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MUTAGENICITY OF CHEMICALLY-TREATED, AFLATOXIN-CONTAMINATED PEANUT MEAL

THE UNIVERSITY OF ARIZONA M.S. 1982

MUTAGENICITY OF CHEMICALLY-TREATED, AFLATOXIN-CONTAMINATED PEANUT MEAL

by

Tomoko Ito

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ABSTRACT

Naturally contaminated peanut meal was treated with $NaHSO_3$, H_2O_2, H_2O_2 + 1% $Ca(OH)_2$, NaOCl, and NaOCl + 1% $Ca(OH)_2$ at concentrations of 0.05 and 2.0% at 22 or 60C, at low and high moisture levels for 24 hours to determine the extent of aflatoxin degradation using the Ames mutagenic test.

All treatments were effective in decreasing mutagenic response of S. typhimurium TA 98 and TA 100 strains. Adding 1% $Ca(OH)_2$ to NaOCl decreased mutagenic activity significantly; whereas adding it to H_2O_2 did not. Of all treatments NaOCl + 1% $Ca(OH)_2$ was the most effective; complete elimination of mutagenicity was not obtained under these treatment conditions.

Increasing the moisture content did not decrease mutagenic activity significantly; the higher reaction temperature did cause a significant decrease.

Comparison of results obtained from chemical analyses with those obtained with the Ames test gave a correlation coefficient of 0.9.

CHAPTER 1

INTRODUCTION

Aflatoxins are secondary metabolites produced by certain toxin-producing strains of Aspergillus Flavus and A. parasiticus grown on substrates when environmental conditions are suitable for development of the molds.[1] They appear to compete best in the microbiological world when the water activity is relatively low (0.84-0.86) and temperature is relatively high (25-40C),[1] however, their requirements are essentially non-specific. On high carbohydrate-content substrates they can grow over a wide range of temperatures. Aflatoxins have been recognized as potential environmental contaminants for agricultural commodities and their products worldwide.[2]

Since aflatoxins were first found in peanut meal, the peanut and its products have been studied extensively as substrates for production of alfatoxin B_1 (AFB_1), B_2, G_1, and and G_2, the main aflatoxins in peanuts.[3]

Most aflatoxins, in particular AFB_1, cause acute toxicity to organisms from microorganisms to plants or to human beings. Doses below those causing toxicity, exhibit carcinogenic potential.[3] Ames and his colleagues

reported that alfatoxin B_1 was one of the most potent carcinogens among those tested.[4-7]

Because of their world wide occurrence, high toxicity and carcinogenicity, many attempts have been tried to develop effective and adequate detoxification of aflatoxin. Until now, chemical inactivation, physical separation, and microbial inactivation have destroyed aflatoxins in contaminated products. Chemical inactivation with a considerable number of chemicals has proven to be effective.[8]

Until now, most of the evaluations determining aflatoxin degradation have been chemical methods, such as thin layer chromatography (TLC), ultra violet (UV), absorbance, or high performance liquid chromatography (HPLC).[2,9] Biological evaluation is necessary when one considers the threat of carcinogenicity posed by even a low dose of aflatoxins or their chemical breakdown products.

Because of the environmental carcinogen problem, the need for a convenient short-term carcinogenicity test has increased, and sizable studies have been carried out utilizing several species of microorganisms or cell culture.[10] Among them, the Salmonella/mammalian-microsome mutagenicity test (Ames test) has been found to be useful. This test has become an integral part in both laboratory and industrial experiments because of its advantages of having no requirement for special facilities, its high reliability, the shortness of time (48 hours), and its economy.[11-14]

In this study, several chemical degradation treatments of peanut meal were evaluated biologically using the Ames test. Comparison of chemical evaluation was also made.

The following possibilities were considered:

1. The effects of chemical treatments evaluated by mutagenicity of aflatoxins.
2. The possibility of degradation products showing mutagenicity.
3. Usefulness of Ames test as a method for evaluating degradation treatments.

$NaHSO_3$, H_2O_2, NaOCl, $Ca(OH)_2$, and their combinations were chosen as degradation treatments because of their wide use and acceptance even in primitive societies.

CHAPTER 2

LITERATURE REVIEW

Aflatoxin in Peanuts

Aflatoxins were discovered as a result of an epizootic outbreak of hepatic necrosis associated with imported Brazilian peanut meal in England in 1960.[2] During the tracing of its cause in 1961, Sargent et al. reported the presence of a new fluorescent compound seen when extracts of the meal were developed on paper chromatography in 1961.[16] Later, this spot was shown to separate into four main components on thin layer chromatography developed in chloroform-methanol. Fluorescent blue spots under UV light were designated AFB_1 and AFB_2 and fluorescent green spots AFG_1 and AFG_2.[17] Their chemical structures are shown in Figure 1.

Although other aflatoxins, such as AFB_{2a}, AFG_{2a}, the 2-hydroxy derivatives of AFB_1 and AFG_1, have been reported to be produced in small amounts by certain *A. flavus* strains in fungal cultures[18], AFB_1, AFB_2, AFG_1, and AFG_2 are the only naturally-occurring aflatoxins in peanuts.[2]

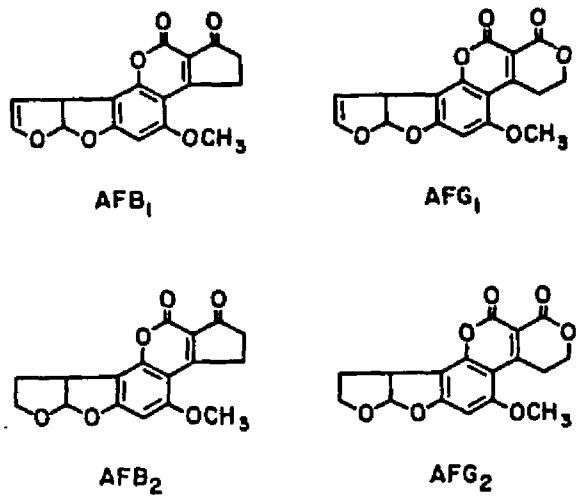

Figure 1. Structures of the Aflatoxins

AFB_1, the most predominant, is always accompanied by AFB_2 and sometimes in lesser amounts with the AFG series.[3] In the United States, AFB_1 has been most often identified in commercial samples in peanuts.[3]

The U. S. Food and Drug Administration regulates total aflatoxin in all food with an "action level" of 20 ppb.

Survey data in the United States and Canada from 1972 to 1975 showed a 19% incidence of aflatoxin contamination with 1 ppb average in consumer products.[1] Tiemstra reported in 1977 that industry surveys have indicated less than 1 ppb aflatoxin content in peanuts sold in the United States.[19]

Biological Effects of Aflatoxins

Biological Activities

Most aflatoxins show strong biological potency and cause various effects upon a wide range of living things. After extensive studies, the following biological activities have been reported.[2,3]

1. Cytotoxicity
2. Carcinogenicity
3. Mutagenicity
4. Covalent binding to macromolecules, including nucleic acid, DNA, RNA, and protein.

5. Inhibition of DNA synthesis
6. Inhibition of RNA synthesis
7. Inhibition of protein synthesis
8. Alternation of steroid hormone mechanism
9. Inhibition of lipid synthesis
10. Inhibition of membrane transport

Metabolism of Aflatoxins

Aflatoxins are primarily metablized by the microsomal mixed-function oxidase system[20], an electron-transport-chain system that requires both NADPH[21] and molecular oxygen.[22] The following are general reactions:

$$RH + NADPH + H^+ + O_2 \longrightarrow ROH + NADP^+ + H_2O$$

This complex enzymatic system has been resolved into three essential components: a flavoprotein (NADPH-cytochrome P-450 reductase), a phospholipid (phosphatidyl choline), and a hemoprotein (cytochrome P-450) that functions as a terminal oxidase.[22,23]

In this enzymatic system, a wide variety of hydroxylated derivatives occur which are conjugated to sulfate or glucuronic acid to form water-soluble glucuronide or sulfate esters. The net result is usually detoxification, however, certain highly reactive derivatives are generated during the metabolic pathway of certain aflatoxins.[20] They may react covalently with various nucleophilic centers and cause biological hazards.[24]

The probable established pathway of aflatoxin B_1 metabolism is shown in Figure 2.[3,21]

Those multi-function oxidases mainly exist on the endoplasm reticulum of liver cells but also may exist in other organs.[3,25]

The differences of susceptibility among species may be due to the different metabolism and disposition of aflatoxins. The rapid conversion of AFB_1 to less toxic and carcinogenic derivatives was reported in aflatoxin resistant species of animals *in vitro* and *in vivo*.[24,26,27]

Carcinogenicity of Aflatoxins

Aflatoxins are very potent carcinogens and are carcinogenic to many species of animals including human beings, rodents, nonhuman primates, birds, and fish.[1,2,3]

Wogan reported that the four natural occurring aflatoxins, AFB_1, AFB_2, and AFG_1, and AFG_2, caused hepatocarcinoma in rats when induced as a mixture, and they were also associated with neoplastic formation in cells of other organs.[28] The dietary levels of AFB_1 which can provoke carcinogenic activity may be very low. According to Wogan et al., dietary levels at 0.1 ppm induced liver carcinoma in rats at an incidence greater than 50% after feeding for 80 weeks. Fisher rats developed tumors and preneoplastic lesions at a dietary level of $1x10^{-3}$ ppm.[29,30]

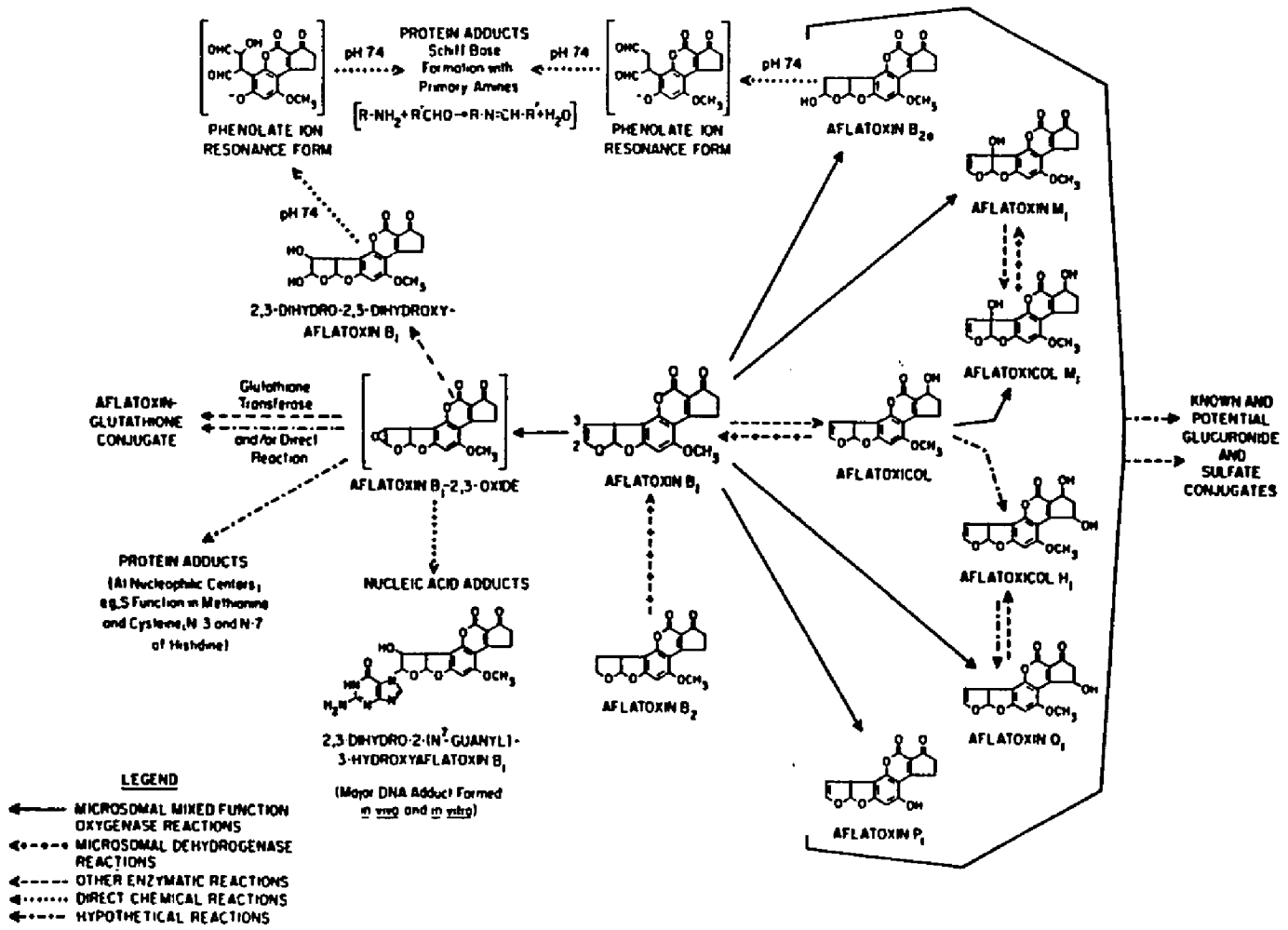

Figure 2. Metabolic Transformations of Aflatoxin B_1

As are most other chemical carcinogens, AFB_1 is a precarcinogen. It enters the body in an inactive form[31] and needs activation to cause carcinogenisis. In the mixed-function oxidase system, it is converted sequentially into "proximate" and "ultimate" carcinogenic derivatives that interact with critical tissue components necessary for carcinogenisis. The electrophilic form has long been suggested to be the "ultimate" form but direct evidence has not yet been shown.[32,33]

Other aflatoxins, including metabolic aflatoxin derivatives, were investigated; some showed carcinogenic activity with metabolic activation and others did not.[3] Those investigations also revealed the importance of the 2,3 carbon-carbon double bond structure[20], which was deduced to be reactive K region of carcinogen of Pulmans' theory for the carcinogenicity of aromatic hydrocarbons.[3] Schoental[34] and Miller et al.[33] believed that the active metabolic derivatives might be the 2-3 epoxide derivatives which were readily formed by epoxidation from the unsaturated 2-3 double bond.

The mechanism of carcinogesis by aflatoxin as yet has not been elucidated. Nucleic acids and/or proteins have been suggested as being reactive sites for carcinogenisis, but recent studies have been in favor of the somatic mutation theory which supposes that a cellular genetic change

may result in tumor induction implicating DNA as the critical target.[3-7,13]

Mutagenicity of Aflatoxins

The close correlation between carcinogenicity and mutagenicity has been shown in extensive studies by Dr. Ames and his colleagues. Those studies have indicated that almost all chemical carcinogens are also mutagens and have provided evidence for the somatic mutation theory of carcinogenicity.[4-7]

Mechanisms of mutation are classified into several different types: base substitutions, frameshift, and larger lesions. Ames deduced the frameshaft mutation theory for aromatic hydrocarbon carcinogens. Polycyclic aromatic compounds, which have a ring structure planar enough to intercalate in the DNA base-pair stack, may stabilize a shifted pairing in a repetitive sequence of bases. During DNA replication or repair, this may result in addition or deletion of base pairs in the DNA sequences. Aflatoxins have been classified as frameshift mutagens.[5,35]

Miller et al. suggested that a 2,3 position epoxidized derivative in electrophilic form may be the active metabolite which causes carcinogenicity and mutagenicity.[20,33] Isolation of the 2,3 epoxide has not been successful; however, an experiment using 2,3 dichloride, a

synthesized analog, showed more potency than AFB_1 with the Ames test.[36]

The direct evidence for the hypothesis that DNA is the main target of aflatoxin has not been reported. However, Garner reported strong binding between DNA and AFB_1 with microsomal enzymes.[37] Garner and Miller also reported that a S. typhimurium strain with a DNA-repair enzyme system was resistant to AFB_1, but the strain without a repair system was very sensitive to AFB_1.[20]

Salmonella/Mammalian-Microsome Mutagenic Test; Methods

Noting a close relationship between mutagenic and carcinogenic activity, a convenient microbial short-term test for carcinogens was developed, which is called the Salmonella/mammalian-microsome mutagenic test or Ames test.[4-7]

In this test, the compounds are tested with several mutants of Salmonella typhimrium on petri plates which contain a minimal amount of nutrients and slight amounts of histidine. Those mutants were originally selected for sensitivity and specificity and are incubated with or without rat or human liver homogenates. Activity is measured by the number of colonies which revert from histidine-requiring mutants back to wild strains by the mutagens.[38] Originally the test was developed as a screening test for chemical carcinogens.[4,6] More recently, it has proven

reliable not only as a screening test but also as a comparative test for carcinogenicity.[13,39]

Four strains of S. typhimurium LT_2 which have histidine-dependent mutations through different genes, his G46, his C207, his C3076, and his D3052 have been used for the Ames test. Since they have different specificity for mutations, appropriate selection should be made.[4] His G46 has been used to detect base-pair substitution mutation and his C207, his C3076, and his D3052 have been used to detect various types of frameshift mutation.[4]

A few types of organisms with sensitivity to different types of mutation or organisms with increased sensitivity have been added gradually. The deletion of the uvrB region of the chromosome, which resulted in elimination of the excision repair system, has improved the sensitivy to mutagenic effects of chemical compounds.[4,20]

TA 1535, TA 1536, TA 1537, and TA 1538 strains which were developed from his G46, his C207, his C3076, and his D3052 respectively, have a deep, rough rfa mutation which removes the polysaccharide side chain of the lipolysaccharide that coats the bacteria surface down to the ketodetoxyoctanoate-lipid core. Rfa mutation makes the bacteria more permeable and has proven to have advantages when the ring system of the compound tested increases in number. The specificity of TA 1537 has been reported to

overlap that of TA 1536 so that TA 1536 is usually omitted from the tester strain.[4,20]

McCann et al. reported the advantages of using TA 100 and TA 98 which were developed from TA 1535 and TA 1538 respectively. Their R factor (plasmids carrying the anti-biotic resistance gene) has proven to be extremely effective in detecting the classes of carcinogens which did not show or showed weak mutagenicity in previous studies. The role of R factor has not yet been elucidated, however, Macphee suggested that its role was to enhance the error-prone repair system.[40] McCann observed the existence of a new endonuclease in R factor plasmid, pk 101-containing strains.[7]

S-9 Activation for Ames Test

Because of the wide variety of chemicals which are precarcinogens,[32] including aflatoxins,[4] activation *in vitro* is very important. If activation is not adequate, the results show a false negative.[12] Most chemical carinogens are metabolized by the mixed-function oxidase system which is located mainly in liver cells. The liver microsome fraction is used for *in vitro* activation. The liver S-9 fraction of rats induced with Aroclor 1254 to increase enzyme activity has been accepted as the standard method.[38]

Meanwhile, several factors which could affect activation have been discussed. When comparing S-9 fraction

activity from other organs, it has been reported that kidney S-9 of rats showed less activity than liver S-9, and bladder S-9 showed only weak activity.[41]

Species differences have also been reported. Sugimura found no marked differences in the preparation of S-9 mix from different species of rodents, such as mice, hamsters, guinea pigs, rabbits, and monkeys.[42] The human liver S-9 fraction showed less activity than that of rat which may have resulted from the difficulty of getting fresh human liver.[7] Because a few chemicals, such as phenacetin, cannot be activated by rat S-9 mix,[43] the results using human liver S-9 mix are very important in screening of new chemical cacinogens.[37,42]

To increase liver enzyme activity, the induction of several chemicals have been reported. Chemically induced rat liver S-9 showed stronger activity than an uninduced fraction, and Aroclor 1254 induced S-9 most favorably over a wide range of chemicals.[37]

NADP and glucose-6-phosphate (G-6-P) were also included as cofactors in S-9 mix preparation. The S-9 fraction consists of microsomes associated with drug-metabolizing enzymes including cytochrome P-450 and P-448 and a supernatant fraction rich in glucose-6-phosphate dehydrogenase. G-6-PD can reduce NADP to NADPH in the presence of G-6-P. With these cofactors the reactions can

proceed and the results are suitable for converting most compounds to reactive forms.[22,42] Because activation with S-9 mix is essential to this enzyme assay, an adequate and effective amount of S-9 should be added for a specific chemical. Excessive S-9 fraction in the preparation may depress the metabolic activity.[37,42]

Mutagenicity of Aflatoxins with Ames Test

Since the initial experiments with the Ames test demonstrated the high mutagenic activity of aflatoxin B_1, many investigations with both naturally occurring aflatoxins and various metabolic derivatives have been conducted. The aflatoxins and their derivatives for which mutagenicity has been reported are as follows:

Aflatoxicol (AFL)--Patterson reported the cyclopentenone ring of AFB_1 was reduced by a NADP-dependent oxidoreductase, and AFL was formed *in vitro* by liver enzyme activation.[21] AFL has been reported to be a potential mutagen, less potent than AFB_1 but more potent than all other forms.[3] According to Wong, AFL showed a potency of 0.228 when the potency of AFB_1 was assumed to be 1.[39]

Aflatoxin G_1 (AFG_1)--McCann reported that AFG_1 was a relatively strong carcinogen.[6] Its mutagenicity was similar to that of aflatoxin M_1 but much less than that of AFB_1.[3] Relative potency as measured by Wong was 0.33.[39]

Aflatoxin M_1 (AFM_1)--Hydroxylation of AFB_1 at the 4 position carbon forms AFM_1. AFM_1, a metabolic derivative, has been detected in the excretion products of the wide variety of animals. It loses some of its mutagenic activity because of detoxification by hydroxylation.[3] Its mutagenic activity is almost equivalent to that of aflatoxin G_1.[39]

Aflatoxin H_1 (AFL H_1)--AFL H_1 is a dihydroxy derivative of AFB_1 and has been reported to be formed from AFB_1 *in vitro*. It has been also reported to be formed from AFL Q_1 and AFL.[44] Wong demonstrated that its mutagenic potency was 0.02 relative to AFB_1.[39] It was, however, reported to be nontoxic to chicken embryos.[3]

Aflatoxin Q_1 (AFQ_1)--AFQ_1 is formed by ring hydroxylation of AFB_1. It has been identified as the major metabolite of *in vitro* activation when monkey and human liver microsomes are used.[3] It showed a relative potency of 0.01.[39]

Aflatoxin B_2 (AFB_2)--AFB_2 is the dihydro-derivative of AFB_1 and is one of the naturally occurring aflatoxins.[3] Although it does not have the 2,3 double bond structure, it has relatively strong mutagenicity; relative potency as measured by Wong was 0.012.[7,39] Roebuck et al. observed the formation of AFB_1 from AFB_2 when the latter was incubated with duckling liver postmitochondrial supernatents.[45] To explain its mutagenicity, Swenson et al. suggested a

conversion of AFB_2 to AFB_1 by desaturation at the 2,3 carbons of AFB_2.[46]

Aflatoxin G_2 (AFG_2)--AFG_2, the dehydro-derivative of AFG_1, is one of the naturally occurring aflatoxins.[3] According to Wong, AFG_2 showed only slight mutagenicity.[39]

Aflatoxin P_1 (AFP_1), aflatoxin B_{2a} (AFB_{2a}), and aflatoxin G_{2a} (AFG_{2a})--These are all the metabolic AF derivatives, and AFP_1 is the major excretory product of AFB_1 in monkeys.[3] AFP_1 is almost non-mutagenic and AFB_{2a} and AFG_{2a} are both non-mutagenic.[39]

The Evaluation of Degradation of Aflatoxin

Several degradation methods have been evaluated using the Ames test. Uwaifo and Bababumi discussed the effect of hydroxylation as a detoxification method by the comparison between AFB_1 and AFM_1 using TA 98, TA 100, TA 1535, TA 1537, and TA 1538. The experiment confirmed previous experiments indicating that hydroxylation of AF decreased mutagenic activity.[47]

Fong et al. reported the effect of heat on AF degradation in peanut oil using TA 98 and TA 100. The results indicated that although the usual method of cooking oil considerably decreased the mutagenicity, this reduction was incomplete. Fong warned of the possibility of carcinogenesis caused by consumption of contaminated peanut oil in China.

Draughon et al. observed a 90-94% decrease of mutagenicity by treatments with NaOH and NH_4OH using the aqueous and DMSO solutions of AFB_1. Further detoxification was limited by increasing amounts of reagents. He also reported that the decrease in mutagenicity corresponded well with the decrease of AFB_1 as evaluated by TLC.[49]

CHAPTER 3

MATERIAL AND METHOD

The purpose of this study was to investigate the effect of the following reagents, $NaHSO_3$, H_2O_2, H_2O_2 + $Ca(OH)_2$, NaOCl (Clorox), NaOCl + $Ca(OH)_2$, at two different concentrations, at two different moisture levels, and at two temperatures on the decrease of mutagenic activity of aflatoxins and their degradation products in naturally-contaminated peanut meals.

Specific treatments were dictated by the multifactorial design shown in Table 1. There were three replications for each treatment condition.

Extraction and chemical evaluation of samples were performed by Wang.[15]

Reagents

Reagents used were: Sodium bisulfite (99.82% purity, ACS grade), hydrogen peroxide (30% concentration, ACS), Clorox (5.25% NaOCl), calcium hydroxide (96.89% purity, ACS).

Table 1. List of Treatment Conditions

Parameter Reagents	Reagent Concentration %			Moisture %		Temperature C	
$NaHSO_3$	0	0.5	2.0	20	30	22	62
H_2O_2	0	0.5	2.0	20	30	22	62
H_2O_2 + 1% $Ca(OH)_2$	0	0.5	2.0	20	30	22	62
NaOCl	0	0.5	2.0	40	50	22	62
NaOCl + 1% $Ca(OH)_2$	0	0.5	2.0	40	50	22	62

Samples

Naturally-contaminated peanut meal was obtained from the National Peanut Research Laboratories, U. S. Department of Agriculture, Georgia.

Hydration of Meals

In general, treatments were initiated by elevating the moisture content of the meal to the desired level by adding the calculated amount of distilled water. For the first three treatments, the final moisture was adjusted to 20% and 30%. For the last two treatments, it was necessary to elevate the final meal moisture to 40% and 50% due to a concentration of only 5.25% of sodium hypochlorite in Clorox.

Treatment of Peanut Samples

For treatments without $Ca(OH)_2$, 100g of the peanut meal was weighed for each replication and treated by placing it into 20cm x 10cm plastic bags (75-Maraflex-American Canning Company) and adding $NaHSO_3$, H_2O_2, or NaOCl (Clorox) at final concentrations of 0.5% or 2.0% based on the dry matter of the peanut meal. These water-soluble reagents were dissolved in the water used to hydrate the meal, thus assuring a homogeneous distribution of reagent throughout the meal.

For treatments including $Ca(OH)_2$, 1% calcium hydroxide based on the dry weight of peanut meal was thoroughly mixed into the hydrated meal and then the reagent (H_2O_2 or Clorox) was added.

After the bags were heat sealed, the contents were mixed by hand, and were allowed to react at 22C or 62C for 24 hours. After the treatment time, samples of the first three experiments were spread on a plastic tray in a hood at ambient temperature for at least 24 hours. Drying of the samples for the last two experiments was done in two phases: (1) air drying for six hours and (2) drying in vacuum oven at ca. 29C for 24 hours. All samples were then stored at 4C until analysis could be completed. Three replicates of each treatment were prepared and analyzed.[15]

Analytical Procedure

Chemical Assay

Meal samples were chemically assayed for aflatoxin by the procedure of Pons et al. with a minor modification.[50] The modification was the addition of hexane to remove oil prior to chloroform (or methylene dichloride) partition. All the chemicals used were ACS grade. Aflatoxin quantitation was done using thin-layer chromatography.

The plates were prepared using Adsorbosil-plus-1 (Applied Science Laboratories) and applying a 0.5mm layer. They were dried overnight at 110C and activated for ca. 30 minutes immediately prior to use at the same temperature. In the second part of the study, precoated plates with 0.25mm Adsorbosil-plus-1 (Applied Science Division, Milton Roy Company Laboratory Group) were used.

Mutagenic Assay

Meal samples were extracted with acetone in the ratio 25g/250ml using the procedure of Pons et al.[50] These extractions were used for the mutagenic assay without further purification. For the preparation of the aqueous sample, 0.1ml of sterilized water was added to the desired portion of extraction and the acetone portion was evaporated under a nitrogen gas stream prior to assay at 60C.

Salmonella typhimurium TA 98 and TA 100 strains were generously supplied by Dr. Leonard Bjeldanes, University of

California-Berkeley. Strains were reisolated to isolate low spontaneous reversion rate colonies, and stored by the procedure of Ames et al.[37]

Standard plate assays were carried out using the procedure of Ames et al.[37] with minor modifications. Shortly before plating the sample was mixed with 0.5ml S-9 mix (Litton Bionetics) and added to 2ml top agar containing a determined amount of histidine, biotin, and 0.1ml bacterial culture. The mixture was mixed thoroughly using a Vortex mixer and poured onto the surface of a base agar plate. The plate was tilted to evenly distribute the top agar which was then allowed to harden. The plates were incubated at 37C for 48-72 hours and revertant colonies were counted. Control plates for determining the spontaneous reversion rate were evaluated with and without S-9 preparation by omitting the mutagen.

S-9 mix was also prepared by the procedure of Ames et al.[37] and checked for activity by using alfatoxin B_1 standard.

Contamination of samples and prepared chemicals were checked by the microbial growth on the nutrient agar plates. Each replica was done in duplicate. Nutrients used were obtained from Difco Company (St. Louis, Missouri) and chemicals from Sigma Chemical Company (St. Louis, Missouri).

Statistical Analysis of Data

Statistical analysis of data was done using a computer program to calculate 3-way analysis of variance and to determine the correlation coefficients. Means were separated using least significant difference at $p<.05\%$.[51]

CHAPTER 4

RESULTS AND DISCUSSION

Aflatoxin contaminated peanut meal was treated with $NaHSO_3$, H_2O_2, H_2O_2 + 1% $Ca(OH)_2$, Clorox (5.25% NaOCl), Clorox + 1% $Ca(OH)_2$, each for 24 hours at 22C and 62C. Effectiveness of the treatments in reducing mutagenic potential was measured using the Salmonella/mammalian-microsome mutagenicity assay.

According to previous studies, the use of Salmonella typhimurium TA 98 and TA 100 strains is effective for the mutagenic assay for aflatoxins. TA 98 is used to detect a frame shift mutation and TA 100 a base-pair substitute mutation. Although it has been shown that aflatoxins are frameshift mutagens, many investigators have reported that TA 100 also shows a good correlation with mutagenic activity of aflatoxins.

For the selection of the suitable conditions for the Ames test several preliminary investigations were done. Strains require a trace amount of histidine to cause back mutation. This trace amount of histidine is also needed for the normal background lawn growth. The toxic effects of some mutagens may kill the microorganisms before growth

and destroy this normal background lawn. In addition to the original amount (10% 0.5mM), several other concentrations of histidine were examined using AFB_1 as a standard mutagen. Fifteen percent of 0.5mM histidine was optimum for the establishment of the normal background lawn. This amount also caused a slight increase of the number of revertant colonies. This amount was used for all further experiments.

Acetone and the other reagents used for extraction or treatment were investigated for mutagenic properties. The results are shown in Table 2. No reagents showed significant mutagenic activity. Sodium hypochlorite showed a strong toxic effect. Acetone showed no mutagenic activity; however, a slight decrease in the rate of spontaneous reversion was observed. Furthermore, an inhibitory effect of acetone on S-9 mix activation had been observed.[52] The result of using the aqueous solutions of AFB_1 standard prepared by the removal of acetone is shown in Table 3. The aqueous solutions of samples were used for the further tests.

The amount of sample extraction necessary to cause an observable effect was also determined using sample solutions. One half ml of acetone extraction concentrated to approximate 0.1ml was judged sufficient for all samples.

The mutagenicity of the original peanut meal was also evaluated. The means were TA 98: 2142 revertants per

Table 2. Mutagenic Activity of Reagents with S. typhimurium TA 98 and TA 100

Revertants/Plate* Reagents	S-9 Mix	TA 98	TA 100
Control	+	106	210
Acetone	-	110	83
	+	132	126
H_2O_2	-	toxic	toxic
	+	121	208
$NaHSO_3$	-	58	184
	+	60	220
$Ca(OH)_2$	-	70	338
	+	109	308

*Mutagenic activity is expressed in the number of revertant colonies per plate.

Table 3. Mutagenicity of Aflatoxin B_1 in Aqueous Solutions

Aflatoxin/Plate Strains	0.1ug	0.05ug	0.01ug
TA 98*	978	578	270
TA 100*	1138	582	262

*Mutagenic activity is expressed in the number of revertant colonies per plate.

plate, TA 100: 2300 revertants per plate. Chemical determination of the original peanut meal indicated 1740ppb AFB_1 and 2220 ppb total AF.

H_2O Treatment

The effects of temperatures and moisture content were investigated separately using H_2O as the only treatment. The results are shown in Table 4. A significant decrease in aflatoxin was observed after 24 hours treatment with H_2O. However, no significant difference in mutagenic activity was recognized between the two different temperatures nor within the two sets (20,30) (40,50) of moisture levels. The significant difference seen between the two sets had no meaning for the chemical degradation experiments.

Aflatoxins are stable up to their melting points above 250C but degrade more easily in the presence of water. Mann et al. reported 80% degradation in oilseed meals containing 20% moisture at 100C for two hours, but saw little effect at 60C and 80C.[54] Fong et al. reported a decrease in mutagenic activity caused by heating peanut oil which contained moisture.[48] Even higher treatment temperature, 62C, may be too low to affect degradation.

The significant decrease of mutagenicity in H_2O treatment of AF contaminated meals for 24 hours may be due to the degradation and/or transformation of AF by

Table 4. Mutagenic Activity of Peanut Meals Treated with H_2O for 24 hours with S. typhimurium TA 98 and TA 100 Strains

Source Strains	Temperature (C) 22	62	Moisture (%) 20	30	40	50
TA 98*	1086	1109**	1205	1156	1059	969
TA 100*	1063	1007	1136	1051	959	958

*Mutagenic activity is expressed in number of revertant colonies per plate.

**Underlined means are not significantly different ($p<0.05$).

microbial or enzymatic attack.[8,55] Marth and Doyle reported the degradation of aflatoxins by the mycelia of Aspergillus flavus and A. parasiticus. They concluded that a heat-stable intracellular substance released by the lysis of cells and peroxidase may have been responsible for the majority of the degradation.[55] The products of AFB_1 degradation were AFB_{2a}, which has been shown to have no mutagenic activity, and other water-soluble materials.[55]

Cliger et al. found the conversion of AFB_1 to AFB_{2a} by an acid-producing mold. This activity was a nonspecific hydration catalyzed by acids and did not require the presence of mold.[8] The decrease of mutagenic activity of AF by mold has not been reported.

Bisulfite Treatment

Sodium bisulfite treatment effectively decreased mutagenic activity in peanut meals. The results of $NaHSO_3$ treatment are shown in Table 5. The decrease may be due to the degradation of AF by sodium bisulfite; the values of correlation coefficient between AFs evaluated with TLC and mutagenicity expressed by the number of revertant colonies of TA 98 and TA 100 were very high. Correlation coefffi-cients were as follows: TA 98-AFB_1 and total AFs, 0.911 and 0.903; TA 100-AFB_1 and total AFs, 0.966 and 0.969, respec-tively. TA 100 showed better association with TLC evaluation.

Increasing treatment temperature decreased muta-genicity in peanut meals in both strains, especially TA 100.

Increasing the concentration of the reagent caused a greater decrease of mutagenic activity, although the greatest difference was seen between the meal tested with $NaHSO_2$ and that tested with only water. The difference be-tween the two moisture contents was insignificant.

Although the treatment was effective in substan-tially reducing mutagenicity, a reduction equal to back-ground could not be reached. The least mutagenic activity was shown in the treatment with 2% $NaHSO_3$ at 30% moisture at 62C for both TA 98 and TA 100.

Several mechanisms have been suggested for the de-gradation of AF by bisulfite. An aqueous solution of

Table 5. Mutagenic Activity of Peanut Meals Treated with $NaHSO_3$ for 24 Hours with S. typhimurium TA 98 and TA 100 and Means of Aflatoxins

Source AFs & Strains	Temperature (C) 22	62	Concentration (%) 0	0.5	2.0	Moisture (%) 20	30
TA 98**	754	686	1181	556	421	733	705*
TA 100**	840	549	1094	556	434	705	684
AFB_1***	734	534	1017	697	571	645	624
Total AF****	871	631	1223	826	679	762	740

*Underlined means are not significantly different ($p<0.05$).

**Mutagenic activity is expressed in the number of revertant colonies per plates.

***Correlation coefficient with TA 98 and TA 100 are 0.911, 0.966 respectively.

****Correlation coefficient with TA 98 and TA 100 are 0.903, 0.969 respectively.

bisulfite and/or sulfite is known to undergo oxidation by a free radical process when it is exposed to air. Doyle and Marth attributed the free radicals initiated the degradation of AF by bisulfite.[56] Dey and Row previously demonstrated that coumarin readily reacted with bisulfite in aqueous solution.[57] Since aflatoxins contain the coumarin moiety, the same reaction is likely to occur, and aflatoxins would be degraded to a sulfonic acid addition product.[56] Such drastic change of the molecular structure of aflatoxins can result in the loss of mutagenic activity.[58]

Doyle and Marth suggested an oxygen-induced anti-Markovnikoff addition of sodium bisulfite to the vinylene group of the dihydrofuran moiety as an alternative mechanism.[56] It has long been suggested that a 2,3-epoxide derivative would be the active form for the mutagenicity of AFs; hence a sulfonic acid addition product which is saturated at 2,3 position carbons would be likely to lose its mutagenicity.

Moerck suggested the possibility of another mechanism which may involve the nucleophilic attack on the carbonyl carbon of cyclopentanone.[59] However, the addition of sulfonic acid at the double bond of cyclopentanone may not cause the loss of mutagenicity when considering the structure of certain derivatives such as aflatoxicol. Further explanation is required for the mechanism.

Hydrogen Peroxide Treatment

Hydrogen peroxide treatment was also effective on decreasing mutagenicity of AF in peanut meal. The loss of mutagenicity expressed by the number of revertants was well-associated with the amount of AF residue evaluated with TLC method. The results are shown in Table 6. The correlation coefficients between mutagenicity and chemical analysis of AF were all above 0.9. The least mutagenic activity was observed at 30% moisture, 2% H_2O_2, and 62C.

An increase of temperature decreased mutagenicity slightly, but increasing the H_2O_2 concentration from 0.5% to 2% had no significant affect. Both strains showed similar results.

Sreenivasamurthy et al. demonstrated 97% degradation of AFB_1 in aqueous solutions of peanut meal with a 6% solution of H_2O_2 at pH 9.5 for 30 minutes at 80C. Treated meals were not toxic to ducklings.[60]

The mechanisms of H_2O_2 degradation of AFs has not yet been elucidated. However, Sreenivasamurthy suggested that opening of the lactone ring and deformation of the carboxylic and hydroxyl groups could be responsible for the degradation.[60]

Beckwith suggested that the terminal double bond in the dihydrofurofuran ring of AFs was susceptible to the attack by reactive oxygen, which would be generated from

Table 6. Mutagenic Activity of Peanut Meals Treated with H_2O_2 for 24 Hours with S. typhimurium TA 98 and TA 100 and Means of Aflatoxins

Source AFs & Strains	Temperature (C) 22	62	Concentration (%) 0	0.5	2.0	Moisture (%) 20	30
TA 98**	923	705	1181	656	606*	827	801
TA 100**	880	618	1094	652	502	757	742
AFB_1***	689	589	1017	651	620	634	637
Total AF****	852	691	1223	798	740	773	766

*Underlined means are not significantly different ($p<0.05$).

**Mutagenic activity is expressed in the number of revertant colonies per plate.

***Correlation coefficient with TA 98 and TA 100 are 0.936 and 0.946 respectively.

****Correlation coefficient with TA 98 and TA 100 are 0.934 and 0.962 respectively.

H_2O_2.[61] Applebaum also suggested that singlet oxygen generated from H_2O_2 would cause oxygenation and saturate the terminal double bond in AF.[62]

The degradation of AFB_2 and AFG_2, which do not have the unsaturated terminal bond, cannot be explained by this mechanism. Even if this mechanism were mainly responsible for the degradation, an alternative degradation mechanism by H_2O_2 which can explain the degradation of AFB_2 and AFG_2 would be needed.

Hydrogen Peroxide plus Calcium Hydroxide Treatment

The results of treating H_2O_2 + 1% $Ca(OH)_2$ are shown in Table 7. A significant decrease of the mutagenic activity was observed with the treatment of H_2O_2 + 1% $Ca(OH)_2$. The loss of mutagenicity agreed with the decrease of AF evaluated with TLC method; all correlation coefficient values were above 0.93. The least mutagenic activity was observed at 30% moisture, and 2% reagent concentration at 62C.

Adding 1% $Ca(OH)_2$ caused a slight decrease in mutagenicity at 2.0% H_2O_2; nevertheless it did not affect mutagenicity at 0.5%. Cleiger et al. suggested that acidic conditions might be required for the initial degradation of AFB_1 with H_2O_2.[63] The higher moisture levels caused a slight decrease in mutagenicity.

Table 7. Mutagenic Activity of Peanut Meals Treated with H_2O_2 + 1% $Ca(OH)_2$ for 24 hours with S. typhimurium TA 98 and TA 100 and Means of Aflatoxins

Source AFs & Strains	Temperature (C) 22	62	Concentration (%) 0	0.5	2.0	Moisture (%) 20	30
TA 98**	929	594	1181	663	441	799	724
TA 100**	863	567	1094	754	447	811	718
AFB_1***	618	295	1017	501	413	457	457*
Total AF****	763	361	1218	612	511	559	565

*Underlined means are not significantly different ($p<0.05$).

**Mutagenic activity is expressed in the number of revertant colonies per plate.

***Correlation coefficient with TA 98 and TA 100 are 0.985 and 0.961 respectively.

****Correlation coefficient with TA 98 and TA 100 are 0.957 and 0.923 respectively.

The effect of H_2O_2 + 1% $Ca(OH)_2$ on the decrease of mutagenicity compared with H_2O_2 treatment alone is not well explained. Larger differences were expected. The function of weak bases on AF destruction has been explored only with ammonia treatment.

According to Beckwith et al., AFB_1 in weak base solutions has two reactive sites, the dehydrofurofuran and the lactone-pentanone ring system.[64] The lactone ring opens easily in alkalin or even aqueous solutions, and demonstrates a reversible electrostatic and/or hydrogen bond interaction. However, prolonged exposure to base in the heterogenous reaction media eventually leads to chemical modification of the difuran ring and results in the covalent interaction of toxin and substrate.[61,64] This interaction may be responsible for the degradation of AFs.

The cleavage of the lactone ring has been reported using a drastic treatment with ammonia hydroxide. Lee et al. demonstrated the formation of AFD_1, a compound formed by lactone ring opening and decarboxylation when AFB_1 was treated with concentrated ammonia hydroxide.[65]

Pohland et al. observed an absorbance band for AFB_{2a} formed from AFB_1 in aqueous ammonium hydroxide solution which suggested an attack at the double bond of dehydrofuran.[58]

Both AFD_1 and AFB_{2a} have been reported to be non-mutagenic.[58]

Clorox Treatment

A decrease in mutagenicity was observed by treatment of the meal with Clorox, a commercial bleach containing 5.25% NaOCl. The results are shown in Table 8. The values of correlation coefficient between chemical and biological evaluations were all above 0.9. The lowest mutagenic activity was observed in the treatment at 50% moisture, 2% NaOCl at 62C. Although treatment by 0.5% NaOCl decreased AF values evaluated with the TLC method, it failed to decrease mutagenic activity as measured by both TA 98 and TA 100. Increasing NaOCl concentration from 0.5% to 2% decreased mutagenic activity measured by both strains. An increase in treatment temperature and moisture significantly decreased mutagenicity as measured by both microbial strains.

NaOCl, including NaOCl-containing commercial bleach, has been reported to be effective in AF destruction. Rhee et al. reported the complete destruction of AF with 0.2% NaOCl using protein isolates and concentrates. Nevertheless, in our experiments a 24 hours-treatment of peanut meal with 2% NaOCl was insufficient to completely destroy the AF. The complexed constituents of a sample may affect the inactivation. This failure may be due to the mutagenic derivatives of AF.[68]

The mechanism of NaOCl degradation of AFs has not yet been elucidated. Castegnaro et al. observed two major degradation products on TLC plates after NaOCl treatment.[68]

Table 8. Mutagenic Activity of Peanut Meals Treated with Clorox for 24 Hours with S. typhimurium TA 98 and TA 100 and Means of Aflatoxins

Source AFs & Strains	Temperature (C) 22	62	Concentration (%) 0	0.5	2.0	Moisture (%) 40	50
TA 98**	1074	651	1014	958*	616	934	791
TA 100**	1195	560	976	1012	669	918	837
AFB_1***	972	375	925	775	571	742	613
Total AF****	1109	463	1091	920	651	856	723

*Underlined means are not significantly different (p 0.05).

**Mutagenic activity is expressed in the number of revertant colonies per plate.

***Correlation coefficient with TA 98 and TA 100 are 0.936 and 0.961 respectively.

****Correlation coefficient with TA 98 and TA 100 are 0.951 and 0.931 respectively.

They identified one spot to be the dichloro derivative which had been previously reported to be very reactive, carcinogenic and mutagenic. According to Swenson et al., the derivative was unstable in the 5% acetone and was quickly converted to the hydroxy derivative which was not carcinogenic nor mutagenic.[36] The mechanism of NaOCl degradation of AF may involve the attack of the 2,3-vinyl double bond with active chlorine. However, the mechanism fails to explain the degradation of AFB_2 and AFG_2 which do not contain the vinyl bond. Other derivatives have not yet been identified.[68]

Clorox plus Calcium Hydroxide Treatment

The addition of 1% $Ca(OH)_2$ to Clorox enhanced the decrease in mutagenicity under all treatment conditions as tested by both strains. Complete reduction of mutagenicity was not obtained. The least mutagenic activity was seen when the meal was treated at 50% moisture, 2% NaOCl + 1% $Ca(OH)_2$, at 62C. Even though correlation coefficient values were the lowest of all five treatments, they were still very high; all four correlation coefficients were above 0.8. Both an increase in treatment temperature and NaOCl concentration significantly decreased mutagenicity. The results are shown in Table 9.

The significant decrease of mutagenicity may be caused by the effect of $Ca(OH)_2$ on the degradation of AF.[61]

Table 9. Mutagenic Activity of Peanut Meals Treated with Clorox + 1% $Ca(OH)_2$ for 24 Hours with S. typhimurium TA 98 and TA 100 and Means of Aflatoxins*

Source AFs & Strains	Temperature (C) 22	62	Concentration (%) 0	0.5	2.0	Moisture (%) 40	50
TA 98**	720	459	1014	571	272	625	555
TA 100**	636	541	976	468	284	672	504
AFB_1***	460	264	925	404	337	395	346
Total AF****	575	323	1091	497	417	492	420

*Means are significant different ($p<0.05$).

**Mutagenic activity is expressed in the number of revertant colonies per plate.

***Correlation coefficient with TA 98 and TA 100 are 0.899 and 0.825 respectively.

****Correlation coefficient with TA 98 and TA 100 are 0.899 and 0.899 respectively.

However, it is not clear whether there is interaction between NaOCl and $Ca(OH)_2$ or whether these reagents affect AF independently. The significant decrease observed at 0.5% NaOCl by adding $Ca(OH)_2$ may be due to the degradation of the dicholoro-derivative.[69]

Treatment Comparison

The means of revertant colonies per plate with five chemical treatments are shown in Table 10. All five treatments caused a significant decrease of revertant colonies in both S. typhimurium TA 98 and TA 100 strains.

In contrast with the report of Rhee et al.,[67] H_2O_2 alone and NaOCl alone treatment showed only slightly greater detoxification effects on the mutagenic activity than did water alone. Adding 1% $Ca(OH)_2$ to NaOCl enhanced the decrease of mutagenicity significantly; adding it to H_2O_2 did not.

The most effective treatment was NaOCl + 1% $Ca(OH)_2$ although mutagenicity with both strains was still evident after the treatment under all conditions. More stringent conditions of longer treatment times are probably necessary. Bisulfite treatment was also effective in reducing but not eliminating mutagenicity.

The effect of reagent concentration is shown in Figure 3 and Figure 4. All treatments were the most

Table 10. Mutagenic Activity of Peanut Meals Treated with Reagents for 24 Hours with S. Typhimurium TA 98 and TA 100*

Strains / Reagents	TA 98**	TA 100**
$NaHSO_3$	719^b	694^c
H_2O_2	814^a	749^b
H_2O_2 + 1% $Ca(OH)_2$	761^b	765^b
Clorox	863^a	886^a
Clorox + 1% $Ca(OH)_2$	588^c	590^d

*Means not having common letter superscripts are significantly different ($p<0.05$).

**Mutagenic activity is expressed in the number of revertant colonies per plate.

effective at 2% and all treatments at 0.5% reagent, except for H_2O_2, decreased the mutagenic activity significantly.

The effect of treatment temperature is shown in Figure 5 and Figure 6. Raising treatment temperature from 22 to 62C decreased mutagenic activity with both TA 98 and TA 100 strains in all treatments.

Although aflatoxins are classified as frameshift mutagens, many investigators have reported good coorelation between residual AF and back mutation of TA 100 which generally detects base-pair mutations.[37,42] This investigation

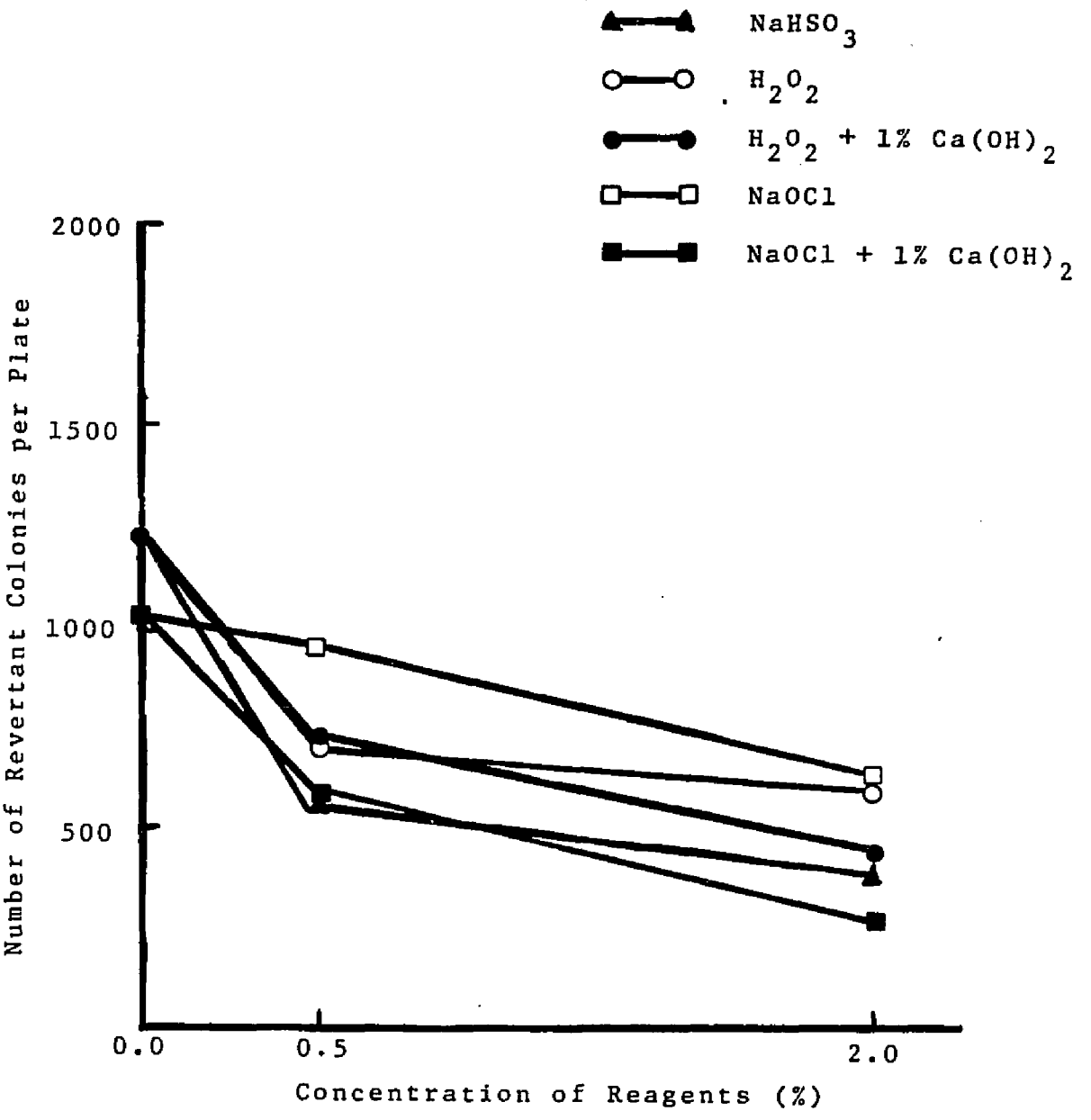

Figure 3. Effect of Concentration on the Decrease of Mutagenic Activity with TA 98 in Peanut Meal by Five Chemical Treatments

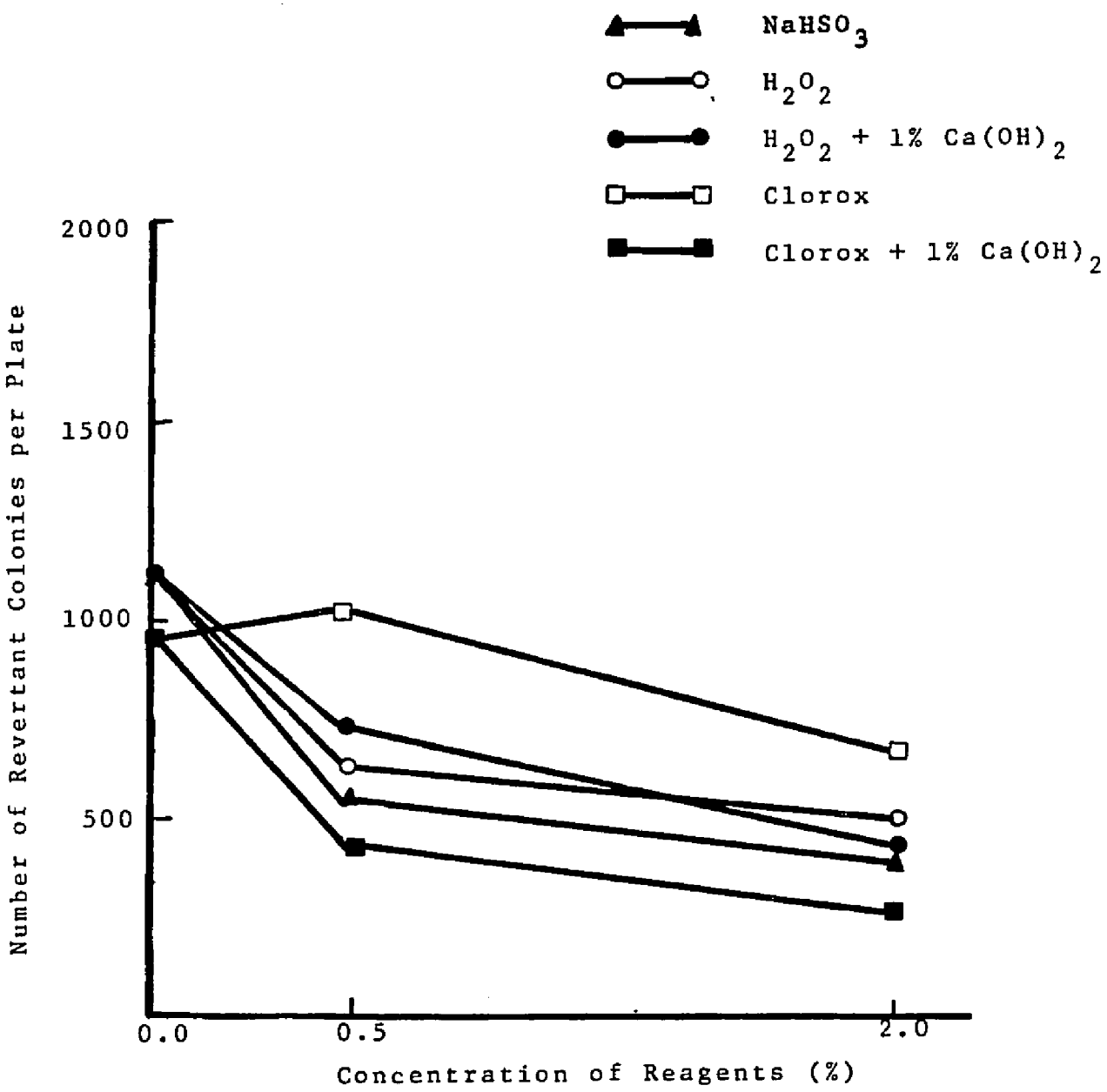

Figure 4. Effect of Concentration on the Decrease of Mutagenic Activity with TA 100 in Peanut Meal by Five Chemical Treatments

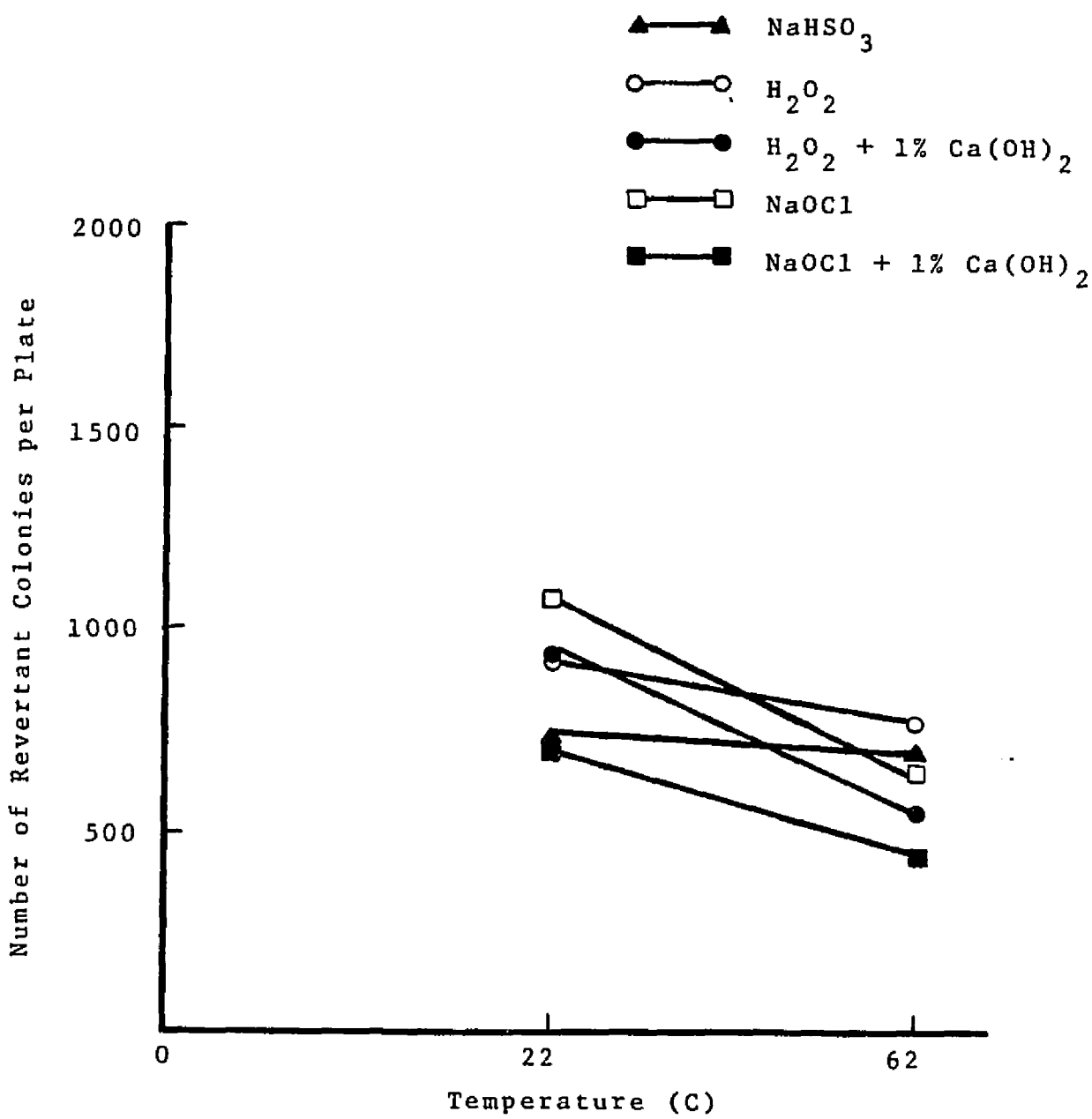

Figure 5. Effect of Temperature on the Decrease of Mutagenic Activity with TA 98 in Peanut Meal by Five Chemical Treatments

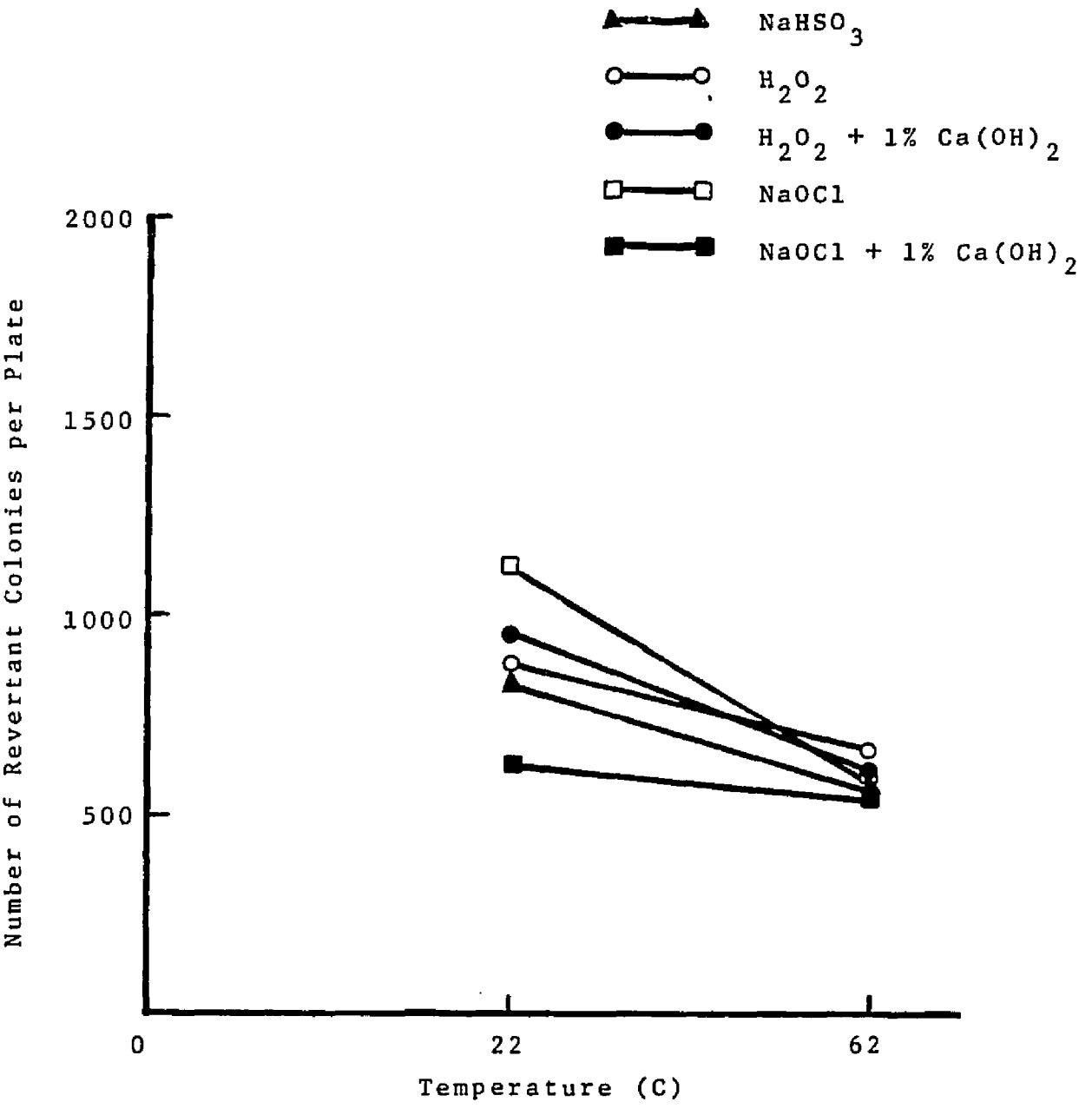

Figure 6. Effect of Temperature on the Decrease of Mutagenic Activity with TA 100 in Peanut Meal by Five Chemical Treatments

has also confirmed that a decrease of AFB_1 and total AF as evaluated with chemical methods can be confirmed by a decrease tested with TA 100.

The higher mutagenicity of the extracts from the meal treated with NaOCl suggests the existence of some mutagenic derivatives, perhaps the chlorinated AF seen by Castegnaro.[68] This effect was not seen as a result of other treatments.

When AFB_1 is used as a standard, an average 1,000 revertant colonies per 0.1ug of AFB_1 has been reported;[42] the higher number of revertant colonies than expected in the original samples and/or samples treated with low concentration of reagents may suggest the contamination by other mutagenic substances. Fong et al. reported the contamination of peanut oils by other mutagenic substances such as nitrosoamine.[48] In their review of mycotoxins, Udagawa and Tsuruta suggested that products may be contaminated by other mycotoxins produced by complexed molds.[69]

There may be the possibility of the enhancing effect by chemical substances which may exist in peanut meals. Shelef and Chin reported an enhancing effect of phenolic antioxidants on mutagenic potenty, whereas several anticarcinogenic effects by phenolic antioxidants have been reported mainly using the tumor inducation.[70]

CHAPTER 5

CONCLUSION

Five chemical detoxification methods for aflatoxin-contaminated peanut meal treated under various conditions were investigated using the Salmonella/mammalian-microsome mutagenic test (Ames test). Conditions used were two different temperatures, 22C and 62C, two different moisture levels and three different reagent concentrations, 0, 0.5, and 2%.

Biological evaluations were compared with the chemical evaluations previously performed by Wang.[15] The loss of mutagenic activity with the Ames test correlated well with the destruction of AF evaluated by TLC, and the Ames test was shown to be useful as a quantitative test.

All conditions for the Ames test were fixed and the same amount of sample extract was used for the test regardless of residual AF without plotting a dose response curve. The results for all treatments showed a high correlation coefficient with TLC chemical evaluations. These high values were consistent within the range of aflatoxin levels found. This method, which omitted the dose response curve, was very useful in the evaluation of the degradation method.

For chemical evaluation by TLC, the acetone extractions were subjected to several purification processes. For biological testing the acetone extracts were not further purified. Because of this the residual AF in a extraction was very low, and concentration was required. This concentration process might have caused concentration of other contaminating substances which could have affected the test results. Mutagenic activity caused by other contaminants could not be determined. When compared with the results using AFB_1 standard, the growth of revertant colonies in samples was poor, which may have been caused by the inhibitory effect of substances in the extraction. A more purified extraction system would give better results but might exclude some AF breakdown products.

With the exception of the NaOCl treatment all treatments showed a greater loss of mutagenic activity than would have been expected from the loss of AF residues evaluated with TLC. This may suggest the existence of other mutagenic substances formed during the chemical treatments.

A significant decrease in mutagenicity of extracts of peanut meal treated with 0.5% NaOCl was not detected by either strain. This suggests either the survival of mutagenic derivatives produced by NaOCl treatment or the inefficiency of treating large amounts of organic matter with weak NaOCl solutions. Since the increased amount of NaOCl did

decrease in metagenicity of the extracts, it is probable that the organic matter decomposed the NaOCl before the NaOCl reacted with the AF. Identification of chlorinated aflatoxin derivatives in the extracts would be desirable.

Of all treatments, Clorox plus 1% $Ca(OH)_2$ showed the most significant decrease of extract metagenicity.

Under all treatment conditions, the higher treatment temperature significantly decreased extract mutagenicity.

The results of this investigation indicated that either relatively high concentrations of reagent or higher temperatures may be required for the complete destruction of mutagenicity in 24 hours by chemicals. Furthermore, chemical detoxification by chlorinated reagents may present the problem of mutagenic derivatives. Depending on the product, the degradation of aflatoxin in contaminated products by chemicals alone may not be effective either chemically or economically. The combination of treatments, such as Clorox + $Ca(OH)_2$, and more favorable conditions such as increasing treatment time or temperature may be a reasonable solution for the detoxification of AF.

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