Aflatoxin Inactivation. Chemical and Biological Properties of Ammonia and Methylamine Treated Cottonseed Meal

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Aflatoxin-contaminated cottonseed meal (334 μg/kg total aflatoxins) was treated with ammonia and with methylamine under two conditions for each reagent. Aflatoxins in the products ranged from 3 to 7 μg/kg, and added nitrogen from 0.43 to 0.85%. The treatments lowered nitrogen solubility less than 10% and available lysine less than 6%. Rat feeding tests (28 days, 14% protein diet) revealed significant lowering of protein efficiency ratios by ammoniation, while the methylamine treated products had ratios about the same as or higher than the ratio for the untreated meal. Ninety-day feeding studies (25% meal in diets) yielded normal body and organ weights, with the exception of the methylamine treated products which increased liver weights significantly. This is a reversible hyperfunctional enlargement rather than a toxic manifestation. Biochemical analyses of plasma and liver were within normal ranges, with the exception of one methylamine product which elevated plasma urea and liver tyrosine transaminase.

Aflatoxins, toxic metabolic products from certain strains of the mold *Aspergillus flavus*, have been found in some lots of peanuts and cottonseed, and in some of the meals prepared from these commodities (Prickett and Salmon, 1964; Whitten, 1969). Many animal species are adversely affected if they consume rations containing oilseed meals having sufficiently high levels of these toxins (Allcroft, 1969; Butler, 1969). Aflatoxin M, a metabolite of aflatoxin B_1, may appear in the milk of dairy cattle if their rations contain high levels of the latter toxin (Masri *et al.*, 1968). Chromatographic evidence has been adduced for the presence of aflatoxins and/or their metabolites in skeletal muscle and/or livers of White Rock chickens fed large doses of crude aflatoxins (Van Zytveld *et al.*, 1970).

The possibility of "rehabilitating" these contaminated meals for feed use by chemical treatments to inactivate aflatoxins has been investigated in this laboratory. A number of reagents have been tested for destruction or inactivation of aflatoxins present in peanut and cottonseed meals (Mann *et al.*, 1970), and a contaminated peanut meal has been treated with several reagents and the products biologically evaluated (Dollear *et al.*, 1968). Treatments with ammonia or with methylamine appeared practical for aflatoxin inactivation, and the purpose of the present investigation was to determine the effects of these two reagents on the chemical and biological properties of a contaminated cottonseed meal.

EXPERIMENTAL

The aflatoxin-contaminated cottonseed meal (CMC) used for the various treatments was prepared by blending a meal having an atypically high aflatoxin content with an uncontaminated meal (CMU) of high quality. Both meals were prepress solvent extracted, and the blend CMC contained 304 μg/kg aflatoxin B_1 and 30 μg/kg aflatoxin B_2. This blending was performed to extend the relatively small quantity of highly contaminated meal available, and to lower the aflatoxin content of this atypical meal to more realistic levels.

The aflatoxin-contaminated meals were handled very carefully to minimize spillage and dusting, and gloves, disposable laboratory smocks, and inhalators were worn by all personnel. All glass and metal containers used for manipulation and treatments of the meal were decontaminated with Clorox, a 5% solution of sodium hypochlorite (Stoloff and Trager, 1965). All glassware used for the aflatoxin assays was also treated with this reagent.

All treatments of meal CMC were carried out using 25-lb batches of the meal in a 10-gal capacity Groen tilting-type reaction kettle described previously (Dollear *et al.*, 1968). The moisture content was adjusted to the desired value by blending with the calculated amount of water in a large Hobart mixer. When sodium hydroxide was included in the treatment, it was dissolved in the water used to hydrate the meal. The hydrated meal was transferred to the preheated reaction kettle, the reagent was added, and the temperature was elevated to the desired value and maintained for the specified time by control of steam pressure in the jacket of the reaction kettle. Constant agitation was maintained during the treatment. Anhydrous ammonia, 99.99% (The Matheson Co., Inc.), was added under pressure, and methylamine (Eastman No. 527) was added as a 40% aqueous solution. The reaction kettle was vented to the atmosphere through a reflux condenser during methylamine treatments. After treatment, the product was spread on trays and dried overnight by exposure to a forced draft of air at ambient temperature.

The reaction conditions used for the ammonia and methylamine treatments of meal CMC were selected on the basis of an earlier report (Mann *et al.*, 1970). For ammoniated preparation A1, the moisture content of the meal was adjusted to 15%, and 45 lb per in.^{2} ammonia pressure was applied for 30 min at 93° C. Identical conditions were used for ammoniated preparation A2, except the moisture content of meal CMC was adjusted to 10%. The conditions for the methylamine treatment yielding product MA1 were: 2.0% methylamine, 1.0% sodium hydroxide, 15% meal moisture, and 30 min at 100° C. Identical conditions were used for methylamine preparation MA2, except no sodium hydroxide was added.

The chemical characteristics of the materials were determined by Official Methods of the American Oil Chemists'

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Table I. Chemical Characteristics of Cottonseed Meals and Treated Products

Characteristic	Meals[a]					
	CMU	CMC	A1	A2	MA1	MA2
Nitrogen, % (MFB)[b]	7.14	7.06	7.91	7.85	7.71	7.49
Nitrogen solubility,[c] %	61.23	60.79	55.81	55.03	59.00	59.66
EAF lysine, g/16 g N	2.74	2.75	2.62	2.60	2.65	2.69
Free gossypol, % (MFB)	0.08	0.07	0.06	0.08	0.08	0.07
Total gossypol, % (MFB)	1.16	1.09	1.03	1.01	0.84	0.83
Lipids, % (MFB)	0.50	0.62	0.40	0.55	0.47	0.45
Crude fiber, % (MFB)	14.4	15.1	15.5	16.8	16.4	17.5
Ash, % (MFB)	6.85	6.3	6.4	6.3	7.4	6.3
Total sugar, % (MFB)		5.90	5.99	5.51	5.51	5.23
Reducing sugar, % (MFB)		0.11	0.00	0.00	0.00	0.00
Aflatoxins B_1	ND[d]	304	3	6	3	7
μg/kg B_2	ND	30	ND	ND	ND	ND
Total	ND	334	3	6	3	7

[a] See text for descriptions. [b] MFB: moisture free basis. [c] In 0.02 N sodium hydroxide. [d] ND: none detected.

Society (1969) and the meals were assayed for aflatoxins by a method developed by Pons (1969).

Briefly, the assay involved extraction of the aflatoxins with acetone/water (85/15, v/v, plus 8 ml of glacial acetic acid per l.), lead acetate purification, extraction into chloroform, purification of the extract on a silica gel column, thin-layer chromatography on plates coated with silica gel, Adsorbosil-1 (Applied Science Laboratories, Inc.), and comparison of the fluorescence of sample spots with the fluorescence of standard spots on developed plates using a Model 520A Photovolt densitometer.

Protein efficiency ratio (PER; grams gain/grams protein intake) and percent digestibility ([feed intake − weight of dried feces] × 100/feed intake) of the meals were determined by the 28-day rat bioassay method of Derse (1960), with the exception that the dietary protein level was 14% rather than 10%. The nonprotein nitrogen added to the meal by the ammonia and methylamine treatments was excluded from the total nitrogen used to calculate the amount of meal fed.

The meals also were fed to rats at the dietary level of 25% for 90 days in an effort to detect toxic effects based on growth, organ weights, hematology, urinalysis, biochemical analysis, and gross and microscopic pathology. Groups of 15 weanling rats (ten males and five females) were fed a commercial Purina rat chow and diets in which each meal replaced 25% of the chow.

RESULTS AND DISCUSSION

The chemical characteristics for the various meals are given in Table I. Both ammonia and methylamine markedly lowered the aflatoxin content of meal CMC. For ammonia treatments, the lowering was favored somewhat by the higher moisture content, 15%, used in preparing A1 as compared with 10% moisture used for A2. For methylamine treatments, aflatoxin lowering was favored by adding 1.0% sodium hydroxide. Enhancement of aflatoxin inactivation by increasing moisture content and by addition of sodium hydroxide during treatments has been noted previously (Mann *et al.*, 1970). Treatments with both reagents yielded products containing additional nonprotein nitrogen, ranging from 0.85% for meal A1 to 0.43% for meal MA2. It is possible that the added nitrogen will be utilized by ruminant animals (Cavanagh and Ensminger, 1969; Hinman *et al.*, 1966), but, as previously mentioned, it was excluded from the calculations of the protein levels in the rations of the rats for the PER assays. All treatments led to some reduction in nitrogen solubility (0.02 N sodium hydroxide solvent) and in EAF lysine ("available" lysine). These results were anticipated from earlier studies on the effects of heat on cottonseed meals (Condon *et al.*, 1954; Frampton, 1965).

The results of the PER assays are given in Table II. The PER values for the ammonia treated meals were significantly lower ($P < 0.01$) than the reference CMC meal value but the values of the methylamine treated meals were about equal to or higher than the PER of the untreated meal. Some reduction in the PER values by the treatments was expected because of the decrease in nitrogen solubility and EAF lysine. The PER values for meals CMU and CMC were identical, indicating that the 334 μg/kg aflatoxins in the latter meal did not affect protein utilization by these animals in the 28-day feeding period. The percent digestibility values were very similar for all six meals tested.

The final body and organ weights of rats fed diets containing 25% of each meal for 90 days are given in Table III. The final mean body weights were all well within range of the mean weight of the group fed the uncontaminated cottonseed meal CMU. A significant increase was observed in liver weights of rats fed diets containing methylamine treated meals MA1 and MA2. Weights of other organs including kidney, spleen, heart, testes, adrenals, and thyroids were well within normal limits.

At autopsy no microscopic lesions were detected which could be related to the treatment of the meals. Examination of slides containing hematoxylin and eosine stained tissues

Table II. Summary of Rat PER Assays[a] (14% Protein in Rations)

Dietary source of protein	Mean weight at 28 days, g[b]	PER[c]		Digestibility,[d] %
		Actual ± standard deviation	Corrected	
Casein	209.1	3.15 ± 0.14	2.50[e]	92.3
CMU	132.0[f]	1.72 ± 0.12	1.37	79.5
CMC	123.7	1.73 ± 0.08	1.37	83.3
A1	98.7	1.35 ± 0.12	1.07[e]	81.2
A2	110.0	1.48 ± 0.10	1.18[e]	82.8
MA1	119.4	1.68 ± 0.06	1.33	82.8
MA2	118.6	1.79 ± 0.15	1.42	82.9

[a] Ten weanling male rats per group in individual wire-bottom cages. [b] Mean starting weight, 50 g. [c] PER = protein efficiency ratio = grams gain/grams protein intake. [d] % digestibility = (feed intake − weight of dried feces) × 100/feed intake during second week of assay. [e] Significantly different from CMC, $P < 0.01$. [f] One rat excluded due to contamination of feed with urine.

Table III. Final Body and Organ Weights of Rats Fed Diets Containing 25% Meals for 90 days

Meal	Sex[a]	Mean body weight, g	Organ weights (g/100 g body weight) Liver	Kidneys	Spleen	Heart	Testes	Adrenals[b]	Thyroids[b]
Purina basal	M	291	3.48 ± 0.13	0.66	0.18	0.33	1.08	9.5	6.4
	F	186	3.65 ± 0.16	0.69	0.20	0.34		23.8	8.0
CMU	M	275	3.45 ± 0.14	0.74	0.18	0.33	1.20	12.5	7.3
	F	191	3.44 ± 0.13	0.74	0.19	0.33		24.5	8.9
CMC	M	288	3.56 ± 0.22	0.69	0.18	0.33	1.11	11.3	6.6
	F	194	3.50 ± 0.26	0.70	0.20	0.35		22.5	8.6
A1	M	274[c]	3.53 ± 0.16	0.71	0.17	0.33	1.19	10.9	7.6
	F	186	3.53 ± 0.36	0.72	0.19	0.36		23.3	10.4
A2	M	278	3.45 ± 0.23	0.70	0.18	0.34	1.22	10.9	6.6
	F	192	3.59 ± 0.19	0.72	0.19	0.34		24.3	9.8
MA1	M	276	3.83 ± 0.25[d]	0.75	0.19	0.34	1.17	10.9	7.4
	F	192	3.90 ± 0.19[d]	0.77	0.21	0.34		23.0	8.2
MA2	M	276	3.69 ± 0.29[e]	0.74	0.19	0.36	1.17	12.4	7.3
	F	185	3.91 ± 0.28[d]	0.77	0.22	0.40		24.0	9.3

[a] Ten weanling male and five female rats per treatment; mean starting weights, male 54.0 g, female 53.8 g. [b] Milligrams per 100 g body weight. [c] Nine rats. [d] Using CMU as control, $P < 0.01$. [e] $P < 0.05$.

Table IV. Liver and Plasma Analyses of Male Rats Fed Diets Containing 25% Meal for 90 Days[a]

Analysis[b]	Meals Purina controls	A1	A2	MA1	MA2	CMC
Liver succinic dehydrogenase	99	101	108	103	109	98
Liver RNA	100	100	99	103	103	99
Liver DNA	103	99	99	101	104	99
Plasma urea	103	103	102	101	120[c]	94
Plasma albumin	100	99	99	101	105	94
Plasma total protein	103	100	100	97	98	98
Liver protein	98	98	99	99	102	99
Liver tyrosine transaminase	96	105	105	111	160[d]	99
Liver nitrogen	102	101	103	103	104	101
Plasma glutamic-oxalacetic transaminase	85	95	98	100	112	104

[a] Ten rats per treatment. [b] Analyses expressed as percent of values obtained from rats on diet containing the uncontaminated cottonseed meal, CMU. [c] Mean for dietary group is significantly greater than that of uncontaminated cottonseed meal CMU, $P < 0.05$. [d] Mean for dietary group is significantly greater than that of uncontaminated cottonseed meal CMU, $P < 0.01$.

from the rats fed the six meals revealed no lesions in any tissues, including liver, after the 90 days of feeding.

All hematology values including red, white, and differential cell counts, hematocrits, and hemoglobin were well within normal limits for all groups. Urinalysis values including specific gravity, occult blood, ketones, glucose, protein, and bilirubin were likewise all within the normal range.

Biochemical analyses of the blood and liver samples from male rats fed diets containing 25% of the meals for 90 days are summarized in Table IV. Changes indicating effects from feeding cottonseed meal containing 334 μg/kg aflatoxins (CMC) were minimal and statistically not significant. In the liver, succinic dehydrogenase, RNA, DNA, and protein, and in the plasma, total protein, albumin, and urea were all slightly decreased (1 to 6%) below control values. These changes, when taken together, are characteristic of those observed in other species after aflatoxin ingestion (Gumbmann and Williams, 1969; Gumbmann *et al.*, 1970). This pattern was not present in any of the treated aflatoxin-contaminated cottonseed meal diets.

Plasma urea and liver transaminase values were significantly elevated ($P < 0.01$) for meal treated with methylamine without NaOH (MA2). Hepatic tyrosine transaminase synthesis is readily induced by numerous factors including starvation, dietary amino acids (particularly tryptophan), dietary protein, and adrenal steroids in response to environmental stress or toxic irritants. Plasma glutamic-oxaloacetic transaminase was also highest in this group, but not significantly so.

With possible exception of the elevated values for plasma urea and liver tyrosine transaminase noted in the rats consuming the methylamine treated meal MA2, no gross changes occurred in the liver and plasma factors for animals fed these meals at 25% dietary levels for 90 days.

The liver enlargement observed in rats fed the methylamine treated meals is not necessarily a toxic response to methylamine or some product formed from the reaction of this reagent with the meal. Rather, the absence of histopathological change in the liver tissue suggests a physiologically-adaptive response to the increased production of microsomal enzyme (Gaunt *et al.*, 1965; Hutterer *et al.*, 1969). Supporting the nontoxic concept, rats were fed the methylamine treated meal for a period of 5 weeks, at which time a significant increase in liver weight was observed, accompanied by a four-fold increase in the output of urinary ascorbic acid. An increased daily output of ascorbic acid is indicative of hyperfunctional liver enlargement as distinguished from toxic liver enlargement. Finally, when the rats were changed to the control diet for 5 weeks after having been on the diet contain-

ing 30% methylamine treated meal, the liver weights were no longer significantly greater, thus confirming the reversibility of the hyperfunctional state of the liver.

Only nonsignificant, borderline evidence of aflatoxicosis appeared in the rats fed the contaminated cottonseed meal CMC for 90 days, even though the diet contained about 84 μg/kg total aflatoxins. Furthermore, the liver tissues of these animals show no lesions. Similar resistance of rats to the toxic effects of 80 μg/kg aflatoxins when fed diets containing naturally contaminated peanut butter for long periods has been observed. Although liver lesions were noted after 90 weeks on the diet, no hepatomas developed (Alfin-Slater *et al.*, 1969).

A more elaborate long-term rat feeding study with ammonia treated aflatoxin-contaminated cottonseed meal is now in progress to obtain some of the data required for clearance of this material as a feed.

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