Schantz, E. J., Mold, J. D., Stanger, D. W., Shavel, J., Riel, F. J., Bowden, J. P., Lynch, J. M., Wyler, R. S., Reigel, B., Sommer, H., *J. Am. Chem. Soc.* **79**, 5290 (1957).
Shimizu, Y., Alam, M., Oshima, Y., Fallon, W. E., *Biochem. Biophys. Res. Commun.* **66**, 731 (1975).
Shimizu, Y., Buckley, L. J., Alam, M., Oshima, Y., Fallon, W. E., Kasai, H., Miura, I., Gullo, V. P., Nakanishi, K., *J. Am. Chem. Soc.* **98**, 5414 (1976).
Sommer, H., Whedon, W. F., Kofoid, C. A., Stohler, R., *Arch. Pathol.* **24**, 537 (1937).
Zwahlen, A., Blanc, M.-H., Robert, M., *Schweiz. Med. Wochenschr.* **107**, 226 (1977).

Received for review December 22, 1977. Accepted April 28, 1978. This work was supported by U.S. HEW Grant Number FD00619 and the University of Rhode Island Sea Grant Program.

Conversion of Aflatoxin B_1 to Aflatoxin D_1 in Ammoniated Peanut and Cottonseed Meals

Louise S. Lee* and Alva F. Cucullu

Cultured and spiked peanut and cottonseed meals, high in aflatoxin, were ammoniated under laboratory conditions paralleling those employed for the model ammoniation of pure aflatoxin B_1, as well as under conditions used in commercial detoxification with ammonia. In the model reaction, 10% aflatoxin B_1 remained after ammoniation, and 10% was converted to aflatoxin D_1. In all ammoniation studies conducted on spiked and cultured peanut and cottonseed meals, an average of 0.36% aflatoxin B_1 was converted to aflatoxin D_1, and an average of 0.37% of the B_1 remained unreacted.

Treatment of peanut and cottonseed meals with ammonia gas at elevated temperature and pressure is a commercially feasible approach to inactivation of aflatoxins that may occur as contaminants of these oilseed meals (Gardner et al., 1971). The success of this approach prompted us to initiate research to isolate and chemically characterize the products formed by ammoniation of aflatoxin B_1 (Lee et al., 1974; Cucullu et al., 1976). Lee et al. isolated the major product formed during a model reaction of pure aflatoxin B_1 with ammonium hydroxide under heat and pressure. They identified this compound as a nonfluorescent phenol of molecular weight 286 that retains the dihydrofurano group but lacks the lactone carbonyl moiety characteristic of aflatoxin B_1. This compound, which derives from opening the lactone ring during ammoniation and subsequent decarboxylation of the resultant β-keto acid, was termed aflatoxin D_1.

Cucullu et al. (1976) estimated a 10% yield of D_1 formed from B_1 during the model reaction after 3 to 4 h ammoniation. Less D_1 was formed after ammoniation at 1 and 8 h. The present report describes the conversion of aflatoxin B_1 to D_1 during ammoniation of peanut and cottonseed meals that are highly contaminated with aflatoxin, under the conditions of this model reaction and also under laboratory conditions that closely simulate those of commercial ammoniation. It also describes the method developed to determine aflatoxin D_1 in ammoniated peanut and cottonseed meals.

EXPERIMENTAL PROCEDURES

Method for Determination of Aflatoxin D_1. Samples of ammoniated peanut or cottonseed meals weighing either 1 or 20 g were soaked for 10 min in 40 mL of 0.1 N hydrochloric acid, and 210 mL of methyl alcohol was added. This acidic methanol solvent mixture is the same as that used by Pons and Franz (1977) for the quantitative extraction of aflatoxins from peanut butter. The samples were shaken on a Burrell wrist action shaker for 30 min and filtered. A 175-mL aliquot of each extract was collected, and 40 mL of a 20% lead acetate solution and 185 mL distilled water were added. Ten grams of acid-washed Hyflo Supercel filter aid was stirred into the solution, and the mixture was filtered. A 340-mL aliquot of each extract was then partitioned with two successive 50-mL portions of chloroform in a separatory funnel, and the chloroform extract was drained through a Butt tube containing the sodium sulfate–silica gel described by Pons and Franz (1977) for assaying aflatoxins in ammoniated cottonseed meals. Extracts were evaporated on a steam bath and further purified on a cellulose partition column prepared according to Pons et al. (1973), except that 11 × 500 mm columns and 4 g of cellulose were used. Interfering substances were removed by elution with 75 mL of hexane–benzene (5:1), followed by 75 mL of hexane–ether (4:1). Unreacted aflatoxin B_1 and the ammoniation product, aflatoxin D_1, were eluted from the column with 100 mL of hexane–chloroform (1:1).

Aflatoxin B_1 in the extract was quantitated according to Pons et al. (1968). Aflatoxin D_1 in the extract was further purified by preparative thin-layer chromatography (TLC) on silica gel G-HR plates developed in chloroform–acetone (9:1). Aflatoxin D_1, R_f 0.46, was eluted immediately from the gel with chloroform–methanol (1:1) into small vials fitted with Teflon-lined screw caps. After removal of the solvent, each sample was acetylated according to Stack et al. (1972). The resultant fluorescent acetate (Lee et al., 1974) was separated by TLC with chloroform–acetone (9:1) and quantitated by comparing the fluorescence intensities with the acetate derivative prepared from known aflatoxin D_1 spotted on TLC plates. Calculations were based on a sample weight of 0.6 of the original meal used, after correcting for the extract removed for estimation of aflatoxin B_1.

Percent recovery of aflatoxin D_1 was estimated by the addition of aliquots of a chloroform solution, containing 100 μg of aflatoxin D_1/mL, to 20-g samples of ammoniated aflatoxin-negative cottonseed meal. Meal samples were spiked with aflatoxin D_1 at levels of 10, 20, 40, 80, 120, 200,

Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, New Orleans, Louisiana 70179.

Table I. Recovery of Aflatoxin D_1 Added to Ammoniated, Aflatoxin-Negative Cottonseed Meal

Aflatoxin D_1		
Added, μg^a	Found, μg^b	Recovery, %
80.0	60.8	76
24.0	18.7	78
16.0	12.0	75
8.0	6.0	75
4.0	2.4	60
2.4	2.3	96
1.6	0.91	57
0.8	0.62	77
0.4	0.34	85
0.2	0.17	85
	Av	77

[a] Added to 20-g samples. [b] Average of duplicate determinations.

400, 800, 1200, and 4000 μg/kg and assayed as described above. The method described was used to estimate aflatoxin D_1 in ammoniated, contaminated peanut and cottonseed meals.

Preparation of Meals. (*a*) *Spiked Meals.* Approximately 1 kg each of blanched peanuts and dehulled, glanded cottonseed was hexane extracted in a Soxhlet and dried. The defatted products were disintegrated in a Waring Blendor and then ground in a Wiley Mill to pass a 20-mesh screen. Meals were adjusted to 15% moisture and equilibrated. Aflatoxin B_1 from a stock solution of chloroform containing 2 mg of B_1/mL was added to 1-g and 20-g samples of each meal at a level of 1 million μg/kg.

(*b*) *Cultured Meals.* Approximately 2 kg each of blanched peanuts and dehulled cottonseed were adjusted to 30% moisture content and autoclaved. Peanuts were inoculated with *Aspergillus parasiticus* (NRRL 2999) and cottonseed with *Aspergillus flavus* (NRRL 5520). Cultures were incubated for 7 days at 30 °C. Excess spores were removed from cultured samples by a current of air. Samples were then treated in the same manner as that described for the noncultured meals except that milling was conducted in a well-vented hood. Assays showed 0.950 and 1.137 million μg of B_1/kg in the peanut and cottonseed meals, respectively.

All laboratory ammoniations were conducted on the above meals.

(*c*) *Naturally Contaminated Meal.* A cottonseed meal containing 330 μg of B_1/kg was ammoniated in the pilot plant with 25 psig anhydrous ammonia gas at 80 °C for 30 min.

Ammoniation Treatments. Two sets of experiments were conducted. The first set utilized a 22-mL Parr bomb, 1-g samples of either spiked or cultured meals, and 2 mL of NH_4OH (30% NH_3) as the ammonia source. The ammoniation conditions (100 °C for 3 h) duplicated those employed by Lee et al. (1974) and Cucullu et al. (1976) for the ammoniation of pure aflatoxin B_1 in a model reaction. In the second set of experiments, 20-g samples of the spiked or cultured meals were ammoniated in a Model 4562 Parr reactor (450-mL capacity) equipped with a thermostatically controlled heating mantle, pressure gauge, mechanical stirrer, and a gas outlet and inlet. When the reactor was charged and securely tightened, ammonia gas was allowed to enter the gas inlet and sweep residual air from the reactor through the outlet. The outlet was then closed, ammonia gas to the desired pressure was added, and the vessel was heated to the desired temperature. Temperature was maintained at ±5 °C, and pressure at ±5 psig. All meals were ammoniated for 30 min at the selected temperature and pressure. Three combinations of temperature and pressure were used: 115 °C, 45–50 psig; 115 °C, 35 psig; and 75 °C, 35 psig. Triplicate ammoniations were conducted for each set of conditions.

All ammoniated samples were air-dried overnight in a forced draft hood. Aflatoxin D_1 and unreacted aflatoxin B_1 were estimated in the entire sample of these ammoniated meals by the methods previously described. For the 330 μg/kg of meal, a 1-kg sample was extracted in 20-g portions. Final purified extracts from the 50 analytical samples were combined and acetylated as one sample. All results are reported as micrograms of aflatoxin B_1 in the sample before ammoniation, and micrograms of aflatoxin B_1 or D_1 detected after ammoniation.

RESULTS AND DISCUSSION

Recoveries of aflatoxin D_1 added at levels ranging from 10 to 4000 μg/kg to ammoniated, negative cottonseed meals are reported in Table I. The average recovery was

Table II. Conversion of Aflatoxin B_1 to Aflatoxin D_1 in Ammoniated Cultured Aflatoxin-Containing Peanut Meals

		Aflatoxin B_1			Aflatoxin D_1	
Conditions of ammoniation	Meal, g^a	Before ammoniation	Remaining after ammoniation		After ammoniation	Conversion from B_1
		μg	μg	%	μg	%
100 °C,[b] 180 min, NH_4OH	1	950	3.3	0.35	1.0	0.11
	1	950	2.1	0.22	2.0	0.21
	1	950	1.8	0.19	1.0	0.11
				Av 0.25		Av 0.14
115 °C,[c] 30 min, 45–50 psig NH_3	20	19000	24.0	0.13	26.0	0.14
	20	19000	24.0	0.13	20.0	0.11
	20	19000	16.0	0.08	20.0	0.11
				Av 0.11		Av 0.12
115 °C,[c] 30 min, 35 psig NH_3	20	19000	28.0	0.15	26.0	0.14
	20	19000	20.0	0.11	24.0	0.13
	20	19000	18.0	0.09	20.0	0.11
				Av 0.12		Av 0.13
75 °C,[c] 30 min, 35 psig NH_3	20	19000	34.0	0.18	50.0	0.26
	20	19000	36.0	0.19	40.0	0.21
	20	19000	32.0	0.17	46.0	0.24
				Av 0.18		Av 0.24

[a] Cultured meal, 0.950 million μg of B_1/kg. [b] Ammoniated in 22-mL Parr bomb. [c] Ammoniated in 450-mL Parr reactor.

Table III. Conversion of Aflatoxin B_1 to Aflatoxin D_1 in Ammoniated Cultured and Natural Aflatoxin-Containing Cottonseed Meals

Conditions of ammoniation	Meal g	Aflatoxin B_1 Before ammoniation μg	Aflatoxin B_1 Remaining after ammoniation μg	%	Aflatoxin D_1 After ammoniation μg	Aflatoxin D_1 Conversion from B_1 %
100 °C,[a] 180 min, NH_4OH	1[b]	1137	2.2	0.19	1.0	0.09
	1	1137	2.0	0.18	0.5	0.04
	1	1137	2.8	0.25	1.0	0.09
			Av	0.21		Av 0.07
115 °C,[c] 30 min, 45-50 psig NH_3	20	22740	35.2	0.15	26.0	0.11
	20	22740	22.4	0.10	24.0	0.11
	20	22740	35.2	0.15	20.0	0.09
			Av	0.13		Av 0.10
115 °C,[c] 30 min, 35 psig NH_3	20	22740	24.0	0.11	26.0	0.11
	20	22740	22.0	0.10	20.0	0.09
	20	22740	15.2	0.07	16.6	0.07
			Av	0.09		Av 0.09
75 °C,[c] 30 min, 35 psig NH_3	20	22740	30.0	0.13	40.0	0.18
	20	22740	32.0	0.14	34.0	0.15
	20	22740	60.0	0.26	50.0	0.22
			Av	0.18		Av 0.18
80 °C,[d] 30 min, 25 psig NH_3	1000[e]	330	10.3	3.12	0.21	0.06

[a] Ammoniated in 22-mL Parr bomb. [b] Cultured meal, 1.137 million μg of B_1/kg. [c] Ammoniated in 450-mL Parr reactor. [d] Ammoniated in pilot plant equipment. [e] Naturally contaminated meal, 330 μg of B_1/kg.

Table IV. Conversion of Aflatoxin B_1 to Aflatoxin D_1 in Aflatoxin-Negative Meals Spiked with Aflatoxin B_1 Before Ammoniation

Meal	Wt, g	Aflatoxin B_1 Before ammoniation μg	Aflatoxin B_1 Remaining after ammoniation μg	%	Aflatoxin D_1 After ammoniation μg	Aflatoxin D_1 Conversion from B_1 %
Peanut[a]	1	1000	15	1.5	20.0	2.0
Peanut	1	1000	17	1.7	11.6	1.2
Peanut	1	1000	10	1.0	15.0	1.5
			Av	1.4		Av 1.6
Cottonseed[a]	1	1000	11	1.1	11.0	1.1
Cottonseed	1	1000	17	1.7	17.0	1.7
Cottonseed	1	1000	13	1.3	13.0	1.3
			Av	1.3		Av 1.3
Peanut[b]	20	20000	42	0.21	33.0	0.16
Cottonseed[b]	20	20000	48	0.24	26.0	0.13

[a] Ammoniated in 22-mL Parr bomb with NH_4OH, 100 °C, 180 min. [b] Ammoniated in 450-mL Parr reactor, 35 psig NH_3, 75 °C, 30 min.

77%, and individual recoveries ranged from 57 to 94%. Incomplete recovery of added aflatoxin D_1 is probably due to the many manipulations involved in the method. Interfering materials in the crude aflatoxin D_1 harvested from the TLC plates may also affect the complete acetylation of aflatoxin D_1. Ten micrograms/kilogram (0.2 μg/20 g) was the lowest level of aflatoxin D_1 detectable in the 20-g analytical sample. Levels below that would not be detected unless the sample size was increased appreciably. Since 20 g was the maximum sample size that could be charged into the Parr reactor, highly contaminated meals, spiked with aflatoxin B_1 or cultured to contain aflatoxin B_1, were used for all the laboratory ammoniation studies. Results are reported in Tables II, III, and IV. In these tables aflatoxin B_1 remaining after ammoniation, and aflatoxin D_1 produced by ammoniation, are reported as microgram in the total sample ammoniated. Table II summarizes data on ammoniation of peanut meals prepared from cultured peanuts, and Table III gives similar data on ammoniation of cottonseed meals from cultured cottonseed. The data in Tables II and III on cultured peanuts and cottonseed meals containing 0.950 and 1.137 million μg of B_1/kg, respectively, ammoniated under four different sets of reaction conditions, show consistently uniform aflatoxin B_1 reductions. Under these diverse conditions, the overall mean percent of B_1 remaining was 0.17% for cultured peanut meals and 0.15% for cultured cottonseed meals.

Results of experiments in which aflatoxin B_1 was added to aflatoxin negative peanut and cottonseed meals prior to ammoniation are shown in Table IV. These experiments were run to compare the results obtained when no mold metabolites other than aflatoxin were present. Therefore they tested the effect of other mold metabolites present in cultured peanuts and cottonseed on the conversion of aflatoxin B_1 to D_1. Conditions for ammoniations conducted in the small Parr bomb duplicated those of the model reaction (Cucullu et al., 1976). Results of ammoniations in the small Parr bomb indicate a tenfold increase in conversion of B_1 to D_1 over those reported for cultured meals in Tables II, III, and IV. The higher percent conversion (1.3 to 1.6) and higher percent unreacted B_1 (1.3 to 1.4) are closer to percent conversions obtained from the

model reaction. Cucullu et al. (1976) report not only 10% remaining aflatoxin B_1, but 10% conversion to aflatoxin D_1 in the reaction of pure B_1 with ammonium hydroxide. Results reported in Table IV, obtained on meal spiked with aflatoxin B_1 and ammoniated in the 450-mL Parr reactor, closely paralleled those obtained on cultured meals ammoniated in the 450-mL Parr bomb (Tables II and III). These results indicate that meal, rather than mold constituents, influence both decrease of aflatoxin B_1 and formation of aflatoxin D_1.

The average conversion of aflatoxin B_1 to D_1 was 0.36% for cottonseed meals. Although the measured amount of D_1 formed from B_1 varies considerably within a given set of conditions, when data are considered on the basis of the amount of toxin in the original meal (approximately 1000 μg of B_1/g), the final content of both B_1 and D_1 are similar. Even more pertinent is the similarity of these results to those obtained for the pilot plant ammoniation of cottonseed meal containing only 330 μg of B_1/kg (Table III).

Feeding studies on ammoniated cottonseed meals (Vohra et al., 1975; Waldroup et al., 1976) indicate that ammoniated cottonseed meals can safely be fed to laying hens and broilers. The meal fed these birds was ammoniated under the harshest conditions of heat and pressure (115 °C, 45–50 psig). Our results show a similar conversion of B_1 to D_1 under all conditions of ammoniation, as well as comparable amounts of unreacted B_1.

Further studies are presently being conducted at this laboratory in which we are evaluating the protein and carbohydrate quality of aflatoxin contaminated meals ammoniated under the three conditions. The results of that study, along with those reported here, will give processors a more complete profile of meal quality.

ACKNOWLEDGMENT

The authors are grateful to A. O. Franz, Jr., for quantitative aflatoxin analyses, S. P. Koltun and E. T. Rayner for the ammoniated cottonseed meal prepared in the pilot plant, and W. A. Pons, Jr., for valuable advice.

LITERATURE CITED

Cucullu, A. F., Lee, L. S., Pons, W. A., Jr., Stanley, J. B., *J. Agric. Food Chem.* **24**, 408 (1976).
Gardner, H. K., Jr., Koltun, S. P., Dollear, F. G., Rayner, E. T., *J. Am. Oil Chem. Soc.* **48**, 70 (1971).
Lee, L. S., Stanley, J. B., Cucullu, A. F., Pons, W. A., Jr., Goldblatt, L. A., *J. Assoc. Off. Anal. Chem.* **57**, 626 (1974).
Pons, W. A., Jr., Cucullu, A. F., Franz, A. O., Jr., Goldblatt, L. A., *J. Am. Oil Chem. Soc.* **45**, 694 (1968).
Pons, W. A., Jr., Cucullu, A. F., Lee, L. S., *J. Assoc. Off. Anal. Chem.* **56**, 1431 (1973).
Pons, W. A., Jr., Franz, A. O., Jr., *J. Assoc. Anal. Chem.* **60**, 89 (1977).
Pons, W. A., Jr., Franz, A. O., Jr., *J. Assoc. Off. Anal. Chem.* **61**, 793 (1978).
Stack, M. E., Pohland, A. E., Dantzman, J. G., Neshiem, S. J., *J. Assoc. Off. Anal. Chem.* **55**, 313 (1972).
Vohra, P., Hafez, Y., Earl, L., Kratzer, F. H., *Poult. Sci.* **54**, 441 (1975).
Waldroup, P. W., Hazen, K. R., Mitchell, R. J., Payne, J. R., Johnson, Z., *Poult. Sci.* **55**, 1011 (1976).

Received for review December 19, 1977. Accepted March 7, 1978. Presented in part at the 29th Annual Southeastern Regional Meeting of the American Chemical Society, Tampa, Fla., Nov 9–11, 1977. Names of companies or commercial products are given solely for the purpose of providing specific information; their mention does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

Nitramines as Thermal Energy Analyzer Positive Nonnitroso Compounds Found in Certain Herbicides

Joseph H. Hotchkiss,* James F. Barbour, Leonard M. Libbey, and Richard A. Scanlan

Certain dialkyl nitramines have been shown to elicit a false positive response on the thermal energy analyzer (TEA). The TEA is a detector specifically employed in the analysis of samples for trace amounts of nitrosamines. Dipropylnitramine (DPNO) was responsible for a TEA false positive peak in the analysis of a widely used herbicide formulation containing α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine. In addition, the presence of dipropylnitrosamine at a concentration of 130 mg/kg in the herbicide was confirmed. The molar response ratio of DPNO compared to dipropylnitrosamine was 0.50. In addition, DPNO was Griess reagent positive, UV sensitive, and had a similar retention time to dibutylnitrosamine on Carbowax 20M. Nitramines are almost certainly responsible for unknown TEA positive peaks in other herbicide formulations. Nitramines might be mistakenly identified as *N*-nitroso compounds, especially where mass spectral confirmation is not made.

The thermal energy analyzer (TEA) is widely employed as a specific detector for the analysis of nitrosamines (NAs) in a variety of substances. The TEA was first used as a GLC detector (Fine and Rounbehler, 1975) and more recently as a LC detector (Fine et al., 1976). The principles of operation have been described in detail elsewhere (Fine et al., 1975).

The high selectivity of the TEA detector over more conventional detectors is well established, but recent work indicates that certain samples contain non-NAs which can elicit a positive TEA response. Unidentified TEA positive peaks which elute prior to dimethylnitrosamine (DMN) have been reported (Stephany and Schuller, 1978) as well as unidentified peaks eluting after DMN in tobacco smoke

Department of Food Science and Technology, Oregon State University, Corvallis, Oregon 97331.

0021-8561/78/1426-0884$01.00/0