

IV. SALMONELLA SYSTEM FOR DETECTING CARCINOGENS AS MUTAGENS

The Use and Storage of Bacterial Tester Strains TA98 and TA100

Storage of tester strains was at -30° C after freezing fresh nutrient broth cultures (0.8 ml) with dimethylsulfoxide (0.07 ml) in small screwcapped vials. Tester strains were obtained from Bruce N. Ames (University of Calfornia at Berkeley). Fresh cultures were prepared by adding 0.5 ml freezer storage culture to 15 ml nutrient broth and incubating overnight at 37° C. Before freezing routine ampicillin (8.0 mg disk) and crystal violet (10.0 mg disk) resistence tests were performed (6) to determine if the strains retained their R factor plasmid pkM101 and "rfa" markers.

The *S. typhimurium* strains TA98 and TA100 were originally constructed from *S. typhimurium* strain LT-2. Important characteristics of the two strains include: (a) elimination of the excision repair system of the DNA including deletions of the nitrate reductase (chi) and biotin (bio) genes, (b) and "rfa" mutation which eliminates the lippopolysaccharide coat down to the ketodeoxyoctanoate core, (c) frameshift mutation of TA98 in the hisG46 operon (3) and base pair mutation of TA100 in the his3052 operon (3), and (d) an R factor plasmid, pkM101, which greatly increases mutagenesis of aflatoxin B_1.

Preparation and Use of the Liver Homogenate

a. Source of liver. Male Holtzman rats were maintained on

Lab bloc laboratory chow. A week before sacrifice their drinking water was made 0.1% sodium phenobarbital (4). The rats (250-350 gram) were sacrificed by cervical dislocation and the livers removed. The livers were placed in an ice cold beaker and covered with plastic wrap until preparation.

b. Preparation of the liver homogenate. Liver homogenate was prepared according to the procedure of Garner et al. (35). All steps were performed at 0—4° C. The livers were washed with equal volumns of 0.15 M KCl, minced with scissors in three volumns of 0.15 M KCl, and homogenized using a VirTis homogenizer for ten seconds at high speed. The homogenate was centrifuged at 4° C for ten minutes at 9000 x g. The supernatant was filtered through glass wool and frozen at -30° C in Whirlpak bags. This homogenate was referred to as the S-9 fraction.

c. Preparation of the S-9 mixture. The S-9 mixture contained the following per ml: 0.3 ml of S-9 fraction, 8mM $MgCl_2$, 33mM KCl, 5mM glucose-6-phosphate, 4mM TPN, and 100mM sodium phosphate (pH 7.4). The S-9 mixture was sterilized using a 0.45 micron Falcon disposable millipore filter. The S-9 mixture was kept in ice at all times during use. Leftover S-9 mixture was discarded at the end of the day.

Standard Plate Assay

The standard plate assay was performed according to the procedure of Ames et al. (3). Bacterial cultures were grown up daily in nutrient broth for 13 hours from a 1/30 inoculum. Aqueous solutions of mutagen were prepared in sterile screwcap tubes with sterile water or sterile dimethylsufoxide (sterile as is, Sigma). Not more than 0.3 ml

dimethylsulfoxide per plate was used. "Top agar" (0.6% NaCl and 0.6% agar) and "base agar" (1.5% agar and 2.0 % glucose) were autoclaved and stored in bottles in volumns of 100 ml at room temperature.

Before use, top agar was melted in a steam cabinet and 10 ml of a sterile 0.5mM L-histidine • HCl-0.5mM biotin solution was added. The top agar was mixed by gentle swirling and distributed in sterile 13 x 100 mm test tubes by adding 2 ml per tube with a sterile pipet. Before use base agar was melted in a steam cabinet and 2 ml Vogel-Bonner "E" medium (72) added and quickly mixed. Disposable petri dishes were poured using 15 ml base agar per plate.

Pour plates were made by adding 0.1 ml of the tester strain, 0.5 ml of S-9 mixture, and an appropriate volume of mutagen by Ependorf pipet to 2 ml top agar. The tube was quickly mixed on a Vortex and poured onto the surface of a base agar plate. The plate was tilted to cover the surface of the base agar and allowed to harden.

Dose response values were established by evaluating the mutagenicity of 0.0, 0.4, 1.0, 2.0, 4.0, and 10.0 μg aflatoxin B_1 in the two tester strains TA98 and TA100. Five replicates were performed with ten observations per replicate.

Toxicity data were obtained using the standard plate assay as described previously with the following exceptions: (1) dilutions of the tester strain were made using sterile water before plating and 1 ml of the dilution was used and (2) nutrient agar was used without exception when top agar or base agar was called for in the procedure. The toxicity of 0.0, 0.4, 1.0, 2.0, 4.0, 10.0, and 20.0 μg aflatoxin B_1 was evaluated. Five replicates were performed with five observations per

replicate. The experimental design of the Salmonella tester system for detecting the carcinogenicity of native and detoxified aflatoxin B_1 is shown in Table 9. Ten replicates were performed. Control plates for spontaneous reversion rate were performed for each tester strain by omitting the mutagen. Sterility checks of each mutagen solution were performed with each replicate. Ethyl methyl sulfonate (EMS) was used as a positive mutagen control. The negative control (requiring S-9 for activation) was 2-aminofluorene (2-AF). Routine reversion tests with ampicillin disks (8 mg) were performed with each replicate. Sodium hydroxide controls were performed with each replicate. All plates were incubated upside down at 37° C for two days, after which the number of revertant colonies could be counted. An analysis of variance was performed to establish significant differences between means using a "student t test".

TABLE 9

Experimental Design of One Replicate for Assay of Mutagenicity in *S. typhimurium* Tester Strains TA98 and TA100 Measuring Number of Revertants Per Plate. Each Petri Plate Equaled One Observation.

SYSTEM	CONTROLS	B_1 4 µg	B_1 2 µg	50%[a] DETOX	90%[b] DETOX	2-AF	EMS
TA98	5x[c]	5x	5x	5x	5x	2x	2x
TA100	5x	5x	5x	5x	5x	2x	2x
2-AF--negative	2x						
EMS--positive	2x						
B_1--sterile	x						
S-9 mixture	x						
Ampicillin-rev.	5x						
S-9 + TA98 (Control)	5x						
S-9 + TA100(Control)	5x						

[a] In the 50% detoxification system, there are 2 µg native aflatoxin B_1 and 2 µg detoxified aflatoxin B_1.

[b] In the 90% detoxification system, there are 0.4 µg native aflatoxin B_1 and 3.6 µg detoxified aflatoxin B_1.

[c] x = 1 observation; 2x = 2 observations; and 5x = 5 observations

CHAPTER III

EXPERIMENTAL RESULTS

I. EFFECT OF CHEMICAL TREATMENT OF AFLATOXIN B_1

NaOH Treatment of Aflatoxin B_1

Treatment of aflatoxin B_1 with 0.0075 milliequivalents (meq) of NaOH reduced the fluorescence of 10 μg aflatoxin B_1 by 50%. Sodium hydroxide treated aflatoxin B_1 continued to decrease in fluorescence until approximately 85% of the fluorescence had been eliminated (Table 10). At this point, treatment with NaOH became increasingly ineffective in lowering the fluorescence of aflatoxin B_1. After treatment with 20 meq NaOH (2000-fold the amount required to eliminate 50% of the fluorescence) 2% fluorescence remained.

NaOCl Treatment of Aflatoxin B_1

Treatment of aflatoxin B_1 with 0.00032 meq NaOCl reduced the fluorescence of 10 μg aflatoxin B_1 by almost 70% (Table 11). There was little or no loss in effectiveness of treatment as fluorescnece was eliminated. The fluorescence was reduced by 96.2% using 0.0032 meq NaOCl (Table 11). This treatment was highly effective in eliminating the fluorescence of aflatoxin B_1.

NH_4OH Treatment of Aflatoxin B_1

Treatment of aflatoxin B_1 with 3.0 meq NH_4OH at room temperature reduced the fluorescence of 10 μg aflatoxin B_1 by 50% (Table 12).

TABLE 10

Reaction of NaOH with Aflatoxin B_1 at Room Temperature. Reduction in Fluorescence as a Function of Meq Base Added was Monitored by Spectrofluorometer Measurement at an Excitation Wavelength of 365 nm and an Emission Wavelength of 435 nm. All Data are the Mean of Five Replicates

MEQ NAOH	% FLUORESCENCE B_1
00.0000	100.00
00.0025	78.53
00.0050	66.60
00.0075	51.40
00.0100	47.10
00.0125	36.50
00.0150	34.50
00.0175	24.65
00.0200	24.47
00.0225	16.43
00.0250	13.70
20.0000	2.00

Ammonium hydroxide became increasingly ineffective as greater than 5.0 meq were required to cause a 60% reduction in the fluorescence of aflatoxin B_1. Treatment of aflatoxin B_1 with 0.035 meq NH_4OH in an autoclave at 121° C and 15 psi reduced the fluorescence of 10 μg aflatoxin B_1 by 50%. The fluorescence was reduced by 94.4% using 0.5 meq NH_4OH (Table 13).

A comparison of the efficiency of treatment of aflatoxin B_1 using NH_4OH at room temperature and NH_4OH at 121° C and 15 psi is shown in Figure 2. Treatment of aflatoxin B_1 with NH_4OH at an elevated temperature was much more efficient than at room temperature. Of all treatments of aflatoxin B_1 performed, NaOCl was the most effective in lowering the fluorescence of aflatoxin B_1.

II. TOXICITY OF NATIVE AND BASE TREATED AFLATOXIN B_1 TO BRINE SHRIMP

Brine shrimp were exposed to 10 μg native aflatoxin B_1 and to a combination of 10 μg native aflatoxin B_1 + 10 μg detoxified aflatoxin B_1. The percent death of brine shrimp as a function of μg aflatoxin B_1 was observed at one hour intervals. Ten μg aflatoxin B_1 caused 0.0% death in the first hour, 7.7% death in the second hour, and 20.1% death in the sixth and final hour (Figure 3). A combination of 10 μg native + 10 μg detoxified aflatoxin B_1 caused 3.5% death in the first hour, 6.7% death in the second hour, and 30.1% death in the sixth and final hour (Figure 3).

Added amounts of aflatoxin B_1 (0-20 μg) increased toxicity to brine shrimp as a function of μg aflatoxin B_1 added (Table 14). Linear regression analysis of percent dead brine shrimp (y) as a function of

TABLE 12

Reaction of NH_4OH with Aflatoxin B_1 at Room Temperature. Reduction in Fluorescence as a Function of Meq Base Added was Monitored by Spectrofluorometric Measurement at an Excitation Wavelength of 365 nm and an Emission Wavelength of 435 nm. All Data are the Mean of Five Replicates.

MEQ NH_4OH	% FLUORESCENCE
0.0	100.00
0.5	80.00
1.0	81.80
1.5	64.10
2.0	60.50
2.5	51.60
3.0	49.80
3.5	42.70
4.0	40.90
4.5	39.20
5.0	39.20

TABLE 13

Reaction of NH_4OH with Aflatoxin B_1 at 121° C and 15 psi. Reduction in Fluorescence as a Function of Meq Base Added was Monitored by Spectrofluorometric Measurement at an Excitation Wavelength of 365 nm and an Emission Wavelength of 435 nm. All Data are the Mean of Five Replicates.

MEQ NH_4OH (14 4 16 1 = 35)	mg	% FLUORESCENCE
0.000	0	100.00
0.025	61.25	50.00
0.050	~~100~~ 87.5 mg	19.04
0.100	175 mg	12.30
0.150	262.5	9.00
0.200	350	9.00
0.250	437.5	7.80
0.300	525	6.70
0.350	612.5	6.70
0.400	700	6.70
0.450	787.5	6.70
0.500	875	5.60

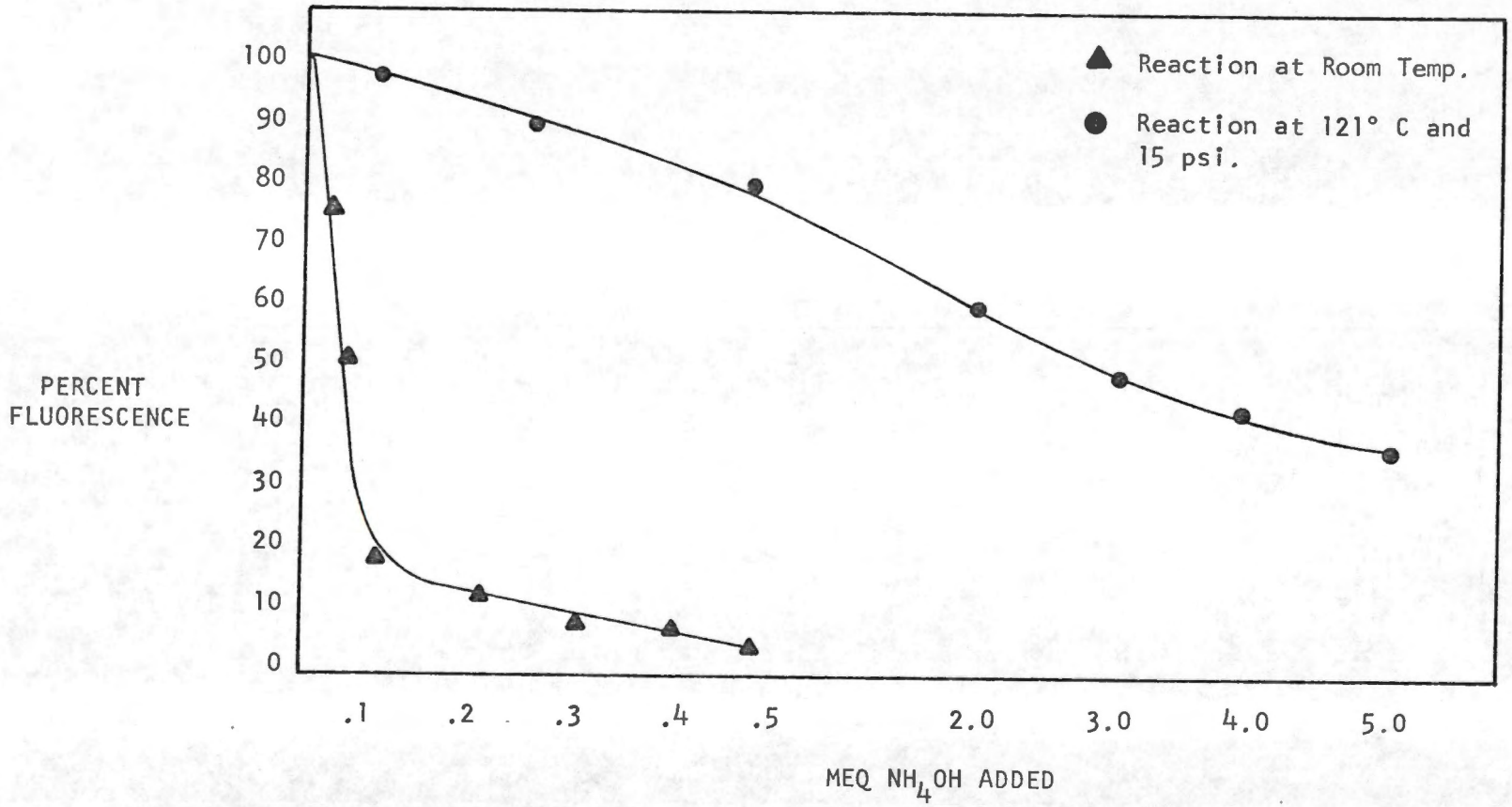


Figure 2. Comparison of reaction of NH_4OH with aflatoxin at room temperature and NH_4OH at 121° C and 15 psi. Reduction in fluorescence as a function of meq base added was monitored by spectrofluorometric measurement at an excitation wavelength of 365 nm and an emission wavelength of 435 nm. All data are the mean of five replicates.

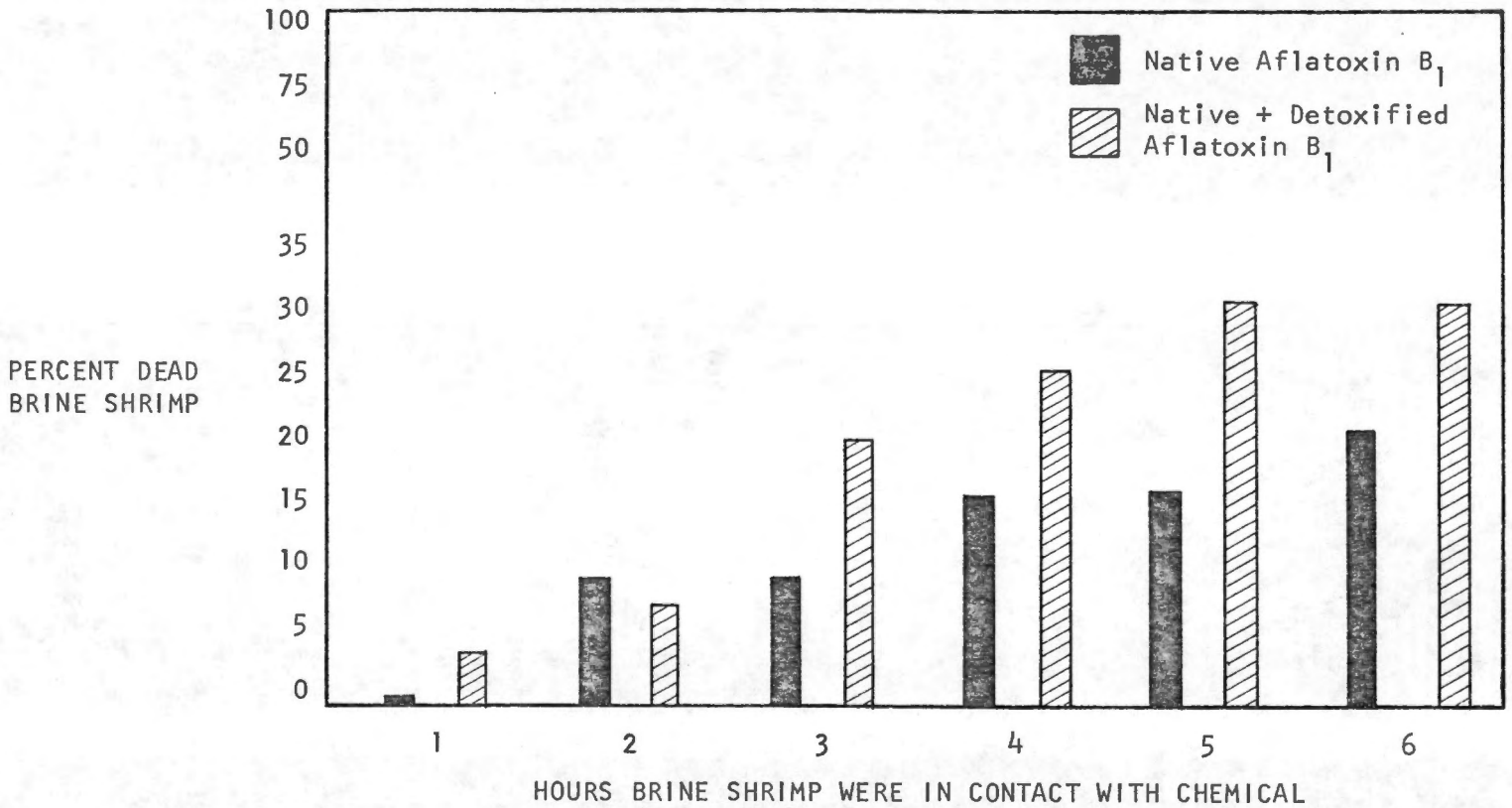


Figure 3. Comparison of the toxicity of 10 μg aflatoxin B_1 and a combination of 10 μg aflatoxin + 10 μg detoxified aflatoxin B_1 as a function of time. Values are the mean of five replicates.

TABLE 14

Percent Brine Shrimp Death as a Function of μg Aflatoxin B_1 Added. All Data are the Mean of Five Replicates.

AMOUNT AFLATOXIN (μg)	AVERAGE PERCENT MORTALITY
0	2.00 ± 4.47
2	15.10 ± 15.90
4	22.30 ± 20.50
6	22.90 ± 20.30
8	23.70 ± 9.97
10	20.10 ± 16.50
12	25.72 ± 13.70
14	30.60 ± 16.20
16	31.14 ± 9.40
18	28.80 ± 18.14
20	56.78 ± 19.30

μg aflatoxin B_1 to which they were exposed (x) yielded a correlation coefficient of 0.850 and a regression equation defined as (Figure 4):

$$y = 1.64x + 9.16$$

Plotting the means and their standard deviations (Figure 5) revealed two data characteristics: (a) although the data as a whole were linear, there was little variation in percent death from 2 to 18 μg aflatoxin B_1 and most of the slope of the regression line was due to the data obtained at 0 and 20 μg aflatoxin B_1 and (b) the standard deviation of the data ranged from 25 to 90% of the mean indicating a great deal of variation in response of brine shrimp to defined aflatoxin B_1 doses.

A combination of aflatoxin B_1 (0-20 μg) and detoxified aflatoxin B_1 (20-0 μg) was also toxic to brine shrimp (Table 15). Regression analysis of percent dead brine shrimp (y) as a function of μg aflatoxin B_1 to which they were exposed (x) yielded a correlation coefficient of 0.880 and a regression equation defined as (Figure 4):

$$y = 2.18x + 11.49$$

The slope of this regression equation is greater than that of the equation for aflatoxin B_1 alone. This suggests that a mixture of aflatoxin B_1 and detoxified aflatoxin B_1 is more toxic than aflatoxin B_1 alone. This suggestion is given further credence by scrutiny of the means and standard deviation of the data. The percent death of brine shrimp was greater for the aflatoxin B_1 + detoxified aflatoxin B_1 combination at all but two dosages of aflatoxin B_1. In addition the lower limit of the standard deviation is as much as twice as high for the combined molecules as for aflatoxin alone.

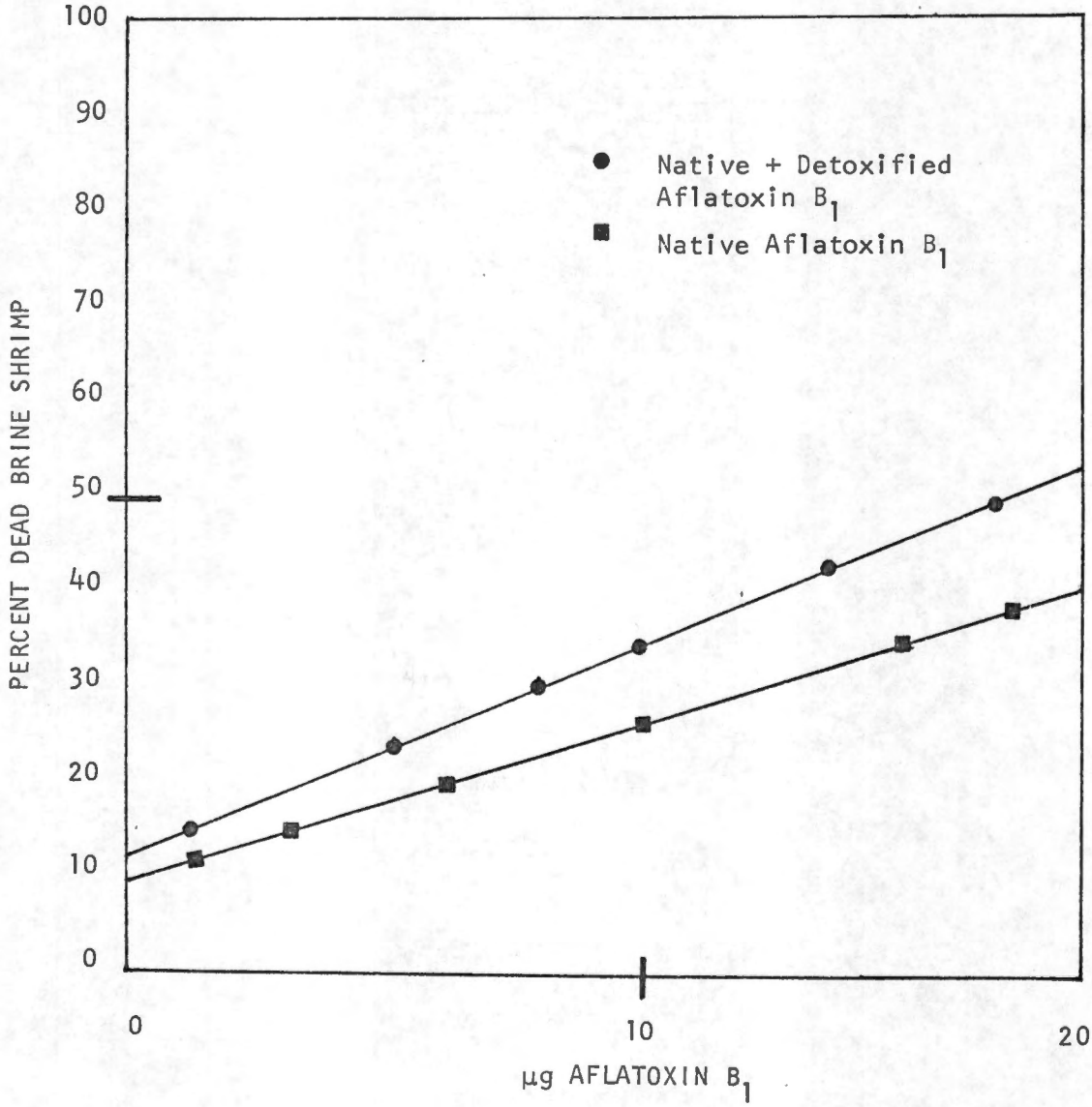


Figure 4. Linear regression analysis of the toxicity of aflatoxin B_1 to brine shrimp ($y = 1.64 + 9.16$) and the toxicity of a combination of aflatoxin B_1 + detoxified aflatoxin B_1 to brine shrimp ($y = 2.18x + 11.49$). Percent death of brine shrimp (y) was a function of µg aflatoxin B_1 (x). Graph points are calculated from the regression equation.

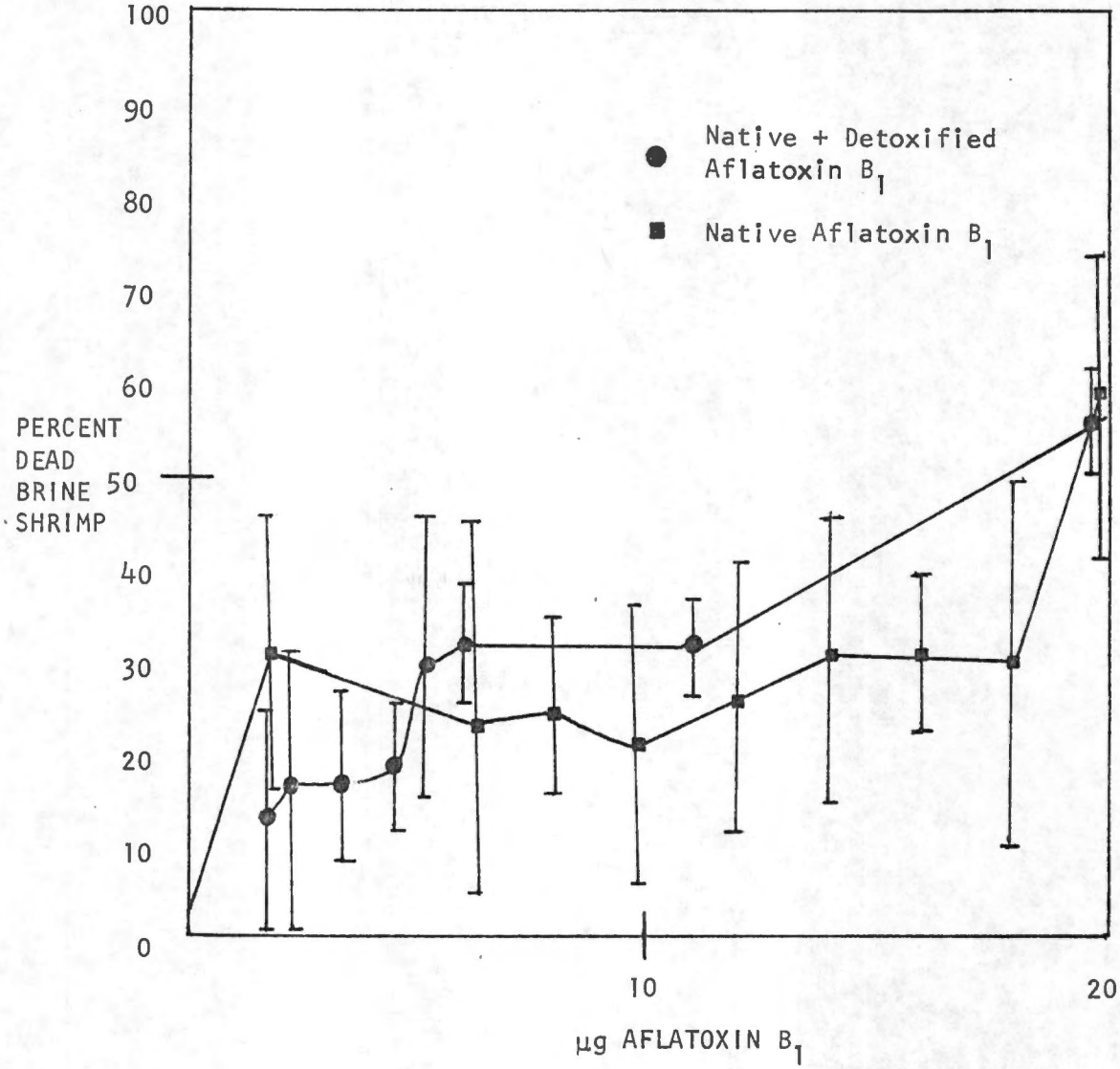


Figure 5. Toxicity of aflatoxin B_1 to brine shrimp and toxicity of a combination of aflatoxin B_1 + detoxified aflatoxin B_1 to brine shrimp. Percent death of brine shrimp (y) was a function of µg aflatoxin B_1 (x). These data are the means and their standard deviations of five replicates.

TABLE 11

Reaction of NaOCl with Aflatoxin B_1 at Room Temperature. Reduction in Fluorescence as a Function of Meq Base Added was Monitored by Spectrofluorometer Measurement at an Excitation Wavelength of 365 nm and an Emission Wavelength of 435 nm. All Data are the Mean of Five Replicates.

22+16+35=73

MEQ NAOCl		% FLUORESCENCE B_1
.00000		100.00
.00032	1.17	69.20
.00064	2.34	44.10
.00100	3.50	35.60
.00128	4.67	23.00
.00160	5.84	23.00
.00192	7.01	19.20
.00220	8.18	17.20
.00260	9.34	9.60
.00290	10.51	9.60
.00320	11.68	3.80

TABLE 15

Measurement of Percent Brine Shrimp Death as a Function of µg of Active Aflatoxin B_1 and Detoxified Aflatoxin B_1 Remaining after Treatment of Aflatoxin B_1 with NaOH. All Data are the Mean of Five Replicates.

MEQ NAOH[a]	mg NaOH	AFLATOXIN B_1-µg	DETOXIFIED AFLATOXIN B_1-µg	% FLUORESCENCE	PERCENT DEAD BRINE SHRIMP
.00000	0	20.0	0.0	100.0	54.6 ± 5.0
.00125	2.5	11.3	8.7	56.4	31.0 ± 3.5
.00250	5 10	6.5	13.5	32.8	31.0 ± 6.5
.00375	~~10~~ 7.5	5.5	14.5	27.3	36.3 ± 3.5
.00500	10	5.6	14.4	28.2	29.5 ± 16.0
.00625	~~20~~ 12.5	4.5	15.5	22.8	18.3 ± 7.6
.00750	~~25~~ 15	4.4	15.6	21.9	16.4 ± 9.7
.00875	~~30~~ 17.5	3.3	16.7	10.9	11.0 ± 19.0
.01000	~~35~~ 20	2.2	17.8	10.9	16.0 ± 28.8
.01250	~~45~~ 22.5	1.8	18.2	9.1	13.3 ± 23.4

[a]The meq of NaOH indicated were added to 0.5 ml of 5% NaCl. In this system 0.00125 meq NaOH reduced the fluorescence of 20 µg aflatoxin B_1 by 44.6%. In earlier chemical studies 0.0125 meq NaOH reduced the fluorescence of 10 µg aflatoxin B_1 by 63.5% in 10 ml distilled water. The moles of NaOH in each system are equal since in the brine shrimp testing 0.00125 meq NaOH x 20 µg aflatoxin B_1/ 0.5 ml of 5% NaCl = the amount in earlier chemical testing 0.0125 meq NaOH x 10 µg aflatoxin B_1/ 10 ml distilled water. Taking into account the dilution factor and µg aflatoxin B_1 in each system, the two values for detoxification of aflatoxin B_1 with NaOH are relatively close with a mean of 54.05 ± 13.36.

The y-intercept term in the two regression equations represents the percent death of brine shrimp in the presence of 0 μg aflatoxin B_1. In the aflatoxin B_1 + detoxified aflatoxin B_1 experiment, this would also mean 20 μg of the detoxified molecule were present. The y-intercept and standard deviation ranges are very similar for the two experimental systems. This suggests, but does not prove, that the detoxified molecule has no inherent toxicity.

III. "IN VITRO" TESTING OF NATIVE AND BASE TREATED AFLATOXIN B_1 IN THE SALMONELLA TESTER STRAINS TA98 AND TA100

Toxicity of Aflatoxin B_1 to S. typhimurium strains TA98 and TA100

Excessive bacterial killing by a mutagen may cause a decrease in the number of revertants observed on a test plate. Incubation of S. typhimurium TA98 and TA100 at 37° C for 13 hours resulted in 10^8-10^9 cells/ml. When bacterial were stressed with concentrations of 0 to 20 μg aflatoxin B_1 the number of viable cells decreased by four to five orders of magnitude. Twenty μg aflatoxin B_1 reduced the number of viable cells of S. typhimurium TA100 to 9.0×10^4 cells/ml (Table 16). Four μg aflatoxin B_1 resulted in 3.6×10^6 cells/ml. S. typhimurium TA98 was also sensitive to the toxic effects of aflatoxin with 20 μg of aflatoxin B_1 reducing the number of viable cells to 4.0×10^4 cells/ml and 4 μg reducing the number of viable cells to 1.52×10^6 cells/ml

Dose Response For S. typhimurium TA98 and TA100 When Exposed to Aflatoxin

Since aflatoxin B_1 was toxic to S. typhimurium it became important to evaluate the dose response for the two tester strains to aflatoxin B_1 (Table 17). In tester strain TA98 a mean of 21.4 revertants

TABLE 16

Evaluation of Toxicity of Aflatoxin B_1 to *S. typhimurium* Tester Strain TA98 and TA100 as a Function of µg Aflatoxin B_1 Added. Values were Obtained in a Standard Plate Count Using Nutrient Agar Media. All Data are the Mean of Five Replicates.

BACTERIAL STRAIN	AMOUNT AFLATOXIN B_1 µg	# COLONIES/PLATE AT THE 10^4 DILUTION
TA100	0.0	TNTC[a]
TA100	0.4	TNTC
TA100	1.0	TNTC
TA100	2.0	TNTC
TA100	4.0	360
TA100	10.0	44
TA100	20.0	9
TA98	0.0	TNTC
TA98	0.4	TNTC
TA98	1.0	343
TA98	2.0	182
TA98	4.0	152
TA98	10.0	81
TA98	20.0	4

[a] TNTC = Too Numerous Too Count (> 500)

TABLE 17

Dose Response for S. typhimurium TA98 and TA100 When Exposed to Aflatoxin B_1 Measuring the Number of Revertants/Plate. All Data are the Mean of Ten Observations

BACTERIAL STRAIN	μg AFLATOXIN B_1						
	0.0	0.1	0.4	1.0	2.0	4.0[a]	10.0[b]
TA98	28	91	96	138	181	466	278
TA98	42	111	142	161	151	440	308
TA98	11	92	101	138	198	249	201
TA98	16	53	87	161	234	291	186
TA98	10	109	190	338	445	756	658
TA100[c]	20	86	111	142	201	471	311
TA100	12	141	139	162	199	342	214
TA100	46	101	142	171	203	229	214
TA100	59	98	195	233	481	912	411

[a]The mean and standard deviation of TA98 = 440.4 ± 199.5. The mean and standard deviation of TA100 = 488.5 ± 299.1.

[b]The mean and standard deviation of TA98 = 326.2 ± 192.4. The mean and standard deviation of TA100 = 287.5 ± 97.2.

[c]One replicate of TA100 was lost due to bacterial contamination.

per plate were observed with no aflatoxin in the test system. The number of revertants increased with increasing amounts of aflatoxin B_1 until at 4.0 μg aflatoxin B_1 a mean of 440.4 reversions/plate were observed. Ten μg of aflatoxin B_1 reduced the mean number of reversions to 326.2 revertants/plate. However, there was an overlap in the standard deviation of 4.0 μg aflatoxin B_1 (440.4 $\pm$ 199.5 revertants/plate) and 10.0 μg aflatoxin B_1 (326.2 $\pm$ 192.4 revertants/plate) indicating: (a) wide variations may be observed in the mutagenicity of aflatoxin B_1 and (b) the dose response for TA98 when exposed to 4.0 and 10.0 μg aflatoxin B_1 was somewhat similar.

Tester strain TA100 showed a similar response to aflatoxin B_1. Increasing dosage of aflatoxin B_1 from 0.1 to 4.0 μg aflatoxin B_1 increased the mean number of revertants observed per plate from 106.5 to 488.5. Ten μg aflatoxin B_1 increased the toxicity such that the mean number of revertants observed per plate was reduced to 287.5. However, again the standard deviations of these means were large (Table 17) indicating an overlap in the response of S. typhimurium when exposed to 4.0 and 10.0 μg aflatoxin B_1.

Mutagenicity of Native and Base Treated Aflatoxin B_1

Base treatment of aflatoxin B_1 resulted in a significant decrease in the mutagenicity and potential carcinogenicity of aflatoxin B_1 in the TA98 tester system (Table 18). Detoxification of 4 μg aflatoxin B_1 by 50% reduced the mean number of revertants/plate from 269.9 to 62.3 (Table 19). Ninety percent detoxification of 4 μg aflatoxin B_1 reduced the mean number of revertants to only 29.68 per plate. However, the mean

TABLE 18

Mutagenicity of 4 μg and 2 μg Aflatoxin B_1, 4 μg Aflatoxin B_1 Detoxified by 50 and 90%, and Bacterial Controls in S. typhimurium Tester Strain TA98. Values are the Number of Revertants/Plate. All Data are the Mean of Five Observations.

	AFLATOXIN B_1				
REPLICATE #	4 μg	2 μg	50% DETOX.	90% DETOX.	BACTERIAL CONTROLS
1	107.4	35.8	53.8	82.0	12.6
2	359.4	271.4	19.4	33.6	12.5
3	409.0	308.0	28.2	24.0	14.0
4	272.4	261.3	54.6	26.6	12.0
5	244.4	43.6	39.8	35.0	20.0
6	264.0	200.6	98.4	14.0	12.4
7	256.6	308.8	99.9	25.2	11.0
8	234.0	186.8	80.6	17.4	12.7
9	350.0	193.8	93.0	18.0	19.0
10	201.8	186.2	55.6	21.0	14.0

TABLE 19

These Data are the Mean Number of Reversions/Plate of All Experimental Systems tested using Tester Strains S. typhimurium TA98 and TA100. All Data are the Mean of Five Observations.

EXPERIMENTAL SYSTEM (Purpose Used)	MEAN NUMBER OF REVERSIONS PER PLATE
TA98--4 μg (Mutagenicity)	269.90 ± 86.28
TA98--2 μg (Mutagenicity)	199.63 ± 96.71
TA98--50% Detoxification (Mutagenicity)	62.33 ± 29.21
TA98--90% Detoxification (Mutagenicity)	29.68 ± 19.58
TA98--Bacterial Control (Spontaneous Reversion)	14.01 ± 2.87
TA98 + NaOH (Spontaneous Reversion)	15.50 ± 8.71
TA98--2-AF(Confirm microsomal activation)	450.00 ± 152.90
TA98--EMS (Confirm tester strain reversion)	49.00 ± 31.28
TA98 + S-9 (Spontaneous Reversion)	14.02 ± 3.51
S-9 Control (Test for Sterile Solution)	000.00 ± 000.00
B_1 Control (Test for Sterile Solution)	000.00 ± 000.00
EMS Control (Test for Sterile Solution)	000.00 ± 000.00
2-AF Control (Test for Sterile Solution)	000.00 ± 000.00
TA100--4 μg (Mutagenicity)	317.68 ± 112.71
TA100--2 μg (Mutagenicity)	258.06 ± 135.70
TA100--50% Detoxification (Mutagenicity)	79.36 ± 26.72
TA100--90% Detoxification (Mutagenicity)	31.40 ± 12.77
TA100--Bacterial Control (Spontaneous Reversion)	15.00 ± 5.44
TA100--2-AF (Confirm microsomal activation)	272.00 ± 151.68
TA100--EMS (Confirm tester strain reversion)	202.00 ± 124.20
TA100 + NaOH (Spontaneous Reversion)	17.10 ± 6.32
TA100 + S-9 (Spontaneous Reversion)	14.19 ± 4.33

mutagenicity of 4 μg of 90% detoxified aflatoxin B_1 (29.68 revertants/plate) was still significantly greater (at the 0.05 level) than that of the S-9 + bacterial controls (spontaneous revertants = 14.02/plate).

Base treatment of aflatoxin B_1 also resulted in a decrease in the mutagenicity and potential carcinogenicity of aflatoxin B_1 in the TA100 tester system (Table 20). Fifty percent detoxification of 4 μg aflatoxin B_1 caused a decrease in mutagenicity from 317.68 to 79.36 revertants/plate. Ninety percent detoxification of aflatoxin B_1 with base resulted in a significant decrease at the 0.10 level to only 30.78 revertants/plate. This was greater than a 90% decrease in mutagenicity. However, the mutagenicity of 4 μg of 90% detoxified aflatoxin B_1 was still greater than the bacterial controls (15.00 revertants/plate) which detected spontaneous reversion or zero mutagenicity at the 0.05 level.

TABLE 20

Mutagenicity of 4 μg and 2 μg Aflatoxin B_1, 4 μg Aflatoxin B_1 Detoxified by 50 and 90%, and Bacterial Controls in S. typhimurium Tester Strain TA100. Values are the Number of Revertants/Plate. All data are the Mean of Five Observations.

REPLICATE #	AFLATOXIN B_1 4 μg	2 μg	50% DETOX. of 4 μg	90% DETOX. of 4 μg	BAC. CONT.[a]
1	607.0	550.8	15.2	8.6	19.0
2	233.6	265.2	86.4	46.4	24.5
3	311.8	324.0	90.2	41.4	10.0
4	324.8	214.0	95.8	46.4	10.0
5	348.6	293.0	75.0	42.2	19.5
6	320.0	134.6	70.8	28.8	10.0
7	262.0	328.4	113.8	33.0	11.5
8	299.0	276.8	90.8	24.2	12.2
9	287.0	96.4	93.0	24.2	12.2
10	183.0	97.4	62.6	18.6	21.1

[a]Bac. Cont. = the number of spontaneous revertants

CHAPTER IV

DISCUSSION

The experimental data in this study have shown that alkaline treatment of aflatoxin B_1 did not destroy all native aflatoxin molecules. After 90-95% of the fluorescence had been eliminated, the aflatoxin molecule became resistant to further detoxification. Only treatment with sodium hypochlorite was found to have the potential to eliminate all fluorescent molecules. Since all native aflatoxin B_1 molecules were not destroyed by base treatment, evaluation of the toxicity and biological activity of the remaining native molecules as well as the detoxified molecules became important considerations.

Toxicity studies with brine shrimp indicate that a mixture of aflatoxin B_1 and detoxified aflatoxin B_1 were more toxic than aflatoxin B_1 alone. The procedure used was a modification in the procedure of Brown et al. (11), in which the testing period was shortened from twenty-four hours to six hours with an increased aflatoxin dosage. Testing time was shortened because excessive evaporation occurred in twenty-four hours and death of controls resulted from the increased osmotic pressure. With the shorter testing period and greater aflatoxin dosage a corresponding loss in sensitivity was expected. In a twenty-four hour testing period, Brown et al. (11) found 100% death using two μg aflatoxin B_1. Although this test was constructed in a less sensitive manner in these experiments, it was shown to be highly accurate. The experimental data have shown a high correlation between

percent death and amount of aflatoxin present (0.85 for native aflatoxin alone and 0.88 for native + detoxified aflatoxin B_1). Five replicates of the toxicity experiment have demonstrated the increased toxicity of a mixture of native and detoxified aflatoxin, therefore the technique of the experiment was accurate although it did not have the sensitivity of Brown's experiment. The increased toxicity of a mixture of native and detoxified aflatoxin B_1 is important since experimental data have shown that chemical treatment detoxified only 90-95% of the aflatoxin B_1 molecules. Therefore, one could suggest that since incomplete detoxification of aflatoxin B_1 would lead to increased toxicity of remaining native molecules, it would be preferable to have no detoxification unless assurance of virtually complete detoxification were possible.

A highly significant reduction in mutagenicity (0.05) and potential carcinogenicity was found in detoxified aflatoxin B_1 when compared with native aflatoxin B_1 in an "in vitro" assay system. However, the mutagenicity of 90% detoxified aflatoxin was not reduced to zero when compared to all controls. There are several questions as to the applicability of an "in vitro" assay for detection of biological activity of carcinogens such as: (a) is the carcinogen activated in the bacterial system in the same manner it would be in the body, (b) can a comparison be made in the mutation of a unicellular organism and a multicellular organism such as man, and (c) can a comparison be made as to the occurrence of revertants on a petri plate and carcinogenesis in the human body? In this study the bacterial assay for carcinogens has been used very conservatively. The S. typhimurium tester

system was not used to detect carcinogens, but to compare changes in the mutagenicity of a known carcinogen during detoxification. The use of bacterial mutants as a test system for carcinogen detection would appear practical because so many carcinogens appear to be mutagens acting on DNA and all DNA is basically the same (3). Ames et al. (3) have theorized that many of the more powerful carcinogens such as aflatoxin B_1, are biologically active due to a planar aromatic ring system which intercalates in the DNA base pair stack and a reactive side chain which increased the activity of an intercalator by orders of magnitude. Since there is an opening in the lactone ring of the aflatoxin molecule and loss of the most reactive side group during base treatment, a corresponding loss in mutagenicity, as seen in this study, would seem reasonable. However, the mutagenicity of the detoxified aflatoxin was still significantly greater (.05 level) than the level of spontaneous reversion of the bacterial controls—indicating that the carcinogenicity of the detoxified aflatoxin is not reduced to zero. The "Delaney Clause" (24) advises that any substance added to a food product must have been found to have zero carcinogenicity. However, if the substance is in the food or feed naturally and is detoxified such that the carcinogenicity is greatly reduced (FDA has established a level of less than 30 ppb for aflatoxin) should this food or feed be prohibited by the "Delaney clause" and by law? More important, is the presence of any trace amount of carcinogen in a food product safe, or is it impractical to insist upon complete freedom of feeds and foods from contamination with carcinogens.

The results of this study have indicated: (a) the aflatoxin

molecule became resistant to chemical detoxification after 90-95% of the fluorescence had been eliminated, (b) a mixture of native and detoxified aflatoxin B_1 was more toxic to brine shrimp than native aflatoxin alone, and (c) a significant decrease in biological activity occurred when aflatoxin B_1 was detoxified by 90%, however, the mutagenicity and potential carcinogenicity were not reduced to zero.

These data would suggest that before the addition of aflatoxin detoxified foodstuffs to the animal or human diet matrix, intensive and long range studies must be undertaken to fully evaluate the toxicity and carcinogenicity of detoxified foods and feeds containing aflatoxin B_1

CHAPTER V

SUMMARY

This experimental study was undertaken to determine testing proceedures which would be applicable to studies concerned with detoxification of aflatoxin in feeds and foodstuff. Experiments were undertaken to ascertain: (1) the decrease in fluorescence of aflatoxin B_1 when chemically treated with NaOH, NH_4OH, and NaOCl, (2) the toxicity of native and base treated aflatoxin B_1, and (3) the carcinogenicity of native and detoxified aflatoxin B_1 in an "in vitro" assay system.

The results of this study indicated: (1) there may be difficulties in complete detoxification of the aflatoxin B_1 molecule since the molecule becomes resistant to detoxification after 90--95% of the molecules have been detoxified, (2) toxicity studies have demonstrated an effect in which the detoxified molecule increased toxicity of remaining native molecules, and (3) "in vitro" carcinogenicity assays show that the carcinogenicity of aflatoxin B_1 is greatly reduced by base treatment, however, it is not completely destroyed.

Since chemical studies indicate that all of the aflatoxin molecules may not be completely eliminated by base treatment and biological studies show that even low levels of aflatoxin remain biologically active, it would appear that intensive and longrange studies to evaluate safety of detoxified foods and feeds are needed. Until these studies are performed, the safety of detoxified aflatoxin containing foods and feeds remains questionable.

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VITA

Frances Ann Draughon, the former Frances Ann Moore, was born in Canebrake, West Virginia, on April 30, 1952. She attended elementary school in Canebrake and junior high school in Berwind, West Virginia. She graduated from Cocke County High School in Newport, Tennessee, in 1970. The following June she entered the University of Tennessee in Knoxville, receiving a Bachelor of Arts degree in Microbiology in December, 1973.

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She and her husband, Mr. Kenneth Earl Draughon, were married on December 15, 1973. She will be entering the University of Georgia Graduate School in Athens, on March 25, 1976, to further her studies in Food Science.