Mutagenic Potential of Ammonia-Related Aflatoxin Reaction Products in Cottonseed Meal

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ABSTRACT

In a joint research effort, the Food and Drug Administration, the National Toxicology Program and the US Department of Agriculture studied the mutagenic potential of aflatoxin reaction products following ammoniation of contaminated cottonseed meal under conditions approximating those approved for commercial ammoniation of nonaflatoxin-contaminated meal. Uniformly ring-labeled ^{14}C-aflatoxin B_1 was added to cottonseed meal that contained ca. 4000 μg naturally incurred aflatoxin B_1/kg. Distribution of the radiolabeled compound was used to trace the modification of aflatoxin B_1 after treatment with ammonia. The radioactivity-to-weight ratio of the fractions isolated by solvent extractions and chemical and enzymic treatments was used to measure the relative concentration of an aflatoxin decontamination product. All extract fractions having a radioactivity-to-weight ratio ≥1 were tested for mutagenic activity using the *Salmonella*/microsome mutagenicity test (Ames test). Purified aflatoxin B_1 was mutagenic at a concentration of ca. 0.005 μg/plate. The methylene chloride extract of the ammoniated meal after Pronase digestion exhibited a similar response when 180 μg of this fraction was applied to each plate. This fraction represented 0.16% of the original added radioactivity. The other fractions produced no detectable mutagenic response at the concentrations tested (10-1000 μg/plate) on *Salmonella* tester strain TA100. Ammonia treatment of aflatoxin-contaminated cottonseed meal significantly decreased aflatoxin levels, and the aflatoxin decontamination products formed by the treatment had little or no mutagenic potential.

INTRODUCTION

The mutagenic activity of aflatoxins has been demonstrated using cell-free preparations, microorganisms, cultured rodent and human cells and in vivo model systems (1-3). Fabry and Roberfroid (4) reported the capacity of aflatoxin B_1 to produce micronucleated cells and chromosomal aberrations in mouse bone marrow cells.

The Ames assay (5,6) and other in vitro tests (7) are useful tools in evaluating the mutagenic activity of aflatoxins (8,9). Based on the results of in vitro mutation data, the mutagenic potential of aflatoxins and their animal biotransformation products correlated well with in vivo carcinogenicity data (10).

From the late 1960s through 1973, the Food and Drug Administration (FDA) authorized the use of ammoniated aflatoxin-contaminated cottonseed meal for animal feed. FDA approved the Food Additive Petition for the ammoniation of noncontaminated cottonseed meal for use as feed for ruminants in 1976 (11). In 1979, the National Cottonseed Products Association petitioned FDA to allow the use of the ammoniation decontamination process for lowering aflatoxin contamination levels in cottonseed and cottonseed meal.

Nutritional trials and short- and long-term toxicological studies (lifetime and reproduction studies in rodents and a lifetime study in laying chickens) showed a nutritionally acceptable product; however, questions on adverse toxicological effects were not fully resolved. Park et al. (12) reported the distribution of aflatoxin decontamination by-products in cottonseed meal following ammoniation. The present study was undertaken to evaluate the mutagenic potential, using the Ames procedure, of those isolated ammoniation-related aflatoxin reaction products separated in the Park et al. (12) study.

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MATERIALS AND METHODS

Sample Preparation

The fractions tested in this study were isolated and purified as reported by Park et al. (12).

Mutagenicity Determination

The Ames test (preincubation method) was conducted essentially as described by Haworth et al. (13). In preliminary experiments TA98 and TA100 gave essentially equivalent histidine revertant recovery with purified aflatoxin B_1 in the presence of Aroclor 1254-rat liver S-9. As limited amounts of meal extract were available, only TA100 (obtained from B. N. Ames, University of California, Berkeley, California) was used for evaluating the mutagenicity of the meal extracts. Each meal extract was dissolved in dimethyl sulfoxide (DMSO) and subsequently was serially diluted with DMSO. Fifty μl aliquots from the appropriate dilutions were then plated in triplicate, as were the appropriate concurrent positive and solvent controls.

At least five dose levels of each extract were plated; the maximum dose level plated for each extract was 500 or 1000 μg/plate, depending upon the amount of extract available. Half of the extracts exhibited some cytotoxicity to the tester strain over the dose range tested.

RESULTS AND DISCUSSION

The scheme used for separating the decontamination by-products is presented in Figure 1. Distribution of the radiolabel following methylene chloride extraction, methanol extraction, acid or base treatment, as well as Pronase digestion, and a detailed presentation of the distribution of the ammoniation reaction products were reported by Park et al. (12). A major portion (23.3%) of total radioactivity was extracted with nonpolar methylene chloride. Methanol and acid and base treatment accounted for 4.0 and 4.1% of the total radioactivity, respectively. The activity-to-weight ratio was high for the methylene chloride and methanol extracts (5.1 and 1.5); however, for the acid and base residues, the ratios were very low. The highest activity-to-weight ratios were observed following the Pronase digestion procedure, where the ratio for the methylene chloride extract of the aqueous layer was 5.1, and the highest ratio, 20.1, was for the methylene chloride extract of the residue following Pronase digestion. A ratio of 20 for this fraction suggests a heavy concentration of aflatoxin decontamination products

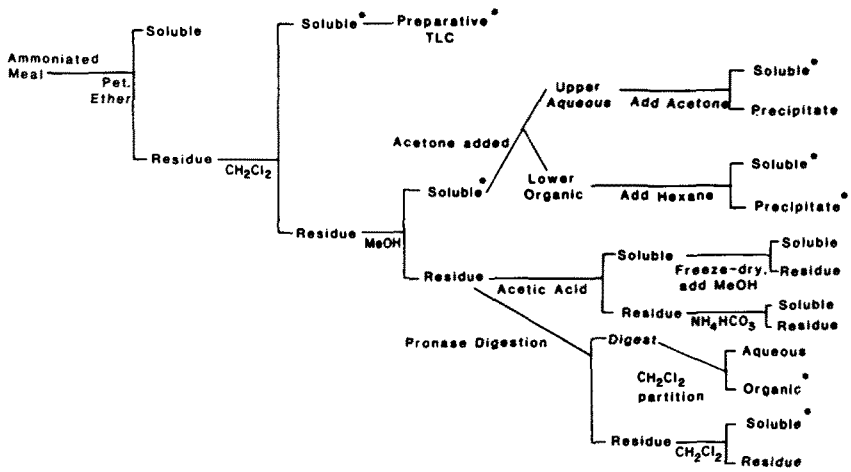

FIG. 1. Separation of aflatoxin-related decontamination by-products in cottonseed meal following ammoniation. * indicates that Ames test was performed.

in a small portion of the total sample (0.16% of the total weight). Since aflatoxin B_1 was the only radiolabeled material added in these experiments, the radioactivity-to-weight ratio is a measure of relative concentration of the aflatoxin decontamination by-products. All subfractions with a radiolabel-to-weight ratio ≥1 were tested for mutagenicity in the Ames test.

The results of the *Salmonella*/microsome mutagenicity test on the various fractions are summarized in Tables I and II. All isolates were tested with metabolic activation at the concentrations indicated. Figure 2 shows the dose response curves for aflatoxin B_1, the methylene chloride extract of the nonammoniated meal and the methylene chloride extract of the residue after Pronase digestion. This is the only fraction isolated from the ammoniated meal that exhibited a positive response to the Ames test. Approximately 180 μg extract following Pronase digestion is required to show the same response exhibited by 0.005 μg aflatoxin B_1, and the concentration (0.006 μg/g) of this isolate is 0.16% of the original aflatoxin B_1 concentration level (4000 μg/g).

Although additional studies will be necessary to elaborate the safety of the ammoniation procedure fully, these studies show that the ammoniation of aflatoxin-contaminated cottonseed meal significantly reduces aflatoxin levels, and aflatoxin decontamination products formed as a result of the process contain relatively low or no mutagenic potential.

TABLE I

***Salmonella*/Microsome Mutagenicity Assay (TA100 revertants/plate) of Aflatoxin B_1[a] and Isolated Ammoniation Decontamination By-Products (Methylene Chloride Extract)**

Fraction	^{14}C-Activity-weight ratio[b]	Aflatoxin B_1 equivalent (ng/g)[c]	Solvent control	Extract concentration (μg/plate)[d] 10	33	100	333	500	1000
CH_2Cl_2 extract (untreated meal)	–	4000	106 ± 14	186 ± 15	256 ± 24	328 ± 16	182 ± 15	148 ± 50[e]	
CH_2Cl_2 extract	5.1	932	85 ± 6	90 ± 9	96 ± 6	92 ± 13	113 ± 4	108 ± 23	
TLC fractionation									
Rf 0.0-0.125	4.3		107 ± 10	105 ± 21	104 ± 11	111 ± 20	112 ± 12[f]		111 ± 14[g]
0.125-0.25	2.1		113 ± 24	109 ± 7	105 ± 17	116 ± 8	107 ± 10		155 ± 7
0.25-0.375	3.4		117 ± 14	100 ± 9	102 ± 2	116 ± 10	98 ± 5		72 ± 10[f]
0.375-0.5	2.4		102 ± 6	98 ± 3	98 ± 4	99 ± 17	48 ± 2		31 ± 9[e]
0.5-0.675	3.9		124 ± 4	90 ± 8	103 ± 4	95 ± 11	104 ± 20		77 ± 7[e]
0.675-0.75	2.2		104 ± 28	112 ± 20	108 ± 16	111 ± 14	96 ± 14		104 ± 16

[a]Aflatoxin B_1 tested at 0.0018, 0.0056, 0.010, 0.032, 0.056 and 0.10 μg/plate caused 172 ± 3, 270 ± 28, 544 ± 13, 742 ± 34, 728 ± 129 and 421 ± 64 TA100 revertants per plate.

[b]Calculated by dividing percentage of radioactivity by percentage of weight.

[c]Based on specific activity of aflatoxin B_1, 560 dpm/ng.

[d]Mean ± S.E.M. (3 plates).

[e]Slight thinning of background bacterial lawn.

[f]Moderate thinning of background bacterial lawn.

[g]Slight precipitate.

TABLE II

***Salmonella*/Microsome Mutagenicity Assay (TA100 revertants/plate) of Aflatoxin B_1 [a] and Isolated Ammoniation Decontamination By-Products (Methanol Extract Following Methylene Chloride Extraction)**

Fraction	^{14}C-Activity-weight ratio[b]	Aflatoxin B_1 equivalent (ng/g)[c]	Solvent control	Extract concentration (μg/plate)[d] 10	33	100	333	500	1000
CH_3OH extract Aqueous/acetone and CH_2Cl_2 partition	1.6	189	99 ± 6	102 ± 20	99 ± 12	105 ± 19	101 ± 1	103 ± 12	
Aqueous soluble	1.5		99 ± 7	98 ± 5	108 ± 21	115 ± 7	107 ± 12		121 ± 2
Organic fraction									
Hexane-soluble	2.1		97 ± 6	97 ± 6	120 ± 14	126 ± 21	128 ± 10		123 ± 2[e]
Precipitate	8.9		101 ± 6	109 ± 9	115 ± 17	109 ± 10[f]	119 ± 20[f]		120 ± 14[f]
Enzymic digestion									
CH_2Cl_2 extract of aqueous layer	5.0	11	117 ± 15	121 ± 5	114 ± 17	117 ± 10	132 ± 18		124 ± 8[g]
CH_2Cl_2 extract of residue	20.1	6	117 ± 15	124 ± 20	132 ± 12	196 ± 24	362 ± 29		783 ± 60[g]

[a]Aflatoxin B_1 tested at 0.0018, 0.0056, 0.010, 0.032, 0.056 and 0.10 μg/plate caused 172 ± 3, 270 ± 28, 544 ± 13, 742 ± 34, 728 ± 129 and 421 ± 64 TA100 revertants per plate.

[b]Calculated by dividing percentage of radioactivity by percentage of weight.

[c]Based on specific activity of aflatoxin B_1, 560 dpm/ng.

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[e]Moderate thinning of background bacterial lawn.

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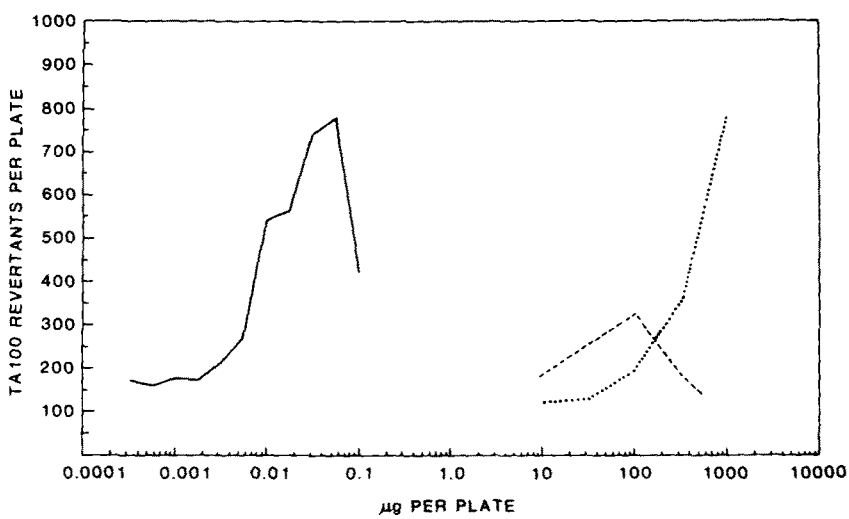

FIG. 2. Mutagenic response of *Salmonella typhimurium* to aflatoxin B_1 and aflatoxin-related decontamination by-products isolated from cottonseed meal. ———, aflatoxin B_1; - - - - - -, CH_2Cl_2 extract of untreated cottonseed; ....., CH_2Cl_2 extract of Pronase digestion residue of ammoniated cottonseed meal.

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[Received February 16, 1985]