The effects of such additives on enzymatic hydrolysis were examined as shown in Table IV. Although sodium ion has no effect, calcium ion appears to act as an inhibitor. The addition of .003% albumin gives essentially no improvement after 8 hr and 16 hr, as does the addition of 0.01% of a nonionic ether alcohol-type of surfactant (Neodol 91-6). However, as the concentration of the latter is increased to 0.1%, strong inhibition is evident during the first 8 hr of reaction time. Understanding the mechanisms that causes the reaction rate to increase dramatically after 8 hr is difficult.

TABLE IV

Effect of Various Additives on Olive-Oil Hydrolysis[a]

Additive	Percentage of hydrolysis after				
	1 hr	2 hr	4 hr	8 hr	16 hr
None	15.4	24.8	39.1	54.7	72.3
.1 M NaCl	14.5	23.8	45.1	60.5	74.0
.1 M $CaCl_2$	5.0	18.0	31.9	45.1	58.0
.003% Albumin	17.8	25.3	41.0	58.1	79.7
.01% Neodol	12.6	16.2	43.5	65.3	81.1
.1% Neodol	0.5	6.7	7.1	20.5	72.6

[a]pH 5.4, .1 M phosphate buffer, 26 C, enzyme concentration = 6 U/meq.

REFERENCES

1. Potts, R.H., and V.J. Mukerheide, Production of Fatty Acids, in Fatty Acids and Their Industrial Application, edited by E. Scott Pattison, Marcel Dekker, Inc., New York, NY, 1968, pp. 28-29.
2. Mattson, F.H., and L.W. Beck, J. Biol. Chem. 214:115 (1955).
3. Mattson, F.H., and L.W. Beck, Ibid. 219:735 (1956).
4. Yamada, K., and H. Machida, Meito Sangyo Kabushiki Kaisha, U.S. Patent 3,189,529 (1965).
5. Centraal Bureau voor Schimmelculture, List of Cultures, Inst. Royal Netherlands Acad. Sciences and Letters, Baarn, Delft, Netherlands, 1978.
6. Benzonana, G., and S. Esposito, Biochim. Biophys. Acta 231: 15-22 (1971).
7. Shinota, A., H. Machida and T. Azuma, Meito Sangyo Co., Ltd., Japan Patent 71:16, 509 (1971).
8. Kokusho, Y., H. Machida and S. Meito Sangyo Co., Ltd., Japan Patent 79:95, 607 (1979).
9. Tros'ko, U.I., G.I. Meerov, K.G. Mikhal'skaya, S.N. Norkina and G.S. Savinova, Vses Nauchno Issled. Inst. Zhirovoi Prom. Leningrad, USSR, Maslo-Zhir Prom-st, 1977 (7), 27-9 (Russ) CA 87:86656Z.
10. Schønheyder, F., and K. Volqvartz, Acta Physiol. Scand. 9:57 (1945).
11. Schifreen, R.S., and P.W. Carr, Anal. Lett. 12(B1):47 (1979).
12. Benzonana, G., and P. Desnuelle, Biochim. Biophys. Acta 164:47 (1968).
13. Brockerhoff, H., J. Biol. Chem. 246:5828 (1971).
14. Martinek, K., Stabilization of Enzymes, Enzyme Engineering, edited by L.B. Wingard, Jr., I.V. Berezin and A.A. Klysov, Plenum Press, New York, NY, 1980, pp. 33-36.
15. Gomori, G., Preparation of Buffers for Use in Enzyme Studies, in Methods in Enzymology, Vol. I, edited by S.P. Colowick and N.O. Kaplan, Academic Press Inc., New York, NY, 1955, pp. 138-146.

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Distribution of Ammonia-Related Aflatoxin Reaction Products in Cottonseed Meal

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ABSTRACT

The fate of aflatoxin during ammoniation of contaminated cottonseed meal was studied under conditions approximating those approved for commercial ammoniation of nonaflatoxin-contaminated meal. Uniformly ring-labeled ^{14}C-aflatoxin B_1 was added to 27.7 kg meal (14% moisture) 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 4% ammonia at 40 psi, 100 C for 30 min. This treatment reduced the chemically detected aflatoxin B_1 to less than 4 μg/kg. In control nonammoniated meals, 90% of the radiolabeled material was accounted for in the methylene chloride extract. Duplicate 2-kg samples of the ammoniated meal were fractionated and the distribution of radioactivity was determined. Ca. 86% of the radioactivity was detected in the meal after initial air-drying. Ca. 25% of the added radioactivity was extracted from the air-dried meal with methylene chloride and another ca. 5% was extracted from this residue with methanol. Weak acid released 3% of the added radioactivity from the residue after methanol extraction, bicarbonate released 1% and Pronase digestion, including methylene chloride extraction of the residue, accounted for nearly 19% of the total added radioactivity. Only 37% of the added radioactivity remained in the meal matrix following solvent extractions and chemical and enzymic treatments.

INTRODUCTION

In 1969, based on the Food and Drug Administration's analytical capability at the time and with little knowledge of the risk from aflatoxin ingestion, an action level of 20 parts per billion (ppb) total aflatoxins (B_1, B_2, G_1 and G_2) was established. This action level remains in effect today and applies to commodities (raw or processed) that are susceptible to contamination, including feeds. A level of 300 ppb is allowed in whole cottonseed that will be used only for beef, swine and poultry rations.

Treatment with ammonia gas under pressure is an effective procedure for chemical inactivation of aflatoxins in cottonseed meal (1). Whole cottonseed has been decontaminated at ambient temperatures (2). Pressure inactivation reduced the aflatoxin content to less than 1 μg/kg but did not account for products formed from the toxin by the ammoniation treatment. Lee et al. (3) and Cucullu et al. (4) studied the products formed in a model reaction of aflatoxin B_1 and ammonium hydroxide with heat and pressure. They identified trace amounts (<0.1%) of one of the major products of this model reaction in ammoniated peanut

meal (5). Later Lee et al. (6) ammoniated peanut meal spiked with radiolabeled B_1 in a small laboratory ammoniator. Similar chemical detoxification experiments were conducted by Park et al. (7) on peanut meal. Beckwith et al. (8) used radiolabeled B_1 to trace binding of aflatoxin to corn during ammoniation at room temperature.

The present study was undertaken to determine the distribution of ammoniation-related aflatoxin reaction products based on radioactivity concentrations of ^{14}C-aflatoxin B_1 added to cottonseed meal containing a high level of naturally incurred toxin. The meal was ammoniated in pilot plant equipment and ^{14}C-activity was measured to monitor fractionation.

METHODS AND MATERIALS

Sample Preparation

Fuzzy cottonseed, potentially high in aflatoxins B_1 and B_2, was obtained from Arizona. This seed was decorticated on a huller-shaker (Carver Gin Co., East Bridgewater, MA), and the hulls, fines and meats were separated. Fines and meats were extracted separately with hexane and assayed by the official AOAC Pons method (9,10). Because Lee et al. (11) reported that the fines were more highly contaminated than the whole meats, the high toxin level defatted fines were mixed by blending with the lower toxin level defatted meats. Three 50 g samples of this meal were assayed by the Pons method. Uniformly ^{14}C-ring-labeled aflatoxin B_1 (sp. act. 50 mCi/mmol, Moravek Biochemicals, Inc., Brea, CA) was used to spike the meal. Radiolabeled toxin was received in a methanol solution. The methanol was evaporated and the residue was redissolved in chloroform (50 μCi/10 mL); 5.5 mL of this chloroform solution was distributed on 100 g meal with naturally incurred toxin, and the meal was air-dried in the dark. The 100 g spiked meal was mixed in a Vortex laboratory mixer with 900 g unspiked meal. The 1 kg spiked meal was mixed with 26.73 kg meal in a pilot plant stainless-steel Vortex mixer for 5 hr, yielding 27.73 kg meal with radiolabeled aflatoxin B_1 at a calculated level of 1 μCi/kg. Radioactivity concentration and distribution purity of the mixture were confirmed using 2 systems, a Searle Isocap/300 liquid scintillation system and a Packard sample oxidizer, with subsequent count and disintegrations per minute (dpm) computed using a Searle Mark III 6880 liquid scintillation spectrometer. Ca. 5 kg spiked meal was reserved as a nonammoniated control and 15.8 kg was ammoniated in pilot plant equipment previously described by Gardner et al. (1). The meal was treated with 4% ammonia for 30 min at 100 C and 40 psi. After the ammoniated meal was air-dried, 3, 50 g samples were removed for aflatoxin assay (9). Additional portions of the meal were sequentially subjected to extraction with organic solvents, treatment with acid and base and enzymic digestion. Radioactivity was measured to monitor this fractionation.

Fractionation of Ammoniated Meal by Solvent Extraction

Two 1 kg samples of ammoniated meal were each extracted first with petroleum ether, and then with methylene chloride followed by methanol. Before each successive solvent was used, the samples were air-dried and then dried at 87 C in a forced-draft oven for 2 hr to remove the previous solvent. The dried residues were weighed before each addition of solvent. Weights were taken without a moisture determination. Water was added, 50 mL/100 g meal, to the air-dried, petroleum ether-extracted meal before the first methylene chloride extraction. The meal was equilibrated ca. 15 min before methylene chloride was added. No water was added before methanol extraction. The meal was extracted by soaking in cold solvent followed by hot extraction in a side-arm Soxhlet apparatus. The solvent-to-meal ratio was ca. 10:1 for each solvent used. Extractions were exhaustive, i.e., until negligible solids were detected as each solvent was evaporated under reduced pressure.

Enzymic Digestion

A portion of the ammoniated meal after methanol extraction was treated with Pronase following the procedure of Park et al. (7). One hundred mg Pronase (45,000 PUK/g, Calbiochem-Behring Corp., La Jolla, CA) was suspended in 250 mL Tris buffer, 0.1 M, pH 7. After 2 hr at 37 C, the enzymic mixture was added to 100 g meal in a water (250 mL) slurry adjusted to pH 7. The mixture was then incubated at 37 C for 24 hr. After digestion, the solubles were removed from the residue by suction-filtration and partitioned with methylene chloride, yielding an aqueous and an organic fraction. The aqueous fraction was freeze-dried and the solvent removed from the organic portion under reduced pressure with the water-bath temperature less than 50 C. The residue from the digestion was air- and oven-dried (82 C) before extraction with methylene chloride. The methylene chloride extract was concentrated under reduced pressure and transferred to a tared vial, and the solvent was removed under a stream of nitrogen.

Acid and Base Treatment

Another portion of the ammoniated meal was treated with weak acid after methanol extraction. Fifty g was refluxed at ca. 100 C with 200 mL 0.1 N acetic acid for 2 hr. The mixture was centrifuged and the supernatant decanted and lyophilized. Methanol was added (100 mL) to the lyophilized acid extract, and after stirring, the mixture was separated by filtration into a solid residue and a methanol-soluble portion. The latter was concentrated under reduced pressure, followed by evaporation of the residual methanol at ca. 50 C under a stream of nitrogen. Solid residue from the acid treatment was extracted with 10% ammonium bicarbonate. The alkali-soluble portion was separated from the residue by filtration; volatile $(NH_4)HCO_3$ was removed under reduced pressure. All extracts were dried in a 40 C vacuum oven to remove any residual solvent. All extracts and residues were weighed. Weights are approximate because some of the material was hygroscopic.

Methylene Chloride Extraction

The methylene chloride extract from 1 kg petroleum ether-extracted ammoniated meal was concentrated under reduced pressure and transferred to a 250-mL volumetric flask. One hundred mL was stripped onto precoated preparative thin layer chromatographic (TLC) plates (silica gel 60 without fluorescent indicator, 2 mm thick) at a load rate of ca. 2 mL/plate. Plates were developed in chloroform/acetone (9:1) and viewed under ultraviolet (UV) light. Eight zones, marked by their appearance under UV light, were scraped from the plates and collected on fritted disc filters. Material from each zone was extracted first with methylene chloride, then with acetone, followed by methanol. Material from each of the middle 6 zones was rechromatographed 3 times to try to achieve separation into distinct zones. Solvent was removed from the eluants from each of the 8 zones under reduced pressure; the eluants were divided into 2 portions in tared vials. Solvent was removed under a stream of nitrogen followed by evaporation at room temperature in a vacuum oven.

Methanol Extraction

The methanol extract was concentrated to 500 mL under reduced pressure. A 50-mL aliquot was added to a separatory funnel and 100 mL acetone/water (30:70) and 50 mL

methylene chloride were added. After vigorous shaking and equilibration, the organic and aqueous phases were separated. Acetone was added to the aqueous phase, causing a precipitate, which was separated from the acetone-soluble portion by filtration. The initial organic layer was concentrated under reduced pressure to ca. 20 mL and poured into ca. 100 mL hexane. Again the precipitate was separated by filtration. Precipitates from the acetone and hexane additions were dried by lyophilization and the solvents were removed from the soluble portions first under reduced pressure and then under a stream of nitrogen. The precipitates (both acetone and hexane) and the corresponding soluble fractions were weighed.

Radioactivity Analyses

^{14}C-Radioactivity concentrations of the aflatoxin-contaminated meal before and after ammoniation and after each extraction step, as well as for each extract obtained throughout the separation scheme, were determined using a Packard sample oxidizer with subsequent count and dpm computed using a Searle Mark III 6880 liquid scintillation spectrometer. Permafluor V (Packard) scintillation fluid was used for all oxidized samples. Radioactivity was counted in the meal during sample preparation and selected fractions after ozonization using a Searle Isocap/300 liquid scintillation system with suspensions of the meal in Kodak Scintillation Fluid I.

RESULTS AND DISCUSSION

Assays on 3, 50 g aliquots of the nonammoniated control meal (4456 μg B_1/kg and 789 μg B_2/kg; 4261 μg B_1/kg and 706 μg B_2/kg and 4160 μg B_1/kg and 638 μg B_2/kg) showed uniform distribution of the toxin in the meal preparation. Uniform mixing of the radioactivity was evidenced by counts of 2148, 2205 and 2364 dpm in 3, 1 g aliquots of the control portion of the meal. The concentration of aflatoxin in the ammoniated meal was 4 μg B_1/kg and a trace of B_2, showing a reduction of extractable aflatoxin of greater than 99.9%. However, even with the cleanup procedure used, the aliquots spotted on TLC plates showed streaking that was not evident with the analyses of the meal before ammoniation. No clear B_1 spot was detected, but a fluorescent zone at the Rf of B_1 was judged to be B_1. This was confirmed by the formation of the trifluoroacetic acid derivative of B_1 (5). Results of the rechromatography zones, done to ensure that all of the unchanged B_1 would be contained in one zone, pointed toward less residual toxin than the chemical assay indicated. Unchanged B_1 that was in the zone that appeared blue under UV light (Table I, Rf 0.5-0.675) represented only 3.1% of the total dpm of the fraction. This zone from a 1 kg sample contained ca. 30 μg aflatoxin-related decontamination by-products, of which 26 μg was not aflatoxin. A total of 53 mg of material was isolated from this fraction.

The procedures used for separating the decontamination by-products are presented in Figure 1. Distribution of the

TABLE I

Radioactivity Distribution in the Methylene Chloride-Soluble Fraction Separated by Preparative TLC

Rf of zone	Color under UV	Radioactivity concentration(%)[a]	Weight (%)[b]	^{14}C-Activity/weight ratio[c]
0.925-1.00	Red	1.7	59.0	0.03
0.800-0.925	Green	0.4	7.0	0.06
0.675-0.800	Purple	8.7	4.0	2.2
0.5-0.675[d]	Blue	3.1	0.8	3.9
0.375-0.5	Yellow	7.8	3.3	2.4
0.25-0.375	Tan	11.4	3.4	3.4
0.125-0.25	Brown	26.6	12.9	2.1
0.00-0.125	Dk. brown	40.4	9.4	4.3

[a]Percentage of radioactivity of the total ^{14}C-activity recovered from the preparative TLC plate.
[b]Percentage of weight of the total amount recovered from the preparative TLC plate.
[c]Calculated by dividing the percent radioactivity by percentage of weight.
[d]Contained aflatoxin B_1.

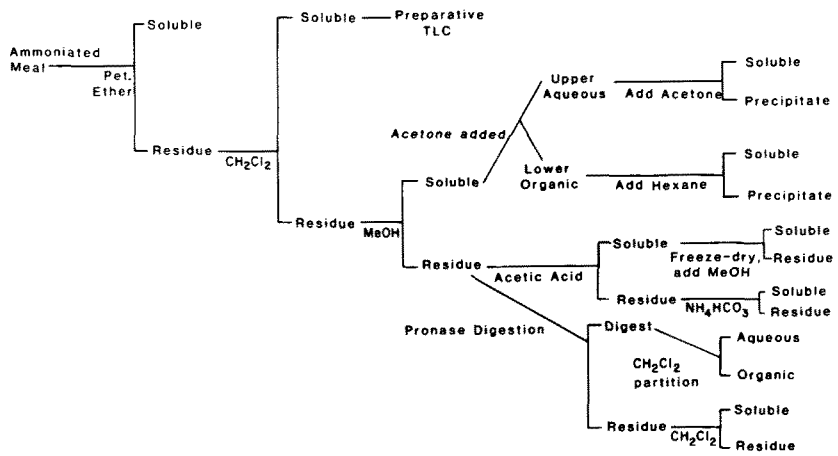

FIG. 1. Separation of aflatoxin-related decontamination by-products in cottonseed meal following ammoniation.

radioactivity in the meal residues after fractionation is presented in Table II. Ca. 86% of the radioactivity added to the cottonseed meal was detected in the meal after the initial air drying. A negligible amount of radioactivity was extracted from the air-dried meal by ethyl ether. After extraction with methylene chloride and methanol, and treatment with acid and base, 45.1% of the total radioactivity remained in the residue. After Pronase digestion, only 37.5% of the added radioactivity remained in the meal matrix. In control nonammoniated meals, 90% of the radiolabeled material was accounted for in the methylene chloride extract.

Distribution of radioactivity after methylene chloride extraction, methanol extraction and acid or base treatment, as well as Pronase digestion, is given in Table III. Nearly one-fourth of the labeled compound (23.3% of total radioactivity) was extracted with nonpolar methylene chloride and 4.7% with more polar methanol. Acid and base treatment accounted for 4.1% of the total radioactivity. The activity-to-weight ratio of the acid- and base-treated meal residues was low, indicating a lack of concentration of radiolabeled material. This ratio was higher for the methylene chloride and methanol extracts (5.1 and 1.5, respectively). After Pronase digestion, a high activity-to-weight ratio of 5 was observed for the methylene chloride extract; however, the highest ratio, 20, was for the methylene choride extract of the residue after Pronase digestion. Because the radioactivity-to-weight ratio is a measure of relative concentration of the alfatoxin decontamination by-products, and aflatoxin B_1 was the only radiolabeled material added in these experiments, the ratio of 20 for this fraction indicates a high concentration of aflatoxin decontamination products in a small portion of the total sample (0.16% of the total weight).

REFERENCES

1. Gardner, H.K., Jr., S.P. Koltun, F.G. Dollear and E.T. Rayner, JAOCS 48:70 (1971).
2. Jorgensen, K.V. and R.L. Price, J. Agric. Food Chem. 29:556 (1981).
3. Lee, L.S., J.B. Stanley, A.F. Cucullu, W.A. Pons, Jr. and L.A. Goldblatt, J. Assoc. Off. Anal. Chem. 57:626 (1974).
4. Cucullu, A.F., L. Lee, W.A. Pons, Jr. and J.B. Stanley, J. Agric. Food Chem. 24:408 (1976).
5. Lee, L.S. and A.F. Cucullu, Ibid 26:66 (1978).
6. Lee, L.S., E.J. Conkerton, R.L. Ory, and J.W. Bennett, Ibid 27:598 (1979).
7. Park, D.L., M. Jemmali, C. Frayssinet, C. LaFarge-Frayssinet, and M. Yvon, JAOCS 58:995A (1981).
8. Beckwith, A.W., R.R. Vesonder and A. Ciegler, J. Agric. Food Chem. 23:582 (1975).
9. Pons, W.A., Jr., L.S. Lee and L. Stoloff, J. Assoc. Off. Anal. Chem. 63:899 (1980).
10. "Changes in Methods", Ibid. 63:391 (Secs. 26.A01-26.A09) (1980).
11. Lee, L.S., S.P. Koltun and S. Buco, JAOCS 60:1548 (1983).

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TABLE II

Radioactivity Distribution in Meal Residues Following Fractionation Procedure Outlined in Figure 1

Sample	Weight (g)	Specific activity (dpm/g)	Total radioactivity (dpm) (× 10,000)	Radioactivity concentration (%)	Weight (%)	^{14}C-Activity/weight ratio[a]
Meal	1000	2240	224	100	100	1
Ammoniated meal	1000	1930	193	86.2	100	0.86
Residue-CH_2Cl_2 extraction	808	1710	138	61.8	80.8	0.76
Residue-MeOH extraction	765	1870	143	63.7	76.5	0.83
Residue-HOAc and $(NH_4)HCO_3$ extraction	524	2180	101	45.1	52.4	0.86
Residue-Pronase digestion	328	2560	84	37.5	32.8	1.1

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

TABLE III

Radioactivity Distribution in Meal Extracts Following Fractionation Procedure Outlined in Figure 1

Extract	Weight (g)	Radioactivity concentration (dpm)	Total radioactivity (dpm) (× 1,000)	Radioactivity concentration (%)	Weight (%)	^{14}C-Activity/weight ratio[a]	Aflatoxin B_1 equivalent (ng/g)[b]
Methylene chloride	45.8	11400	522	23.3	4.6	5.1	932
Methanol	29.6	3600	106	4.7	3.0	1.5	189
Acetic acid-ppt.	53.5	670	36	1.6	5.3	0.3	64
-sol.	61.2	570	35	1.6	6.1	0.26	62
Ammonium bicarbonate	26	790	21	0.9	2.6	0.35	37
Enzymic digestion water	436	950	414	18.5	43.6	0.42	739
-CH_2Cl_2	0.6	9880	6	0.3	0.06	5	11
Methylene chloride extract of Pronase residue	0.076	47000	3.6	0.16	0.008	20	6
						Total	2040

[a]Calculated by dividing percentage of radioactivity by percent weight.
[b]Original aflatoxin B_1 concentration ca. 4000 ng/g; calculations based on specific activity of aflatoxin B_1, 560 dpm/μg.