Preparation of Aflatoxins and Determination of Their Ultraviolet and Fluorescent Characteristics

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Cultures of *Aspergillus flavus* grown on sterilized shredded wheat were extracted with chloroform. The crude mixtures of aflatoxins were precipitated with petroleum ether and partially purified by use of a silica gel cleanup column. Highly pure aflatoxins B_1 and G_1 were isolated by liquid-partition column chromatography followed by recrystallization of appropriate fractions from chloroform–petroleum ether and chloroform–methanol. Aflatoxins B_2 and G_2 were obtained by hydrogenation of aflatoxins B_1 and G_1, respectively. Ultraviolet spectra were determined for the crystalline aflatoxins in methanol and in acetonitrile; the major differences between the two solvents were that in acetonitrile, the cutoff point was lower, the first maximum occurred at lower wavelengths, and the molar absorptivity of this maximum was lower. The relative fluorescent intensity was determined for the four aflatoxins in methanol, 95% ethanol, and chloroform. The order of relative fluorescent intensity was the same only for the two alcohols, and even in these solvents the ratios differed.

Numerous laboratories have been concerned with the preparation of aflatoxins, the highly toxic metabolites of *Aspergillus flavus*. Various investigators have produced them in high yields on several types of substrates, including crushed wheat, rice, corn meal, peanuts, cottonseed, and liquid media (Armbrecht *et al.*, 1963; Codner *et al.*, 1963; Davis *et al.*, 1966; Mayne *et al.*, 1966; Merwe *et al.*, 1963; Shotwell *et al.*, 1966). Sargeant and Carnaghan (1963a) isolated 20 mg. of a mixture of crystalline aflatoxins from 30 kg. of toxic groundnut meal. Later, they obtained crystalline aflatoxin in larger quantities by growing a toxigenic strain of *A. flavus* on sterile groundnuts (1963b). Hartley *et al.* (1963) isolated crude aflatoxin both from cultured groundnuts and from various liquid synthetic media that had been inoculated with *A. flavus*. The crude aflatoxin was fractionated by column chromatography. Aflatoxin B_1 was recrystallized from trichloroethylene–chloroform, B_2 and G_1 from methanol, and G_2 from ethanol. Van der Zijden *et al.* (1962) isolated aflatoxin B_1 in crystalline form from cultures of *A. flavus* grown on sterile groundnuts. Asao *et al.* (1965) isolated aflatoxin B_1 and G_1 from extracts of *A. flavus* cultures grown on crushed wheat. The toxins were precipitated from chloroform extracts by addition of petroleum ether, the crude mixtures separated by TLC, and aflatoxins B and G crystallized from chloroform–methanol. Aflatoxins B_2 and G_2 have been produced by catalytic hydrogenation of aflatoxin B_1 and G_1 (Chang *et al.*, 1963; Dorp *et al.*, 1963; Merwe *et al.*, 1963). Recently aflatoxins M_1 and M_2 have been isolated from the urine of sheep fed mixed aflatoxins and their structure elucidated (Holzapfel *et al.*, 1966).

Although considerable quantities of aflatoxins are needed for standards and for experimental purposes, few specific details of procedures for their isolation and crystallization have been reported in the literature. The present paper is concerned with the stepwise procedure for the isolation and crystallization of aflatoxins B_1, B_2, G_1, and G_2 and a comparison of their ultraviolet spectra and relative fluorescence.

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MATERIALS AND APPARATUS

The Merck silica gel used for column chromatography was dried by heating overnight in $^1/_2$-inch layers at 110° C.

Glass columns (400 × 24 mm. I.D.), fitted with a fritted-glass plate at the bottom and a 2000-ml. reservoir at the top, were used for column chromatography.

Ultraviolet absorption spectra were determined with a Beckman Model DK-2A spectrophotometer. Molar absorptivities reported here were determined in either methanol or acetonitrile solvents at concentrations of 10 μg. per ml. (1-cm. cell) or 100 μg. per ml. (0.1-cm. cell). Fluorescence spectra were determined with the Baird-Atomic fluorescence spectrophotometer.

The following strains of *A. flavus* were used to produce aflatoxins: *A. flavus* British strain 3734/10 obtained from the Food and Drug Administration, Washington, D.C., labeled M-3; and *A. flavus* strain SU-15 isolated in this laboratory from peanuts.

Safety. Since aflatoxins have been shown to be toxic substances and may also be present in mold spores, precautions were taken during the isolation and crystallization steps to avoid contaminating the operator and the laboratory with aflatoxins or with airborne spores (Fischbach and Campbell, 1965; Hesseltine *et al.*, 1966).

PREPARATION OF AFLATOXINS B_1 AND G_1

Production and Extraction. The procedure of Armbrecht *et al.* (1963), slightly modified, was used for the production and the extraction of the aflatoxins. In typical fermentations, the highly toxigenic *A. flavus* strains were grown on 150 grams of sterilized shredded wheat, wetted with 75 ml. of tap water, in a 4-liter Fernbach flask for 7 to 9 days at 30° C. The aflatoxins were extracted and the mold spores killed by refluxing with 750 ml. of chloroform on a steam bath for 10 minutes. After cooling to room temperature, the extract was filtered successively through a double thickness of Whatman No. 1 filter paper

and through 400 grams of anhydrous sodium sulfate in a coarse fritted-glass funnel. The extraction was repeated twice with 750-ml. portions of chloroform. After the extracts were combined, the chloroform was evaporated to approximately 10 ml. on a steam bath under a stream of nitrogen. Then 20 volumes of petroleum ether (b.p. 30° to 60° C.) were added slowly and were stirred vigorously to precipitate the aflatoxins. The mixture was cooled to 5° C., and the precipitate was filtered on a small Büchner funnel. From 150 grams of shredded wheat, approximately 500 mg. of crude aflatoxin was obtained (50 to 60% purity by ultraviolet absorptivity).

Preliminary Column Cleanup. Seventy grams of Merck silica gel (0.05 to 0.20 mm.) was added dry to the glass column, which was tapped with a rubber mallet to settle the silica gel, and capped with 25 grams of anhydrous sodium sulfate. Crude aflatoxin powder (400 to 500 mg.) was dissolved in approximately 10 ml. of chloroform and pipetted onto the column. The column was eluted with 500 to 600 ml. of ethyl ether to remove a bright yellow-colored material from the column. This fraction, which normally does not contain aflatoxin, was discarded. The aflatoxins were eluted in one band with approximately 600 ml. of 3% methanol in chloroform. The movement of the toxins was followed by intermittent inspection of the column with a hand model long-wave ultraviolet lamp. When the blue fluorescent band of aflatoxin had almost reached the bottom of the column, collection of fractions was begun and was continued until most of the aflatoxin had eluted. The volume of the first fraction was 300 ml. and of the following fractions, 100 ml. The combined fractions were concentrated with a rotary evaporator, transferred with chloroform into a tared beaker, and dried on a steam bath under a stream of nitrogen.

Partition Chromatography of Purified Aflatoxins. Initially, the chromatographic method for the separation of crude aflatoxin mixtures employed activated silica gel or silicic acid as the adsorbent and 0.25% methanol in chloroform (Hartley *et al.*, 1963) or 1.0% ethanol in chloroform (Zijden *et al.*, 1962) as the elution solvent. With these columns, resolution of aflatoxin G_1 was poor, the aflatoxin B_1 fractions were often highly contaminated with non-aflatoxin impurities, and tailing of the aflatoxins was excessive. These problems were largely overcome by fractionating the partially purified aflatoxins by a slightly modified version of the liquid-partition chromatographic procedure developed by Frankel *et al.* (1961) for the analysis of lipids. Sixty-five grams of Merck silica gel (less than 0.08 mm.) and 52 ml. of 20% methanol in benzene (immobile solvent) were mixed thoroughly with a mortar and pestle. The adsorbent was slurried with 1.75% methanol in benzene (mobile solvent), poured in two portions into a 24 × 400 mm. column, and packed to a constant height under approximately 3 pounds of nitrogen pressure. Approximately 10 ml. of the slurried mixture was retained to be added to the column after the sample was added.

Since the purified aflatoxins were not very soluble in the mobile solvent, 250 mg. of a partially purified aflatoxin mixture (about 60% pure) was dissolved in about 2 ml. of chloroform and added to the column in 1-ml. portions after the solvent layer was adjusted to just below the surface of the adsorbent. After all the sample had been added, the remainder of the slurried adsorbent was carefully added and was rinsed with two 10-ml. portions of 1.75% methanol in benzene. The column was then eluted with mobile solvent at a flow rate of 80 ml. per hour under approximately 3 pounds of nitrogen pressure, and 20-ml. fractions were collected.

Five-microliter aliquots of the fractions were then spotted on a TLC plate along with a standard containing all four aflatoxins. The TLC plates were prepared as previously reported (Robertson *et al.*, 1965) and were developed in chloroform–acetone (85 to 15 v./v.) in unlined and unequilibrated chambers (Pons *et al.*, 1966). The aflatoxin content of selected fractions was determined by visual comparison of the fluorescent intensity of each individual aflatoxin with that of the aflatoxin standards. Based on the TLC results, appropriate fractions were combined, and the solvent was removed with a rotary evaporator.

Yields of individual aflatoxins in pooled fractions from the chromatographic separation are listed in Table I. At this stage of the preparation, the combined aflatoxin B_1 fractions (36 through 42), and the aflatoxin G_1 fractions (58 through 72), were 66 and 77% pure, respectively, on the basis of dry weight. Approximately 91% of aflatoxin B_1 and 94% of aflatoxin G_1 were eluted free of the other toxins and very little tailing of the aflatoxins was encountered (Figure 1). Recoveries of 90% or greater were usually obtained from silicic acid columns.

It was necessary to rechromatograph the fractions containing either B_1 or G_1 when a high concentration of impurities was present. Some of these impurities can be removed on another partition column or on an activated silica gel column eluted with chloroform containing approximately 0.5% ethanol as a preservative. However, most of these materials were removed in the first crystallization with chloroform–petroleum ether.

The aflatoxin B_1 and G_1 fractions that contained low concentrations of B_2 and G_2 were rechromatographed on another partition column to obtain additional quantities of B_1 and G_1 for recrystallization.

Crystallization. Purified aflatoxin B_1 or G_1 from the silica gel column was dissolved in a minimum amount of chloroform necessary to achieve solution, approximately 1 ml. per 25 mg., and filtered through a fine fritted-glass

Table I. Chromatographic Separation of Partially Purified Aflatoxins on a Silica Gel Liquid-Partition Column

Fraction[a] No.	Weight,[b] Mg.	Milligrams of Aflatoxin			
		B_1	B_2	G_1	G_2
1–35	...	Nd[c]	Nd	Nd	Nd
36–42	34.9	23.00	Nd	Nd	Nd
43–54	21.5	2.30	1.23	Nd	Nd
55–57	12.7	Nd	0.02	4.65	Nd
58–72	135.5	Nd	Nd	104.00	Nd
73–81	9.0	Nd	Nd	1.75	2.60
82–110	23.3	Nd	Nd	Trace[d]	4.00
Total	...	25.30	1.25	110.40	6.60

[a] Twenty-milliliter fractions.
[b] Dry weight of combined fractions.
[c] None detected.
[d] Less than 0.01 mg. per fraction.

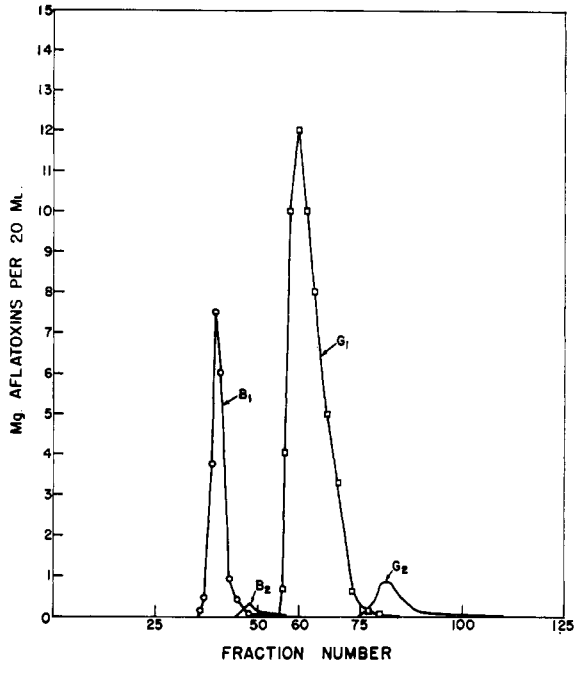

Figure 1. Chromatographic separation of partially purified aflatoxin on a silica gel liquid-partition column

funnel; the filter was washed with chloroform. The solvent was evaporated on a steam bath under a stream of nitrogen until crystals began forming on the sides of the container. Then petroleum ether (b.p. 30° to 60° C.) or *n*-hexane was added dropwise to incipient turbidity. Crystallization was induced by allowing the solution to stand overnight at room temperature. The crystals were collected by decanting the mother liquor through a fine fritted-glass funnel under nitrogen pressure to effect rapid filtration. The aflatoxin crystals again were dissolved in a small volume of chloroform on a steam bath, then the solution was concentrated under a stream of nitrogen as before. For the second crystallization, methanol was added dropwise until the cloud point was reached. The solution was allowed to stand overnight at room temperature for the crystals to develop. Recrystallization from chloroform–methanol was repeated four to six more times. After air drying in the dark for a few hours, the crystals were transferred to a tared vial and dried in an Abderhalden apparatus at 78° C. under reduced pressure. The crystals were considered pure when the molar absorptivity became constant and the material exhibited a single spot when analyzed by TLC.

PREPARATION OF AFLATOXINS B_2 AND G_2

Because most strains of *A. flavus* produce a low concentration of aflatoxins B_2 and G_2, these two aflatoxins were prepared by catalytic hydrogenation of the respective B_1 and G_1 compounds.

Fifty milligrams of aflatoxin B_1 in 30 ml. of 1,4-dioxane was hydrogenated in the apparatus described by Pack *et al.* (1952) with 150 mg. of 5% palladium on calcium carbonate at room temperature. The hydrogenation was stopped after 30 minutes, the catalyst removed by filtration through a fine fritted-glass funnel, and the filter cake washed with chloroform. The crude reduction product was then purified by column chromatography. The solution of hydrogenated aflatoxin B_1 was passed through a column containing 70 grams of Merck silica gel (0.05 to 0.20 mm.) and eluted with chloroform (0.70% ethanol as a preserva-

Table II. Ultraviolet Spectrophotometric Characteristics of the Aflatoxins in Different Solvents

	λ_{max}, mμ(ϵ_{max})		
Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2
199 (31,250)[a]	199 (24,000)[a]	203 (28,800)[a]	203 (24,900)[a]
197 (28,900)[b]	200 (20,300)[b]	201 (28,050)[b]	200 (27,300)[b]
223 (24,550)[a]	222 (20,400)[a]	216 (28,750)[a]	214 (28,600)[a]
223 (21,800)[b]	222 (17,000)[b]	216 (27,100)[b]	214 (28,100)[b]
...	...	242 (8,400)[a]	243 (10,200)[a]
		242 (9,800)[b]	244 (11,600)[b]
...	...	256 (8,500)[a]	256 (8,200)[a]
		257 (9,200)[b]	257 (9,000)[b]
264 (12,500)[a]	264 (12,400)[a]	264 (9,200)[a]	264 (9,200)[a]
265 (12,450)[b]	265 (12,200)[b]	265 (10,000)[b]	265 (9,700)[b]
355 (20,600)[a]	358 (22,500)[a]	356 (17,800)[a]	358 (20,700)[a]
360 (21,800)[b]	362 (23,800)[b]	360 (18,900)[b]	362 (20,900)[b]

[a] Acetonitrile.
[b] Methanol.

Table III. Fluorescence of the Aflatoxins in Different Solvents

	Excitation Wavelength, mμ	Emission Wavelength, mμ			KQ[a]		
Aflatoxin		Methanol	Ethanol	Chloroform	Methanol	Ethanol, 95%	Chloroform
B_1	365	430	430	413	0.6	1.0	0.2
B_2	365	430	430	413	5.3	2.7	0.25
G_1	365	450	450	430	1.0	1.4	6.2
G_2	365	450	450	430	8.7	4.7	6.8

[a] $KQ = \frac{\text{Fluorescent intensity of aflatoxin solution}}{\text{Fluorescent intensity of standard quinine sulfate solution (1 } \mu\text{g./ml.)} \times \text{concentration of aflatoxin solutions (0.2 } \mu\text{g./ml.)}}$.

tive). After a forerun of 800 ml. of chloroform, the main band containing B_2 emerged in the next 600 ml. When this was evaporated to dryness with a rotary evaporator, yellow aflatoxin B_2 crystals were formed. These were recrystallized from chloroform–petroleum ether (b.p. 30° to 60° C.) once and from chloroform–ethanol, usually twice, until the molar absorptivity became constant.

Aflatoxin G_2 was prepared in similar manner except that it was eluted from the silica gel column in 900 ml. of chloroform after a forerun of 1350 ml. of chloroform.

ULTRAVIOLET AND FLUORESCENT CHARACTERISTICS

The crystalline aflatoxins thus isolated had the ultraviolet spectrophotometric characteristics shown in Table II. The ultraviolet maxima between 197 and 203 mμ have not been reported previously. It was difficult to determine the 197- to 203-mμ absorption maxima of the aflatoxins in methanol, which has a cutoff point at approximately 192 mμ; however, acetonitrile was a good solvent for the aflatoxins and was usable to approximately 182 mμ (cutoff point). In methanol, the first peak was at 360 to 362 mμ; however, in acetonitrile it shifted to 355 to 358 mμ. The spectra of the aflatoxins in the two solvents were similar except for minor differences in position of maximum absorption, especially at the longer wavelengths, and in molar absorptivity.

Both the ultraviolet absorption maxima and the molar absorptivities of the crystalline aflatoxins in methanol agree well with those reported in the literature for methanol and ethanol (Asao *et al.*, 1965; Chang *et al.*, 1963; Dorp *et al.*, 1963; Hartley, *et al.*, 1963; Merwe *et al.*, 1963).

The excitation and emission maxima and relative fluorescence (KQ) of the aflatoxins in different solvents were determined as described by Carnaghan *et al.* (1963) and are shown in Table III. The wavelength of the emission maxima was about 20 mμ shorter in chloroform than in the alcohols. Aflatoxin G_2 was the most fluorescent of the toxins in all three solvents. The emission maxima in the alcohols were similar to the emission maxima of 425 mμ for B_1 and B_2 and 450 mμ for G_1 and G_2 reported in the literature for aflatoxins in methanol (Carnaghan *et al.*, 1963).

In both methanol and ethanol, the relative fluorescent intensity was in the order $G_2 > B_2 > G_1 > B_1$ with ratios of 14.5:8.8:1.7:1.0, and 4.7:2.7:1.4:1.0, respectively. Carnaghan *et al.* (1963) reported the same order for the aflatoxins in methanol, but the corresponding ratios were 13.0:8.0:5.0:1.0. In chloroform solution, however, the relative fluorescent intensity was in the order $G_2 > G_1 > B_2 > B_1$ with ratios of 34.0:31.0:1.3:1.0. Both of these orders differ from the relative response of $B_2 > G_2 > B_1 > G_1$ with ratios of 3.3:3.0:1.4:1.0 previously reported from this laboratory for the fluorescent of solid state aflatoxins adsorbed on silica gel (Pons *et al.*, 1966). These striking differences in the fluoresence of the aflatoxins in the same solvent, in different solvents, and in the solid state emphasize the problem of relating fluorescence to concentration and toxicity.

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