CONDITIONS NECESSARY FOR INACTIVATION OF AFLATOXIN B1 AND LOSS OF MUTAGENICITY IN COPRA MEAL ON CHLORINE GAS TREATMENT

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Abstract: Copra meal spiked with aflatoxin B1 (AFB1) at a concentration of 10 μg/g was treated with chlorine gas in evacuated chlorination apparatus. More than 75% degradation of AFB1 occurred on exposure of copra meal at 6.2% moisture content to chlorine gas for 2.5 hr. at a dosage of 16 mg chlorine gas/g copra meal. No visible changes in colour occurred in treated copra meal. Prolonged exposure of copra meal to chlorine gas for 24 hr. with or without continuous stirring did not increase the percentage degradation of AFB1. Chlorination of copra meal with increased moisture percentages up to 52 caused decreased percent degradation of AFB1. The mutagenicity of extracts of chlorine treated copra meal was tested with the Ames Salmonella mutagenicity assay. The percentage decrease in mutagenicity of the extracts showed a high correlation ($r = 0.8856$; $p < 0.01$) with percentage degradation of aflatoxin. No new mutagenic compounds were generated during chlorine induced degradation of AFB1.

1. Introduction

Copra meal, the residue remaining after expulsion of oil from kiln dried coconut kernels (copra) is a nutritionally valuable raw material for animal feed. The aflatoxins produced by fungi *Aspergillus flavus* and *A. parasiticus* in improperly dried copra, is retained partly in the copra meal (poonac) during expulsion of oil.[7] Aflatoxin contaminated meal poses problem to animal health and has no export value.

Inactivation of pure aflatoxin B1 (AFB1) on exposure to chlorine gas has already been reported.[10] The degradation products were non-toxic and non-mutagenic. The possibility of using chlorine gas to inactivate AFB1 in spiked corn meal, spiked copra meal and artificially contaminated peanut has already been shown.[8] In this study the conditions necessary to degrade AFB1 in spiked copra meal and the changes in mutagenicity of the toxic meal on chlorine gas treatment were examined.

2. Materials and Methods

2.1 Copra Meal

Copra meal collected from a commercial oil mill in Sri Lanka was ground in a Waring blender to pass through a 2 mm sieve. The powder was tested for the absence of AFB1. The powdered meal was wetted with acetone, and spiked with pure AFB1 in acetone (1 µg/ml) while stirring magnetically to a concentration of 10 µg/g of AFB1 per gram of meal. Stirring was continued with slight warming to evaporate the solvents as assessed by drop in weight of the contents to the original weight.

2.2 Estimation of Moisture

Moisture was estimated by drying powdered copra meal to constant weight at a dial setting of 40 for 10-20 min. in a I. R. moisture balance (Cenco CSC Scientific Co., Fairfax VA, USA)

2.3 Estimation of Aflatoxins

The copra meal (10g) was blended with 90 ml of 70% aqueous acetone, and filtered. The filtrate was extracted with chloroform, dried in a stream of nitrogen and made up to 500 µl.[6] Separation of AFB1 was done by spotting the samples in benzene-acetonitrile (98:2) on TLC plates (Fisher Rediplate; 250 nm, silica gel G) and developing in chloroform:acetone:propanol (85:15:2.5). The plates were observed under a uv source at 362 nm and the individual AFB1 spots were marked. The estimations were done by scanning the fluorescent spots using a Kratos model SD 3000 spectrodensitometer (Kratos Scientific, Westwood, NJ, USA) at 360 nm and comparing the integrated peak areas for spots of AFB1 with a standard curve.

2.4 Generation of Chlorine Gas

Chlorine gas was generated by reacting potassium permanganate with 3M Hydrochloric acid in a closed system described earlier for chlorination of aflatoxins.[10]

2.5 Estimation of chlorine

Chlorine gas (10ml) at standard temperature and pressure were passed into 50 ml of 0.01 N acidic potassium iodide solution kept under reduced pressure of 40 mm mercury in the chlorination apparatus. The mass of chlorine in this volume was estimated by titrating the liberated iodine with 0.01 N sodium thiosulphate with starch as indicator.[2]

2.6 Chlorination of Copra Meal

Copra meal (10g) was placed in round bottom flasks of total capacity 120 ml and evacuated to a reduced pressure of 40 mm of mercury. Required amounts of chlorine gas was introduced at standard temperature and pressure through a syringe from the chlorination system to copra meal in the flasks. The flasks sealed by closing the gas adapter taps were disconnected from the chlorination system and stored for required durations in dark at $25 \pm 2°C$.

2.7 Mutagenicity Testing

Aliquot of the extracts used to estimate AFB1 in treated and control copra meal were dried under a nitrogen stream and dissolved in dimethyl sulfoxide. Ames Salmonella/microsome assay was performed in the presence of a rat liver S-9 mix.[1,4] The S-9 metabolic activation system was prepared from Arochlor 1256 induced rats. For plate incorporation tests, 0.1 ml of fresh overnight culture of *Salmonella typhimurium* strain TA 98 was added along with the test chemical, and 0.5 ml of S-9 mix to 2.5 ml top agar containing small quantities of histidine and biotin. 2-amino fluorene was used as the positive control. All estimations were done at AFB1 concentrations of 0.4, 0.2 and 0.1 µg per petridish. The extracts from copra meal (chlorinated and unchlorinated) contained equivalent amount of AFB1 originally. The top agar mixture was poured into minimal media agar plates. They were incubated at 37°C for 2 days and the resultant colonies were counted.

3. Results and Discussion

3.1 Recovery of AFB1

The recovery of spiked AFB1 under our experimental conditions was 77%. In all calculations the AFB1 estimated in extracts from untreated copra meal was considered 100%.

3.2 Dose of Chlorine Gas

The dose of chlorine gas required for more than 75% degradation of AFB1 was 160 mg per 10g of copra meal under our experimental conditions. The dose-response plot for degradation showed a linear relationship ($r = 0.980$; $p < 0.001$) (Figure 1). The chlorine requirement for degrading AFB1 in copra meal is slightly higher than the requirement for corn meal and is about $1/4$ for peanuts. No changes in the colour of copra meal was observed on treatment at the suggested concentration of chlorine gas indicating low or negligible visible deleterious effects.

3.3 Duration of Exposure and Stirring

Attempts to increase the interactions by prolonged exposure of copra meal to chlorine gas, as well as by continuous stirring during exposure did not increase the percentage degradation of AFB1 markedly (Table 1). Insufficient interac-

Table 1 : Effect of duration of exposure and stirring on degradation of spiked aflatoxin B1 in copra meal by chlorine gas. (Initial AFB1* = 9.7 µg/g; amount of chlorine used = 160 mg per 10 g. of copra meal).

Treatment	% Degradation in hr.	
	2.5	24
Stirred	70	72
Unstirred	67	69

* As estimated in extracts from untreated samples.

tion between the gas and AFB1 did not appear to be an important factor at the ratio of copra meal to chlorine gas used during chlorination.

3.4 Effect of Moisture

Hypochlorous acid produced by the reaction of chlorine with moisture is the active entity responsible in releasing Cl^+ for electrophillic reaction with AFB1.[3] However, increased moisture content in copra meal during chlorination did not increase, but reduced, the percentage degradation of AFB1, indicating that chlorine is made less available or unavailable at the sites of AFB1 with increased moisture concentrations (Table 2). The storage moisture content of 6.2% in copra meal appeared to be sufficient for the chlorine induced degradation of AFB1.

Table 2: Effect of moisture in copra meal on degradation of spiked AFB1** (10.5 µg/g) on chlorine gas treatment.

Added moisture %	Final AFB1	% Degradation
0*	2.7	75
23	4.6	56
33	5.0	52
48	5.5	48

* Initial moisture content 6.2%.
** As estimated in extracts from untreated samples.

3.5 Mutagenicity

One of the major concerns in the use of chlorine gas in inactivating AFB1 in foods and feeds is the possible interactions of chlorine with food components producing toxic, mutagenic or carcinogenic compounds. The percentage decrease in mutagenicity observed in extracts from chlorinated copra meal showed a high correlation with percentage degradation of AFB1 detected chemically ($r = 0.8856$; $p < 0.01$) (Figure 2) This observation suggests not only a loss of mutagenicity with chlorine induced degradation of AFB1, but also that there is no generation of new mutagenic compounds. However, these observations need to be confirmed with other biological assays.

4. Discussion

The chlorine gas treatment carry several advantages over ammoniation[5] already established for detoxification of animal feeds. The reaction of chlorine gas is instantaneous, and hence the treatment is faster compared with ammoniation which needed exposure for several days or weeks under ambient conditions or a few hours at elevated temperatures and under high pressure.[9] The chlorine treatment done under reduced pressure avoid the risks associated with a high pressure ammoniation system. Chlorine gas is already permitted as a bleach, disinfectant, and in chlorination of water in the food industry.[11] Chlorination of AFB1 yield two products, 8, 9-dichloro-AFB1 and 8, 9-dihydroxy-AFB1. The former is carcinogenic, but possesses a half life of a few minutes and is converted to the latter which is non-mutagenic.[10] Thus the identified chlorinated products of AFB1 appear to be more acceptable from a toxicological point of view than the aflatoxin D1 produced during ammoniation of AFB1. Aflatoxin D1 is shown to be mutagenic at a dosage 450 times that of AFB1 and toxic to chick at a dosage 18 times AFB1 whereas the chlorination products of AFB1 are non-toxic and non-mutagenic. (See Figures 1 & 2 , pages 30, 31).

5. Conclusions

More than 75% of degradation of AFB1 could be attained with 16 mg chlorine gas per g copra meal at 6.2% moisture on exposure for 2.5 hr. Increased duration of exposure, stirring or higher moisture content did not increase degradation of AFB1. Chlorine gas treatment of toxic copra meal did not produce any mutagenic products. Further experiments on chlorination on an expanded scale and investigations using other bioassay methods are suggested.

Acknowledgements
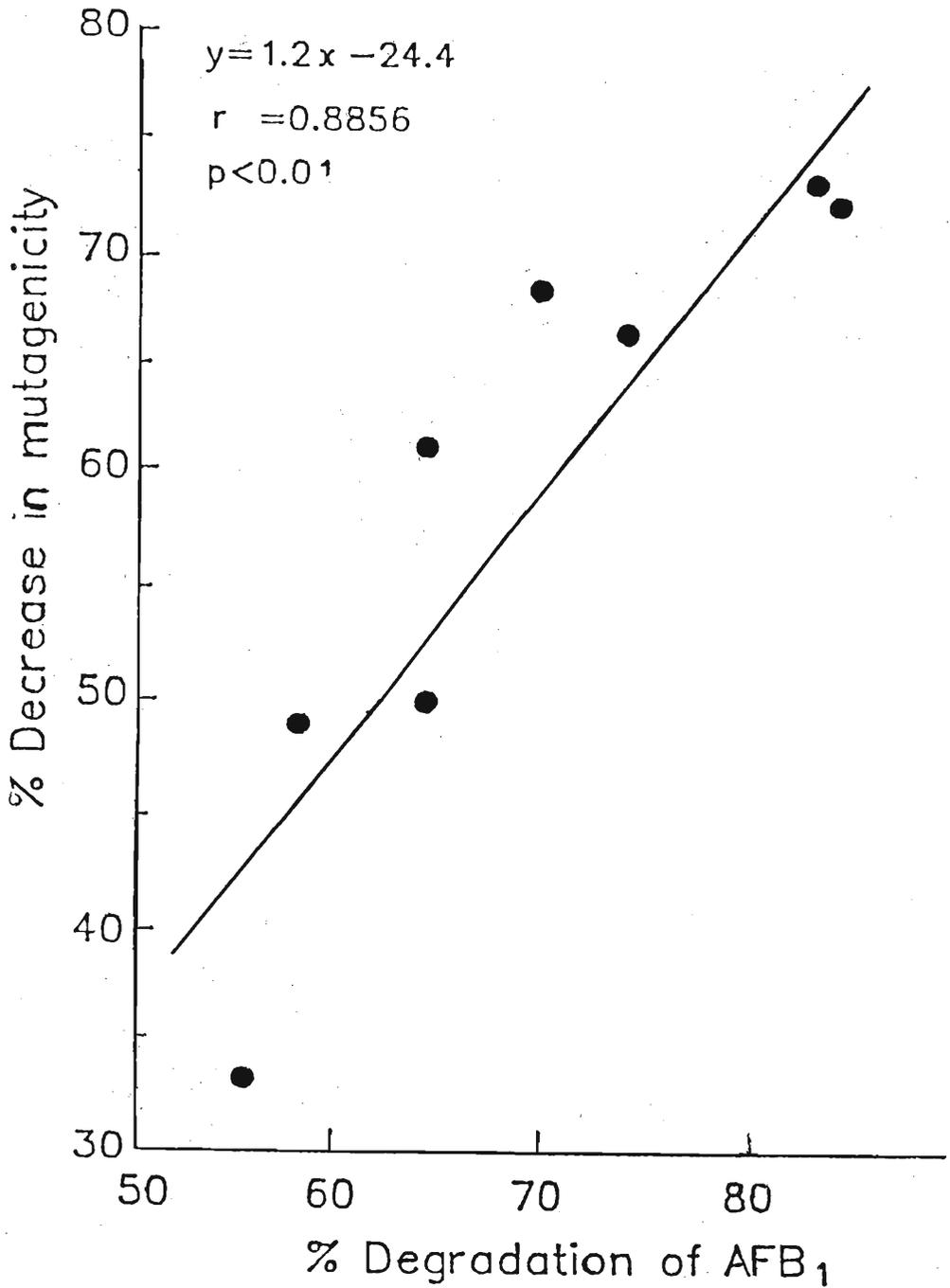
$$y = 1.2x - 24.4$$
$$r = 0.8856$$
$$p < 0.01$$

Figure 2: Relationship between percent decrease in mutagenicity and percent degradation of aflatoxin B_1 in extracts of chlorine treated copra meal.

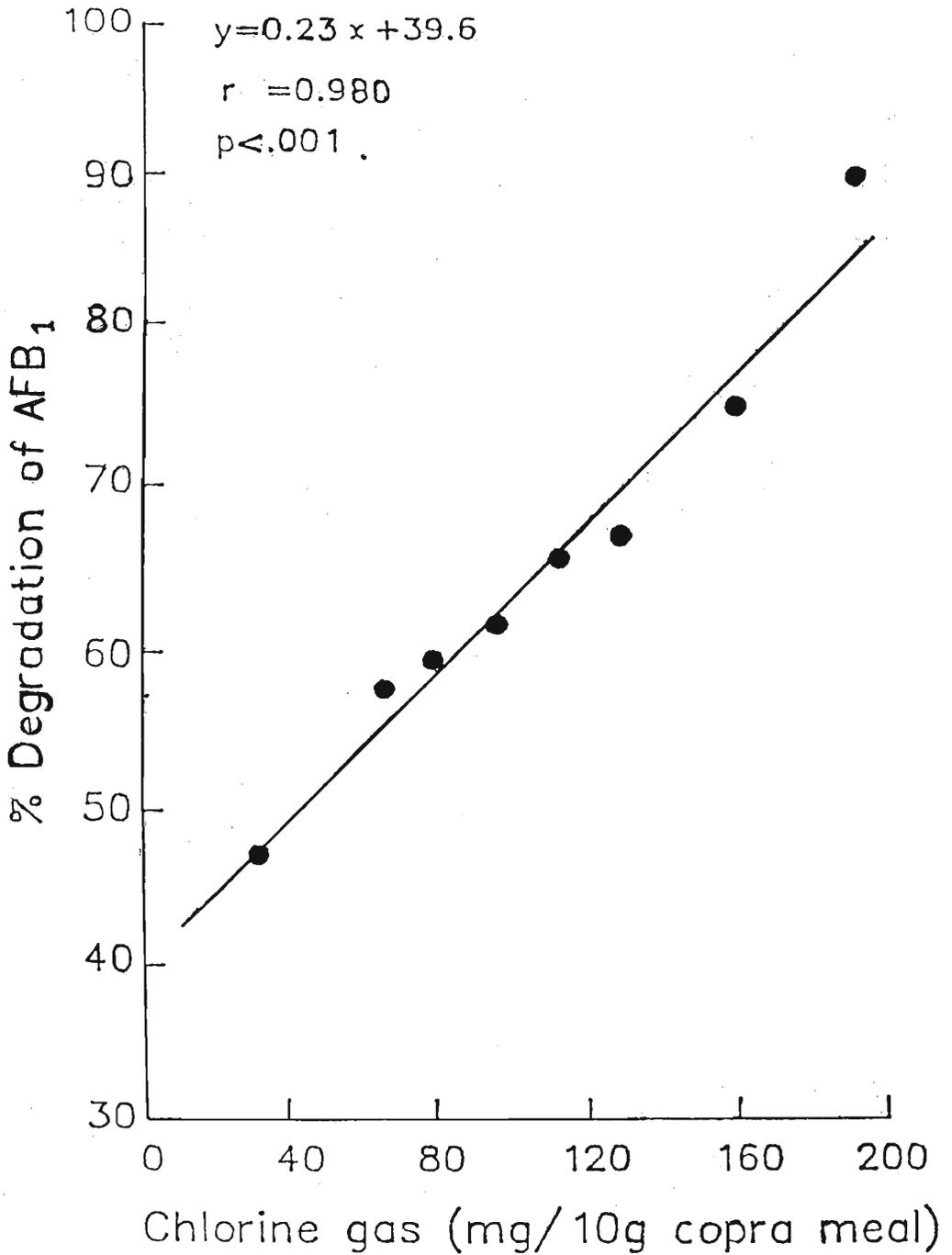$y = 0.23 x + 39.6$

$r = 0.980$

$p < .001$

Figure 1: Relationship between percent degration of Aflatoxin B_1 in copra meal and the dose of chlorine gas when exposed for 2.5 hr. The values are means of triplicate samples.

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