Ojima, K., Gamborg, O. L., *Biochem. Physiol. Plant Growth Subst., Proc. Int. Conf. Plant Growth Subst., 6th*, 857 (1968).
Oleson, A. E., Clark, E. T., Janski, A. M., *Phytochemistry* **13**, 2113 (1974a).
Oleson, A. E., Janski, A. M., Clark, E. T., *Biochim. Biophys. Acta* **366**, 89 (1974b).
Robertson, M. M., Kirkwood R. C., *Weed Res.* **10**, 94 (1970).
Srivastava, O. P., van Huystee, R. B., *Can. J. Bot.* **51**, 2207 (1973).
Steen, R. C., Schultz, I. R., Zimmerman, D. C., Fleeker, J. R., *Weed Res.* **14**, 23 (1974).
Wang, H. C., Hamilton, R. H., Deering, R. A., in *Biochem. Physiol. Plant Growth Subst., Proc. Int. Conf. Plant Growth Subst., 6th*, 685 (1968).

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Occurrence of Aflatoxin B_{2a} in Cottonseed Meal

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Aflatoxin B_{2a}, 17 ppb, was found in cottonseed meal contaminated with aflatoxin B_1 and B_2. Aflatoxin B_{2a} was not detected in feed-grade meal or in meal which had been decontaminated by the ammonia process. Other fungal metabolites such as zearalenone, sterigmatocystin, ochratoxin A, and aflatoxins M_1 and M_2 were not detected.

Since the discovery that a peanut meal containing aflatoxin was responsible for many deaths among turkey poults and ducklings (Allcroft et al., 1961), a massive literature has appeared describing the efforts devoted to aflatoxin and other mycotoxins that affect agricultural products. Aflatoxins, which are secondary fungal metabolites, include at least 16 closely related chemicals with highly substituted furofuran rings of which aflatoxin B_1 is the most important (Rodricks, 1969). The aflatoxins, which are acutely toxic and oncogenic mold products, are elaborated by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus* (Sargeant et al., 1961). These molds can grow readily on feedstuff if favorable conditions of temperature and humidity prevail (Goldblatt, 1969; Borker et al., 1966; Ciegler and Lillehoj, 1968). These compounds fluoresce brightly under long-wave UV light. This important property has been utilized as the basis for an extremely sensitive method for their detection, isolation, and analytical quantitation (Goldblatt, 1969).

Chemical characteristics of the aflatoxins have been determined by various workers and the structures of aflatoxins B_1, B_2, G_1, and G_2 have been elucidated (Asao et al., 1963, 1965; Hartley et al., 1963; van der Merwe et al., 1963). The biotransformation of aflatoxin B_1 results in hydroxylated derivatives, namely aflatoxins M_1 and M_2; these were first isolated from cow's milk (de Iongh et al., 1964). The structures of both of these compounds have been elucidated (Holzapfel et al., 1966). Two new hydroxylated aflatoxins produced by mold cultures of *A. flavus* have been described and designated aflatoxins B_{2a} and G_{2a} (Dutton and Heathcote, 1966, 1968). It has been shown that these two compounds can be formed from aflatoxins B_1 and G_1, respectively, by treatment with strong acid and heat (Pons et al., 1972; Pohland et al., 1968). The occurrence of aflatoxins B_{2a} and G_{2a} in cottonseed meal has not been reported.

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Aflatoxigenic *Aspergilli* are ubiquitous in nature (Hesseltine et al., 1966; Wogan, 1966) and contamination of cottonseed by aflatoxin occurs in the field (Ashworth, 1972). In addition to aflatoxins, the *Aspergilli* can produce other toxic substances on feed materials (Wilson and Wilson, 1964). For this reason we screened aflatoxin-contaminated cottonseed meals for fungal metabolites other than aflatoxins B_1 and B_2. Naturally infected cottonseed meals usually contain only aflatoxins B_1 and B_2 (McMeans et al., 1968). In controlled laboratory conditions, however, a strain of *A. flavus* grown on media containing cottonseed products elaborated all four aflatoxins, B_1, B_2, G_1, and G_2 (Mayne et al., 1966).

This report describes the isolation and quantitation of aflatoxin B_{2a} found in contaminated cottonseed meal. This procedure did not detect zearalenone, sterigmatocystin, ochratoxin A, and aflatoxins M_1 and M_2.

EXPERIMENTAL PROCEDURES

Materials. Three different cottonseed meals were used. One was a meal free of aflatoxin B_1 and B_2 contamination. A second contained 948 μg/kg (ppb) aflatoxin B_1 and 170 ppb aflatoxin B_2. The third was the same contaminated meal, which had been detoxified in the pilot plant by the ammonia process (Mann et al., 1970). All chemicals and solvents were reagent grade. Silicic acid for column chromatography, 100 mesh powder, analytical reagent, was from Mallinckrodt Chemical Works, St. Louis, Mo. Adsorbosil-1 (Applied Science Laboratories, State College, Pa.) was used to coat thin-layer plates 0.5 mm prepared according to methods already described in the literature (AOAC, 1975). Aflatoxin B_{2a} (kept under N_2 at 4 °C) and ochratoxin A were obtained from W. A. Pons, Jr., SRRC, and zearalenone was supplied by Commercial Solvents, Terre Haute, Ind. Sterigmatocystin was purchased from Aldrich Chemical Co. Aflatoxins M_1 (10186-65-A) and M_2 (8578-95-A) were obtained from R. D. Stubblefield, NRRC, Peoria, Ill. Resolution of B_{2a} was evaluated visually in a View Box Spectroline, Black Light, Model 3F, Eastern Corp., Westbury, L.I., New York. Quantitative estimation was made with a Photovolt Model 530A densitometer,,

Photovolt Corp., New York. Estimations were made in duplicate.

Extraction. The cottonseed meals were extracted with acetone–water (85:15 v/v) on a Burrell wrist action shaker both with and without 8 mL of glacial acetic acid per liter of extracting solvent. The outcome of this study was not altered by this slight modification in the solvent. Since it is possible that aflatoxin B_{2a} could arise from hydration of aflatoxin B_1 in the presence of acid, contaminated meal was extracted with the acidulated solvent by shaking continuously for periods of 0.5, 4, 16, and 24 h, then filtering the mixture through filter paper. The clear solution was partitioned into chloroform, and the chloroform solution was concentrated under reduced pressure and purified by column chromatography.

Column Chromatography. The crude material from the above extractions was chromatographed through a silicic acid column (2.5 × 36 cm). The column was developed with ten column volumes of ether–*n*-hexane (3:1), followed with chloroform–acetone (9:1), until all aflatoxins B_1 and B_2 were eluted. Development of columns was carried out in a darkened room, and movement of fluorescent materials was monitored by indirect and infrequent illumination by long-wave UV light. Final elution was carried out with chloroform–acetone (9:4) to chromatograph hydroxylated aflatoxin. All fractions eluted were concentrated under reduced pressure to about 3.0 mL, transferred to screw cap vials, and evaporated to dryness in a stream of nitrogen. All residues were dissolved in chloroform to a final volume of 0.5 mL and analyzed by thin-layer chromatography.

Thin-Layer Chromatography (TLC). Plates (20 × 20 cm) were activated for 1 h at 110 °C and cooled to room temperature in a TLC desiccator before the samples were spotted. Volumes ranging from 5 to 15 μL of eluted column material were spotted, along with authentic samples of aflatoxin B_{2a} and other mycotoxins. Solutions of zearalenone, sterigmatocystin, ochratoxin A, and aflatoxins M_1 and M_2 were used as standards to detect material having similar mobility and fluorescence. Plates were developed by the ascending technique about 12 cm from the origin in unlined, unequilibrated stainless steel Thomas Mitchell tanks. The developing solvent systems used were: chloroform–acetone (9:1), I; chloroform–acetone–2-propanol (850:125:25), II; and chloroform–acetone–2-propanol (8:1:1), III. Plates were removed from the development tank and the solvent was permitted to evaporate. Only aflatoxin B_{2a} was detected by this method. After preliminary and/or quantitative TLC, plates were sprayed gently with 25% sulfuric acid (von Schuller et al., 1967; AOAC, Natural Poisons, 1975).

Quantitative Estimation of Aflatoxin B_{2a}. Estimations were carried out by spotting standard solutions of known concentrations of authentic aflatoxin B_{2a} adjacent to a fixed volume of unknown concentration of extractive material that had eluted from the column. Plates were scanned densitometrically, and quantitation was achieved by comparing known B_{2a} fluoresence with fluorescence of unknown sample spots.

RESULTS AND DISCUSSION

The survey of cottonseed meals for the presence of mycotoxins other than aflatoxin B_1 and B_2 revealed that cottonseed can be contaminated with aflatoxin B_{2a}. Figure 1 shows typical thin-layer chromatographic behavior of the purified extract compared to authentic B_{2a}. Sample extracts containing suspected B_{2a} were chromatographically compared with standard B_{2a} in solvents I, II, and III. In each of these solvents the suspected B_{2a} and the standard

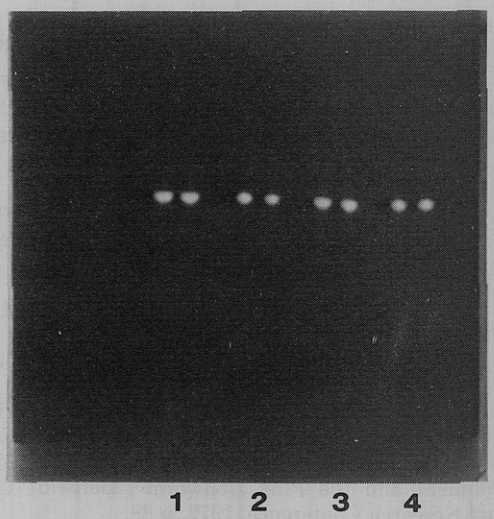

Figure 1. Thin-layer chromatography of aflatoxin B_{2a}. No. 1 and 3 are aflatoxin B_{2a} from cottonseed meal; 2 and 4 are authentic aflatoxin B_{2a}. Eluting solvent was chloroform–acetone–2-propanol (8:1:1; eluent II).

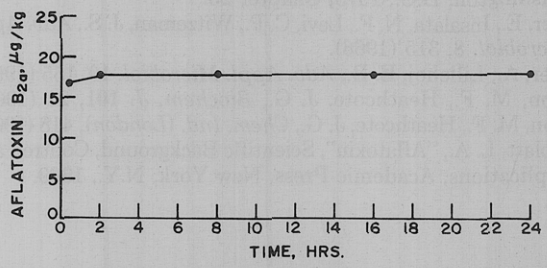

Figure 2. Appearance of aflatoxin B_{2a} in extracting solvent (acetone–water (85:15 v/v) with added 8 mL of glacial acetic acid/L of solvent). Substrate: cottonseed meal. Separate samples extracted for 30 min, 2, 8, 16, and 24 h. Amount of B_{2a} remained the same.

were identical by both cochromatography and by chromatography of adjacent spots. Highly purified suspected material spotted on thin-layer plates and overspotted with authentic B_{2a} chromatographed as single material. The developed thin-layer plates, containing both known and unknown spots, were sprayed with sulfuric acid, and a change in fluorescence color from blue to yellow-green was observed in both spots. This is generally recognized as a chemical test for the presence of aflatoxin-like substances (von Schuller et al., 1967; AOAC, Natural Poisons, 1975).

The kinetics of the appearance of aflatoxin B_{2a} in the extraction solvent is summarized in Figure 2. On prolonged extraction of aflatoxin-contaminated cottonseed meal with acidulated solvent, there was no increase in extractable B_{2a} as time of extraction increased. Recovery was maximum at approximately 30 min and remained constant for at least 24 h. Duplicate densitometric evaluations showed 17 ppb aflatoxin B_{2a}; this indicates that aflatoxin B_{2a} is a contaminant of cottonseed meal, instead of an artifact produced during isolation and assay procedures.

Aflatoxin B_{2a} could not be detected either in cottonseed meal that had been detoxified by the anhydrous ammonia process or in a meal free of aflatoxin B_1 and B_2 contamination. Eluate fractions from column chromatography of several cottonseed meals were tested for sterigmatocystin, zearalenone, ochratoxin A, and aflatoxins M_1 and M_2. These tests were accomplished by spotting on thin-layer plate samples of unknown composition alongside known mycotoxins. Developed chromatoplates were compared under long- and short-wave UV light. Typical

R_f values and characteristic fluorescence showed the above mentioned toxins were not present in the cottonseed meals tested. When impurities were suspected of having a masking effect, further purification by column chromatography was carried out and the process was repeated.

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LITERATURE CITED

Allcroft, R., Carnaghan, R. B. A., Sargeant, H., O'Kelly, J., *Vet. Rec.* **73**, 428 (1961).
Asao, Y., Buchi, G., Abdel Kader, M. M., Chang, S. B., Wick, E. L., Wogan, G. N., *J. Am. Chem. Soc.* **85**, 1706 (1963).
Asao, T., Buchi, G., Abdel Kader, M. M., Chang, S. B., Wick, E. L., Wogan, G. N., *J. Am. Chem. Soc.* **87**, 882 (1965).
Ashworth, L. J., Jr., "Aflatoxins in Cottonseed: The Roles of Environment and the Pink Bollworm", Beltwide Cotton Product Research Conference, 1972, p 88.
Association of Official Analytical Chemists, "Official Methods of Analysis", 12 ed, Washington, D.C., 1975.
Association of Official Analytical Chemists, Natural Poisons, "Official Methods of Analysis", Hortwitz, W., Ed, 12 ed, Washington, D.C., 1975, Chapter 26.
Borker, E., Insalata, N. F., Levi, C. P., Witzeman, J. S., *Adv. Appl. Microbiol.* **8**, 315 (1966).
Ciegler, A., Lillehoj, E. B., *Adv. Appl. Microbiol.* **10**, 155 (1968).
Dutton, M. F., Heathcote, J. G., *Biochem. J.* **101**, 21 (1966).
Dutton, M. F., Heathcote, J. G., *Chem. Ind. (London)*, 418 (1968).
Goldblatt, L. A., "Aflatoxin", Scientific Background, Control, and Implications, Academic Press, New York, N.Y., 1969.
de Iongh, H., Vles, R. O., van Pelt, J. G., *Nature (London)* **202**, 466 (1964).
Hartley, R. D., Nesbitt, B. F., O'Kelly, J., *Nature (London)* **198**, 1956 (1963).
Hesseltine, C. W., Shotwell, O. L., Ellis, J. J., Stubblefield, R. D., *Bacteriol. Rev.* **30**, 795 (1966).
Holzapfel, C. W., Steyn, P. S., Purchase, I. F. H., *Tetrahedron Lett.* **25**, 2799 (1966).
Mann, G. E., Codifer, L. P., Jr., Gardner, H. K., Jr., Koltun, S. P., Dollear, F. G., *J. Am. Oil Chem. Soc.* **47**, 173 (1970).
Mayne, R. Y., Pons, W. A., Jr., Franz, A. O., Jr., Goldblatt, L. A., *J. Am. Oil Chem. Soc.* **43**, 251 (1966).
McMeans, J. L., Ashworth, L. J., Jr., Pons, W. A., Jr., *J. Am. Oil Chem. Soc.* **45**, 575 (1968).
Pohland, A. E., Cushmac, M. E., Andrellos, P. J., *J. Assoc. Off. Anal. Chem.* **51**, 907 (1968).
Pons, W. A., Jr., Cucullu, A. F., Lee, L. S., Janssen, H. J., Goldblatt, L. A., *J. Am. Oil Chem. Soc.* **49**, 124 (1972).
Rodricks, J. V., *J. Agric. Food Chem.* **17**, 457 (1969).
Sargeant, K., Sheridan, A., O'Kelly, J., Carnaghan, R. B. A., *Nature (London)* **192**, 1996 (1961).
van der Merwe, K. J., Fourie, L., Scott, de B., *Chem. Ind. (London)*, 1660 (1963).
von Schuller, P. L., Ockhuizen, Th., Werringgloer, J., Marquardt, P., *Arzneim.-Forsch.* **17**, 888 (1967).
Wilson, B. J., Wilson, C. H., *Science* **144**, 177 (1964).
Wogan, G. N., *Bacteriol. Rev.* **30**, 460 (1966).

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Oxidative Inactivation of Ethylenethiourea by Hypochlorite in Alkaline Medium

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The mechanism of hypochlorite mediated oxidation of ethylenethiourea (ETU) (I) to ethyleneurea (EU) (II) was investigated. Evidence is presented which suggests a sequential oxidation of ETU in aqueous base to a sulfenate, a sulfinate, a sulfonate, and finally to EU and sulfate. Although rapid and complete when using hypochlorite, the reaction is incomplete when using hydrogen peroxide, ETU being oxidized to the level of a sulfonate only. The synthesis of intermediates are detailed. Oxidation in alkaline medium is proposed as a technique for decontaminating surface residues of ETU on agricultural products.

Current concern regarding residues of ethylenebisdithiocarbamate (EBDC) fungicides centers on the possibility that these residues may be converted to ethylenethiourea (ETU) (I), which is a potent teratogen and tumorigen to mice and rats (Graham et al., 1975; Graham, 1973; Graham and Hansen, 1972; Khera, 1973; Innis et al., 1969). Under optimum laboratory conditions the conversion of EBDCs to ETU can be quite efficient (Marshall, 1977; Watts et al., 1974; Newsome and Laver, 1973). ETU has also been demonstrated to form from EBDC field residues during normal food processing techniques (Baron, 1976). During a search for chemical agents which would oxidatively inactivate these residues it was observed that aqueous hypochlorite reacted rapidly and completely with ETU and with nabam (disodium ethylenebisdithiocarbamate). As a prelude to a detailed study of the action of hypochlorite on EBDCs it was deemed necessary to study the oxidation of ETU in some depth. This paper details the mechanism of oxidation of ETU with hypochlorite and with hydrogen peroxide and describes the synthesis of intermediates on the oxidative pathway.

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EXPERIMENTAL SECTION

Materials. Jaffe's base [1-(2′-imidazolin-2′-yl)-2-imidazolidinethione] (III) was synthesized according to the method of Johnson and Edens (1942). Chromatography of the base on silica gel using methanol–water (4:1) as eluent resulted in a colorless crystalline product which was recrystallized from ethanol (mp 236–237 °C). ETU disulfide [bis(2-imidazolin-2-yl) disulfide] (IV) was prepared as the dihydrochloride according to the method of Freedman and Corwin (1949) (mp 231–234 °C). ETU (2-imidazolidinethione) and EU (2-imidazolidineone) were purchased from Fisher Scientific Co. ETU was recrys-