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Aflatoxin Formation by *Aspergillus flavus*

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INTRODUCTION

The occurrence of mycotoxins has been known for several decades. In 1913, Alsberg and Black (5), working in the U.S. Department of Agriculture, studied the biochemistry of toxins of certain molds isolated from corn meal. *Penicillium puberulum* Bainier was found to produce penicillic acid, with the molecular formula of $C_8H_{10}O_4$. When this material was injected subcutaneously into mice at a dosage of 0.2 to 0.3 g per kg of body weight, death resulted. More recently, a great deal of interest has been aroused by the discovery of toxins, which have been named aflatoxins, produced by certain strains of *Aspergillus flavus* Link, *A. parasiticus* Speare, and *P. puberulum* Bainier (22). At least four aflatoxins are known to exist, and structures of B_1, B_2, G_1, and G_2 have been determined (8, 20). Interest in this family of fluorescent mycotoxins arose after the death of a large number of young turkeys in Great Britain in 1960 (36); the toxin was produced by *A. flavus* in a peanut meal incorporated into turkey feed. It also has been demonstrated that aflatoxins, when fed to rats, cause a high incidence of hepatomas and renal damage (30). Furthermore, aflatoxins have been responsible for hepatomas in young trout fed a diet containing cottonseed meal (2).

FUNGI PRODUCING AFLATOXIN AND OTHER TOXINS OF *A. FLAVUS*

Three species of fungi have been reported to produce aflatoxin: *A. flavus*, *P. puberulum*, and the most active aflatoxin producers which are members of the species *A. parasiticus* (10).

The only study on the production of aflatoxin by microorganisms other than those of the genus *Aspergillus* is that of Hodges et al. (22). They found a strain of *P. puberulum*, isolated from moldy peanuts, which formed aflatoxin when cultivated on potato plugs, moist shredded wheat, Sabouraud agar, or potato dextrose agar. In addition to the production of aflatoxins, *A. flavus* strains also produce kojic acid, β-nitropropionic acid, penicillin F (flavicidin, flavicin), flavacol, aspergillic acid, grangegillin, and hydroxyaspergillic acid (26).

Tilden et al. (37) studied strains of *A. flavus* and *A. fumigatus* isolated from Humboldt penguins suffering from aspergillosis. These strains produced endotoxins (found in the mycelium, but only in small amounts in broth). The endotoxins from both species were nephrotoxins, but only the one from *A. fumigatus* also had strong hemolytic properties and marked dermonecrotic activity on rabbit skin. Cultures of these strains were grown on Sabouraud or potato dextrose agar, and spore suspensions were used to inoculate a variety of media, including peptone-beef extract-glucose, glucose-ammonium nitrate medium, and modifications thereof. With *A. fumigatus*, the addition of yeast extract or beef extract increased yields of toxin 20-fold. However, the best medium proved to be a corn steep medium developed for penicillin production, but with glucose substituted for

lactose and adjusted to a *p*H of 7.6. Inorganic salts are also important in the production of endotoxin. Incubation at 25 C was better than that at 37 C. Optimal incubation time for production of nephrotoxin by *A. flavus* varied from 3 to 5 days at 25 C. Toxin was not formed in the quantities produced by *A. fumigatus*. Forgacs (18) reviewed the literature on outbreaks of disease due to mycotoxins in swine foraging on moldy corn in a field in Georgia.

Since *A. oryzae* is a close relative of *A. flavus* (they are distinguished on the basis of minor morphological characteristics), note should be taken of the work of Iizuka and Iida (23), who identified a toxin, maltoryzine, produced by a strain of *A. oryzae* var. *microsporus*. This toxin was found in malt sprouts supplied as food to dairy cattle. The mycotoxin was produced in tank culture on Czapek Dox medium supplemented with malt sprout extract. The fermentation was carried out at 30 C for 2 to 5 days, with yields of 300 mg of crystalline toxin from 150 liters of broth.

NATURAL OCCURRENCE OF AFLATOXIN

Aflatoxin has been found in relatively few commodities. Originally it was identified in turkey feed which contained peanut meal (24). At present, only two commodities are known in which aflatoxin was detected in an article of commerce. Sargeant et al. (31) first reported that the toxic properties of certain samples of peanuts were due to metabolic products of the fungus *A. flavus*. The identification of the fungus was made by J. J. Elphick of the Commonwealth Mycological Institute. Aflatoxin occurs sometimes naturally in cottonseed cake at a level of above 0.5 ppm, as determined by the duckling test (25). On the basis of the world-wide distribution of *A. flavus*, we predict that aflatoxin will be found naturally in other commodities as well. Laboratory studies have shown that growth can occur on any agricultural commodity, provided that temperature, moisture, and aeration are adequate.

The question regarding the fate of aflatoxin ingested by farm animals in milk, meat, and eggs has been inadequately explored, although a toxic substance has been found in milk of cows fed strongly toxic groundnut (peanut) meal (1).

FACTORS AFFECTING AFLATOXIN FORMATION IN NATURE

The most detailed accounts of the conditions and factors affecting growth and formation of aflatoxin by *A. flavus* are those of Austwick and Ayerst (9), Spensley (36), and a working group (1). All agree that invasion of the kernels of peanuts usually occurs during curing and after removal from the soil. Austwick and Ayerst state that, of all the samples of peanuts examined, in only one did *A. flavus* grow prior to curing. According to one report (1), nuts may become toxic while in the ground, especially if left after maturity or if the shells are damaged. Bampton (11) considered damage to shells to be a major contributing factor in aflatoxin formation by *A. flavus*. The two most important factors governing growth of *A. flavus* in peanuts, or in other agricultural commodities, are temperature and moisture. *A. flavus* grows at 30 C and at a relative humidity of 80 to 85% (36).

Usually, only a few kernels in a lot contain aflatoxin. Austwick and Ayerst (9) found that less than 3% of peanut kernels had toxin, but the amount present was sufficient to cause the whole sample to be toxic.

The amount of toxin produced by different strains of *A. flavus* varies appreciably; a few strains examined do not produce aflatoxin. As mentioned below, at the Northern Regional Research Laboratory, we have attempted many times to detect aflatoxin in *A. flavus* NRRL 482 without success.

PRODUCTION OF AFLATOXIN IN CULTURE

Considerable quantities of aflatoxin are necessary for studies on its chemical characteristics and degradation products. Even more aflatoxin would be required to determine the effect of both high and low levels on laboratory and farm animals. The fate of each of the aflatoxins needs to be studied in animals and their tissues, particularly those destined for human consumption. Since aflatoxin is a very potent carcinogen, this effect requires special investigation. It is necessary to produce quantities of the material, and, in doing so, optimal conditions for aflatoxin formation have to be elucidated. These data could be applicable to studies on the prevention of aflatoxin formation in natural products.

Sargeant et al. (31) grew cultures for 7 days at 27 C on Czapek solution agar, and a toxic substance similar to that from toxic peanuts was isolated. Later, they grew the aflatoxin-producing fungus on heat-sterilized, nontoxic groundnuts (peanuts). After 7 days of incubation at room temperature, extracts prepared from the moldy nuts showed the same blue fluorescent material, and proved to be lethal to 1-day-old ducklings. In a later paper, Sargeant et al. (32) reported that material grown under these conditions was 10 times more toxic than any naturally occurring peanut meals; 100 mg of aflatoxin per kg was

consistently obtained. No satisfactory surface or submerged fermentation with either synthetic or complex media has been developed. Other investigators have used sterilized peanuts inoculated with *A. flavus* to produce aflatoxin (27). Diener et al. (17) used autoclaved, shelled, and sterilized peanuts, but allowed the fungus to grow for 3 weeks.

Aflatoxin was produced by growing *A. flavus* on well-crushed sterile peanuts. From this material, crystalline aflatoxin was extracted with methanol by Van der Zijden et al. (39) and by De Iongh et al. (16). Newberne et al. (29) prepared aflatoxin by growing *A. flavus* on shelled crushed peanuts and ground whole wheat. The inoculated material was held in open trays in a closed incubator for 7 days at 30 C. The volume of water was 10% of the weight of the substrate fermented.

Other investigators have used either crushed or shredded wheat. Schumaier et al. (33) grew the fungus on wheat or ground wheat and allowed the material to incubate at room temperature for 15 days. Armbrecht and Fitzhugh (7) likewise used sterilized, moist wheat as a substrate. Their material was incubated for 5 to 7 days, during which time a very heavy mat formed. During sporulation, fluorescence increased. Bixler and Lopez (12) grew *A. flavus* on wheat grains, fed these to chickens, and noted weight loss, poor appetite, and liver damage. Crushed, sterilized wheat was used to produce aflatoxin by Asao et al. (8) and by Chang et al. (13). The latter workers report the production of aflatoxin B_2 by culturing *A. flavus* for 7 days on sterile, cracked wheat incubated at 30 C. The isolated B_2 was produced in very small amounts along with B_1 and G_1.

Newberne et al. (30) prepared aflatoxin by growing *A. flavus* on coarsely ground wheat. Ground wheat (250 g) was soaked in distilled water for 1 hr in 3-liter Fernbach flasks, and the substrate had a 30 to 40% moisture content. Fermentation was carried out for 7 days at 30 C.

At Pretoria, Van der Merwe et al. (38) found *A. flavus* in maize meal, and consequently grew this fungus on sterilized maize meal. Crystalline aflatoxin was obtained by extraction with methanol-chloroform, followed by partition chromatography of the extract.

Armbrecht et al. (6) reported in some detail methods for producing mycotoxins from *A. flavus*. They used the following as fermentation substrates: wheat, corn, oats, rye, buckwheat, rice, soybeans, and peanuts broken into coarse pieces by grinding. The pieces were soaked in water for 1 hr and sterilized. They also used Czapek solution medium and agar with and without the addition of 0.1 g of zinc sulfate per liter, and a glucose-ammonium nitrate medium. Cultures were grown at 25 to 27 C for 5 to 10 days, and the cultures in Fernbach flasks were killed with chloroform. Nesbitt et al. (27) likewise used a Czapek Dox medium to which zinc sulfate was added to increase the yield of aflatoxin.

Among the molds isolated from *Cycas circinalis*, Forgacs and Carll (19) found the most toxic to be *A. flavus*. Cultures were grown for 28 days at 24 C in petri dishes on Czapek solution-agar or for only 13 days on Mycophil agar. The entire minced culture was then fed to mice.

Newberne et al. (29) studied submerged fermentations of *A. flavus* cultures in 100-liter fermentors containing Czapek solution supplemented with 0.1% corn steep liquor. The fermentation was carried out for 6 days at 30 C with both aeration and agitation.

Other synthetic media have been used to produce aflatoxin. For studies of the incorporation of labeled compounds into aflatoxin, Adye and Mateles (4) used a medium composed of the following: glucose, 50 g; $(NH_4)_2SO_4$, 3.0 g; KH_2PO_4, 10.0 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; $Na_2B_4O_7 \cdot 10H_2O$, 0.7 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.5 mg; $Fe_2(SO_4)_3 \cdot 6H_2O$, 10.0 mg; $CuSO_4 \cdot 5H_2O$, 0.3 mg; $MnSO_4 \cdot H_2O$, 0.11 mg; and $ZnSO_4 \cdot 7H_2O$, 17.6 mg. In a second paper, these authors (3) report yields of 25 to 100 mg per liter of aflatoxin in 2 to 4 days. They found that optimal production occurred at *p*H 3, and that none was produced when the *p*H was held at 4. With uncontrolled fermentation, the *p*H dropped to 2, resulting in lower yields of aflatoxin. The best carbon sources were glucose, sucrose, and fructose, whereas little or no aflatoxin was produced with lactose, galactose, maltose, xylose, sorbose, sorbitol, mannitol, glycerol, acetate, succinate, and malate.

Smith and McKernan (35) grew *A. flavus* in penicillin flasks on a glucose-ammonium nitrate medium at 27 C for 9 days. This medium was also used by De Iongh et al. (16).

Probably the most important paper on the production of aflatoxin is the one published by Codner et al. (14), who first used a Czapek Dox medium containing corn steep liquor. They reported yields of 100 to 200 mg per liter in 250-ml flasks on a rotary shaker in 3 days. However, when the same medium and culture were used in 3- to 20-liter, stirred, aerated fermentors, no aflatoxin was produced. Consequently, they chose to produce aflatoxin in 250-ml flasks containing 75 g of pale-skinned peanuts and 18 ml of water. Inoculum was grown on Czapek Dox agar slants incubated at 30 C for 7 days. After inoculation,

flasks were incubated at 30 C for 10 to 13 days. Each flask was shaken for a few seconds every day. When peanuts were packed in aerated columns or in conical flasks, poor yields of aflatoxin resulted. Five strains of *A. flavus* and one strain of *A. parasiticus* were grown under these conditions. The highest yields (265 mg/kg) were obtained from *A. parasiticus*. All of the strains tested produced all four aflatoxins, except the poorest strain which produced only B_1 and B_2. Interestingly, the relative proportions of the four aflatoxins produced on sterilized peanuts by a particular strain were the same as those found in unsterile peanuts from which the strain had been isolated.

During the past few years, a large number of papers dealing with aflatoxin were published, Shau et al. (34) assembling no less than 188 references for the years 1960 to 1964.

The problem of mycotoxins, including aflatoxins, has been reviewed by Forgacs (18). In addition to *A. flavus* toxins, he discusses the mycotoxins produced by *Stachybotrys atra*, *A. chevalieri*, *A. clavatus*, *A. fumigatus*, *Penicillium rubrum*, *Fusarium sporotrichioides*, and *Pithomyces chartarum* (*Sporodesmium bakeri*).

AFLATOXIN STUDIES AT THE NORTHERN REGIONAL RESEARCH LABORATORY

Our objective is to develop a method of producing large quantities of aflatoxin for testing in larger animals, to discover the conditions under which aflatoxin is formed, to study its effect on various microorganisms, and to determine to what extent agricultural substrates would permit its formation. Codner et al. (14) have described the production of aflatoxin on sterilized peanuts with a yield of 265 mg of toxin per kg of peanuts.

Methods

All cultures of *Aspergillus* used were from the ARS Culture Collection and came from the sources shown in Table 1.

For seeding purposes, the strains were grown on potato dextrose agar (21) and incubated at 25 C for at least 5 days, but no more than 3 weeks, before use. To the slants was added 3 to 5 ml of sterile tap water, and the surface was gently rubbed to release the spores, which were used as inoculum. The spores readily floated to the surface of the liquid. For aflatoxin production, liquid potato dextrose medium (as above, without agar) was used.

Fluorescence of materials in solution was measured with a Turner fluorometer, model 110 (3× range, primary filter 7–60, secondary filters, 2A + 2ND; G. K. Turner Associates, Palo Alto,

TABLE 1. *Sources of Aspergillus cultures used*[a]

NRRL no.	Source
482	Centraalbureau voor Schimmelcultures to Thom, 1909
502	Type from Speare, Honolulu, 1913, isolated as a parasite upon the mealy bug of sugar cane
2999	CMI[b] 91019b from peanuts, Uganda, 1961; Austwick strain V. 3734/10
3000	CMI 89717 from peanut seeds, Uganda, 1961; Tropical Products Institute T-12
A-4018b	Forgacs as his No. P1 from toxic bran, in 1952
A-11,606*A*	CMI 911546 from Uganda peanuts, 1962; Austwick's V. 3734/14
A-11,607	CMI 91547 from Uganda peanuts, 1962; Austwick's V. 3734/15
A-11,608	CMI 91548 from Brazilian peanuts, 1962; Austwick's V. 3827/30
A-11,609	CMI 91549 from Brazilian peanuts, 1962; Austwick's V. 3827/31
A-11,610	CMI 91550 from Brazilian peanuts, 1962; Austwick's V. 3827/32
A-11,611	CMI 91552 from Nigerian peanuts, 1962; Austwick's V. 4030/6
A-11,612	CMI 93070 from Nigerian peanuts 1962; Austwick's V. 4104/6
A-11,613	CMI 93080 from South African peanuts, 1962; Austwick's V. 4065/4
A-12,267	F. A. Hodges, Food and Drug Administration M 25 from moldy peanuts
A-12,268	F. A. Hodges, Food and Drug Administration M 26 from turkey feed mix
A-12,353	J. C. Lewis, Western Regional Research Laboratory, No. 6b from cottonseed hulls
A-12,589	F. A. Hodges, Food and Drug Administration as M 51 from peanuts
A-12,590	F. A. Hodges, Food and Drug Administration as M 52 from peanuts
A-12,591	F. A. Hodges, Food and Drug Administration as M 53 from peanuts
A-12,592	F. A. Hodges, Food and Drug Administration as M 54 from peanuts
A-12,593	F. A. Hodges, Food and Drug Administration as M 66 from peanuts

[a] All cultures used were *A. flavus*, except NRRL 502, which refers to *A. parasiticus*, and NRRL A-4018b, which refers to *A. flavus-oryzae* group.

[b] CMI = Commonwealth Mycological Institute.

TABLE 1.—*Continued*

NRRL no.	Source
A-13,071	J. P. Van der Walt, Pretoria, South Africa, 501 from maize meal
A-13,072	J. P. Van der Walt, Pretoria, South Africa, 500 from maize meal

Calif.). Before readings were made, the instrument was set to zero with a solvent blank.

Paper chromatograms were run in descending manner on Whatman no. 1 paper [7 by 18 inches (17.8 by 45.7 cm)]. Solutions were applied to papers with micropipettes. The two solvent systems used were as follows: (A) upper layer of 1-butanol–glacial acetic acid–deionized water (20:1:19) (16), and (B) benzene–toluene–cyclohexane–95% ethyl alcohol–water (3:3:5:8:3) containing 1% glacial acetic acid in upper phase (14). The R_F of aflatoxins in solvent A was 0.68 to 0.75. Solvent B separated aflatoxins G (R_F, 0.34 to 0.43) and B (R_F, 0.49 to 0.58). Zones containing aflatoxins were located by observing their fluorescence in a Chromato-Viewer (Ultra-Violet Products, Inc., San Gabriel, Calif.) with light 366 mμ in wavelength.

Thin-layer chromatoplates were run by use of the method of W. A. Pons, Jr. (*unpublished data*). Plates were coated with silica gel G-HR (Brinkmann Instruments, Inc., Westbury, N.Y.) 0.3 mm thick and developed with 3% methanol in chloroform (v/v). Plates were inspected in the Chromato-Viewer. The four aflatoxins were separated: B_1, R_F 0.47; B_2, R_F 0.43; G_1, R_F 0.38; and G_2, R_F 0.33.

Aflatoxin production in still culture. The inoculum consisted of 0.5 ml of a heavy spore suspension (5 ml of sterile tap water added to a 2- to 3-week-old slant) of *A. flavus* NRRL 2999 and NRRL 3000. The following Fernbach flasks containing 500 ml of media were inoculated with each strain: four flasks of potato dextrose broth (*p*H 6.7), two of Czapek solution (*p*H 6.9), and two of Czapek solution plus 0.5% wheat gluten (*p*H 6.9). Uninoculated flasks of each medium served as controls. Flasks were incubated as still cultures at 28 C. By the 4th day, all flasks with potato broth had excellent growth, solid mats, and uniform green sporulation over the surface of the mat. On the other two media, poor growth resulted, and no sporulation had occurred, although the Czapek solution broth plus wheat gluten showed slightly more growth. This growth became much more apparent as the cultures aged, but sporulation never became as extensive as on the potato dextrose medium. Samples were removed at 4 and 7 days and extracted with equal volumes of chloroform to determine fluorescent materials present. By the 7th day, sporulation had occurred on both Czapek solution media, and there was a cottony type of growth toward the bottom of the liquid. After incubation for 10 days, the cultures were harvested.

All the flasks of each medium inoculated with the same organism were combined, and the mycelium was collected on coarse filter paper (520-B-1-1/2; Schleicher and Schuell Co., Keene, N.H.). Samples (10 ml) of the filtrates were extracted with chloroform, and fluorescence was measured on the Turner fluorometer. Filtrates from whole culture liquors, resulting from the growth of NRRL 2999 and NRRL 3000 grown on Czapek medium with and without 0.5% wheat gluten added, were discarded because very little fluorescent material was extracted.

The filtrate (1,690 ml) from the fermentation of NRRL 2999 on potato dextrose medium was concentrated in vacuo to 505 ml and extracted three times with 500-ml portions of chloroform. The crude product (136 mg) obtained by evaporation of chloroform from combined extracts was chromatographed on silicic acid (Mallinckrodt, St. Louis, Mo., analytical reagent, 100 mesh, $SiO_2 \cdot H_2O$) with 1% ethyl alcohol in chloroform. The product (56 mg) contained 42 mg of aflatoxin, as determined by paper chromatographic comparisons with standard aflatoxin B_1.

Similar yields were obtained when filtered culture liquors obtained by fermentation of NRRL 3000 on potato dextrose broth were extracted.

The mycelia from the filtrates were dried in vacuo. Dried mycelia were extracted with methanol, and the amount of fluorescent material was determined. Paper chromatographic comparison with known aflatoxin B_1 indicated that the mycelium of NRRL 2999 grown on potato dextrose medium contained 1 to 2 mg of B_1. Most of the fluorescent material extracted from mycelium was not aflatoxin.

Production of aflatoxin in spores. Strains of NRRL 2999 and NRRL 3000 were grown on potato dextrose slants, and 1.0 ml of spore suspension was used to inoculate the surface of potato dextrose agar (500 ml) in Fernbach flasks. This quantity of spores was sufficient to seed the entire surface of the agar. Two flasks were seeded with each strain and incubated at 28 C. After 11 days, all of the flasks contained an abundance of spores that could not be broken loose by tapping the flasks. NRRL 3000 showed uniform growth and sporulation, and the medium was slightly yellow. NRRL 2999 had areas of

white, tufted growth, and the medium was more or less tan. To harvest spores separate from conidiophores, 100 ml of distilled water was added to each flask and gently swirled. The water and suspended spores were poured immediately into clean flasks, and were steamed to prevent germination. The spores were washed with water and extracted with methanol to remove any aflatoxin. Paper chromatography showed that part of the fluorescence of spore extracts was caused by aflatoxin.

Survey of aflatoxin production in strains of Aspergillus. A total of 22 *Aspergillus* strains (*A. flavus* and *A. parasiticus*), all reported to produce aflatoxin, were grown in three 300-ml Erlenmeyer flasks, each containing 75 ml of potato dextrose broth. The flasks were placed on a reciprocal shaker and incubated at 28 C. All cultures grew well, and one flask of each strain was harvested on the 2nd, 5th, and 8th day, respectively. Differences in growth of the strains were noted in 5 days. Culture of strain NRRL 502 was deep grass-green and had very small pellets. Almost all of the other cultures showed a light-yellow pigment and bright-tan pellets, 1 to 2 mm in diameter. NRRL A-11,610 had much larger white pellets, and NRRL 482 had small white pellets. All cultures had sporulated on the edge of the flask.

The mycelia from each flask were extracted with 15 ml of methanol; the filtered culture liquors (10-ml sample) were extracted with chloroform (10 ml). Readings were taken on a Turner fluorometer with a machine setting at zero for both chloroform and methanol.

Survey of A. oryzae for aflatoxin production. Erlenmeyer flasks (300 ml) containing 75 ml of potato dextrose broth were inoculated with 53 strains of *A. oryzae* used in food fermentations. All the strains were isolated from commercially available starters from Japan used for the preparation of miso koji, except NRRL 1988 and NRRL 1989, which came from China and are used in making shoyu koji, and NRRL A-13,203 through A-13,206, which were obtained from Chinese black beans from Taiwan. The cultures were started from lyophilized preparations and transferred once before inoculation. A 5-ml amount of sterile tap water was used to wash the slant, and 0.5 ml of a heavy spore suspension was used to seed each flask. After inoculation, the flasks were placed on a reciprocal shaker and incubated at 28 C. *A. flavus* NRRL 3000, a good aflatoxin producer, was run as the control. At the end of 6 days, the flasks were harvested. The *p*H of broths was determined, and cultures were grouped according to color and colony height. Those strains of *A. oryzae* which sporulate lightly and have elongated conidiophores grew as white pellets and produced no pigment in the broth. The deep-greenish colored strains with heavy sporulation produced pigment in both broth and pellets. Interestingly, none of these broths showed any fluorescence. The remainder of the strains varied considerably in pigmentation of broth and pellets. Strain NRRL 3000 produced a reddish-brown pigment in broth and pellets that differed in appearance from that produced by all but a few *A. oryzae* strains. The *p*H values varied considerably, but only a few were as low as those of NRRL 3000. The broth was filtered, and a 10-ml sample of the filtrate was extracted with chloroform (10 ml).

Examination of food products prepared with A. oryzae and other fungi. The food products (5 g) and shoyu (10 ml) were extracted with chloroform (10 ml). Fluorescence of all extracts was measured on the Turner fluorometer, and all of the extracts were subjected to paper chromatography.

Aflatoxin formation in agricultural commodities. The three *A. flavus* strains shown to be the best aflatoxin producers were used in these experiments: NRRL 3000, NRRL 2999, and NRRL A-11,613. The following amounts of substrates and tap water were added to 300-ml Erlenmeyer flasks: 75 g of pearled soybeans (Hawkeye) and 25 ml of water; 100 g of pearled wheat (Conley) and 35 ml of water; 75 g of cracked corn and 50 ml of water; 100 g of cracked sorghum (yellow milo) and 35 ml of water; 100 g of peanuts (unroasted) broken into 6 to 10 pieces and 25 ml of water; and 50 g of polished rice and 25 ml of water. The flasks were allowed to stand for 2 hr with frequent mixing, and then were sterilized at 15 psi 20 min before inoculation. Amounts of water and substrate varied, because each commodity took up a different amount of water, and some commodities, e.g., rice, swelled when water was added. Since molds require oxygen for growth, the amount of substrate in each flask was limited to permit growth throughout the mass of material. Solid substrates were used to reproduce conditions which might be found on a farm or in an elevator.

To each of half of the flasks was added 1 ml of a 0.01% solution of sterile methionine. All flasks were inoculated with our three best aflatoxin-producing strains, and incubated at 28 C. Additional sterile water was added if the substrate appeared too dry. At least every day the flasks were shaken by hand to loosen any clumps of mold growth and to redistribute the moist grain from the bottom. Addition of water to

peanuts and soybeans was unnecessary. Growth on all substrates began promptly, and after 48 hr in most flasks there was sporulation in parts of the culture. NRRL 3000 sporulated on all substrates. After 6 days, the flasks were autoclaved.

Aflatoxins were extracted by mixing the fermented substrates (from 50 g) with 250 ml of methanol–water (55:45, v/v) in a Waring Blendor for 2 min (28). The resulting slurry was centrifuged for 30 min at 3,000 rev/min, and the supernatant fluid was filtered through paper. The filtrate was extracted with 100 ml of hexane in a separatory funnel to remove lipids. The hexane layer was washed once with 50 ml of methanol–water (55:45, v/v). The methanol layer and wash were combined and concentrated in vacuo to remove methanol. The concentrated aqueous solution was extracted once with 75 ml of chloroform and twice with 50-ml portions of chloroform. Combined extracts were concentrated almost to dryness in vacuo, transferred quantitatively with chloroform to 10-ml volumetric flasks, and made up to 10-ml volume with chloroform for thin-layer chromatography.

The experiment described above was repeated, but the Erlenmeyer flasks were placed on a rotating shaker in such a way that the grain was rotated and kept loose. In this instance, 50 g of each commodity was added to each flask, and tap water was added as follows: peanuts, 12 ml; sorghum, wheat, and rice, 15 ml; soybeans, 18 ml; and corn, 36 ml. Each culture was inoculated and incubated as before. In all instances, growth was excellent, even though the surface of the grain was kept from being covered with conidiophores. In some instances, balls of several kernels were bound together by the mycelium. The material was harvested on the 6th day.

Results and Discussion

Aflatoxins were successfully produced in still culture on potato dextrose medium (Table 2). Although Czapek solution has been recommended for the production of aflatoxin, very little was obtained on this medium, even when wheat gluten was added to it. Aflatoxin was isolated from filtered culture liquors, but small amounts were found in the mycelium. It should be noted that aflatoxin was detected in spores of *A. flavus* NRRL 2999 and NRRL 3000, a matter of importance to persons working with these organisms. Also, toxin may remain active after autoclaving, so that even heat-treated spores should be handled with caution.

Several of the *A. flavus* strains examined were especially good producers of aflatoxin: NRRL 2999, NRRL 3000, and NRRL A-11,613. Paper chromatographic studies of culture liquor and mycelium extracts of the following 14 NRRL strains did not reveal the presence of aflatoxin: 482, 502, A-4018b, A-11,607, A-11,608, A-11,610, A-11,612, A-12,267, A-12,268, A-12,353, A-12,590, A-11,591, A-13,071, and A-13,072. The results of studies on toxin-producing strains are summarized in Table 3. The mycelial extracts contained highly fluorescing substances, but these were not necessarily aflatoxin, as shown by paper chromatography, Aflatoxins B and G were detected in both mycelium and culture filtrates of NRRL 2999, NRRL 3000, and NRRL A-11,613. If one assumes that substances responsible for most of the fluorescence in culture

TABLE 2. *Production of aflatoxin in still culture (10 days)*

Medium	NRRL 2999					NRRL 3000				
	Filtrate		Mycelium			Filtrate		Mycelium		
	pH	Fluorometer reading[a]	Weight	Vol of CH_3OH used for extraction	Fluorometer reading	pH	Fluorometer reading	Weight	Vol of CH_3OH used for extraction	Fluorometer reading
			g	*ml*				*g*	*ml*	
Potato dextrose broth	3.3	>100	22.5	125	20	4.0	>100	21.5	125	12
Czapek solution	4.7	0	0.3	10	6	4.7	4	0.3	10	7
Czapek solution + wheat gluten	6.9	7	13.8	125	11	6.7	9	12.3	125	9

[a] Samples (10 ml) of culture filtrates were extracted with an equal volume of chloroform, and the fluorescence of the extracts was determined. Readings taken on a Turner fluorometer, model 110 (3× range, primary filter 7-60, secondary filters, 2A + 2ND). Before readings were made, the instrument was set to zero with a solvent blank.

TABLE 3. *Survey of aflatoxin-producing strains*

NRRL no.	2 days		5 days			8 days	
	*p*H	Fluorescence[a]	*p*H	Fluorescence	Amt of aflatoxin produced[b]	*p*H	Fluorescence
2999	3.6	20.0	2.8	37.5	+++	3.5	69.5
3000	3.7	31.0	3.1	51.5	+++	3.8	19.5
A-11,606	4.0	2.0	3.5	9.0	+	4.2	3.7
A-11,609	4.1	0	4.0	9.2	+	6.2	1.5
A-11,611	4.5	3.0	3.7	17.5	++	4.3	10.0
A-11,613	4.1	8.0	3.8	24.0	+++	6.5	22.0
A-12,589	4.6	3.0	3.9	19.2	+	6.0	2.5
A-12,592	4.1	2.0	3.5	13.2	+	3.9	4.0
A-12,593	4.4	0	3.8	11.5	++	5.5	3.0

[a] As determined by readings on a Turner fluorometer on chloroform extracts of culture liquors.
[b] As shown by paper chromatography in two solvent systems: +++, appreciable amounts; ++, some produced; +, traces found.

filtrates are aflatoxins, then some aflatoxin is produced in 48 hr accompanied by a drop in *p*H and formation of a yellow or brown pigment in the pellets. Highest yields are usually obtained in 5 days, followed by a decrease in the amount of toxin that can be isolated, perhaps because of enzymatic degradation. All the strains that produced aflatoxin, except NRRL 2999 and NRRL A-11,613, appeared to have the detoxification factor. Comparisons by paper chromatography revealed that much more aflatoxin is produced in still culture than in those kept on a reciprocal shaker.

None of the 53 strains of *A. oryzae* investigated produced aflatoxins in quantities that could be detected by the paper chromatographic procedures used. Food products that were fermented by *A. oryzae* were shown not to contain aflatoxin at levels above 25 parts per billion (Table 4).

The three strains of *A. flavus*, NRRL 3000, NRRL 2999, and NRRL A-11,613, grew very well on all the agricultural commodities used as substrates: rice, sorghum, peanuts, corn, wheat, and soybeans. Yellow pigments were produced during growth and were especially noticeable on rice. The amount of aflatoxins produced varied greatly depending on the commodity used (Table 5). None of the strains made much aflatoxin when grown on soybeans, regardless of conditions. High yields were consistently noted when rice was the substrate. From peanuts, as expected, and sorghum as well, substantial quantities were obtained. When wheat and corn were the substrates for the growth of *A. flavus*, very little aflatoxin was produced in standing culture. When wheat and corn were shaken, there was a manifold increase in yields of aflatoxin. In fact, shaking flasks continually during incubation resulted in higher yields on all commodities (Table 6). Addition of methionine, a known aflatoxin precursor, had little effect on the fermentation. Strain NRRL A-11,613 produced much more aflatoxin G_1 than did the other strains.

These investigations revealed great differences in capabilities of *A. flavus* strains to make aflatoxin—even among strains reported to be aflatoxin producers. Aflatoxin can be produced either on solid or in liquid media. Aeration is undoubtedly one of the most critical factors in its production on either medium and will be examined in greater detail. It is interesting that Alsberg and Black (5) observed that penicillic acid is produced under conditions of reduced aeration and at a low *p*H.

The observation that spores of NRRL 2999 and of NRRL 3000 contain aflatoxin is important because spores have a tendency to become airborne, especially since they are not wet by water. Mycelia and media can be readily treated to destroy aflatoxin, and would not constitute a health hazard when properly handled. Precautions must be taken to prevent spores of *A. flavus* strains producing aflatoxin from getting out in the atmosphere before they are treated to destroy the toxin.

A. oryzae and its near relatives are widely used in the preparation of koji for such food fermentations as shoyu (soy sauce), miso, black beans, and sake. In view of the close relationship of *A. flavus* and *A. oryzae*, it was imperative to study the formation of aflatoxin by *A. oryzae* strains and the possible presence of aflatoxin in food products made with these strains. No evidence was found that the toxin was produced by *A. oryzae* or that it was present in food products. However, all the cultures and food products studied came from pure tane koji and not from poor tane koji or starters used in home fermen-

TABLE 4. *Examination of fermented foods for aflatoxin*

Sample	Source	Fluorescence color	Turner reading of fluorescence[a]	R_f values of fluorescing substances in chloroform extracts[b]
Shoyu	From Japan prepared under a PL 480 project	Brilliant blue	>100	0.15; 0.35?
Sendai miso	From commercial market, Japan	Brilliant blue	>100	0.13
Miso no. 13	Made at NRRL	Brilliant blue	>100	0.15
Chinese black beans	Commercial sample from Taiwan	None		
Soybeans	U.S.	Greenish	2.5	
Soybean tempeh	Made at NRRL	Greenish	4.0	
Wheat tempeh	Made at NRRL	Light-blue	1.0	
Rice tempeh	Made at NRRL	Light-blue	1.5	
Wheat-rice-soybean tempeh	Made at NRRL	Greenish	8.0	
Aflatoxin B				0.60
Aflatoxin G				0.37

[a] Readings taken on a Turner fluorometer, model 110 (3 × range, primary filter 7-60, secondary filters, 2A + 2ND). Before readings were made, the instrument was set to zero with a solvent blank.

[b] The solvent system was benzene-toluene-cyclohexane-ethyl alcohol-water (3:3:5:8:5) containing 1% glacial acetic acid in the upper phase.

TABLE 5. *Production of aflatoxin by strains of Aspergillus flavus on a number of agricultural commodities*

Substrate	NRRL 3000				NRRL 2999				NRRL A-11,613			
	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
Sorghum	24	7	35	6	48	12	23	5	21	8	22	6
Sorghum + methionine	24	6	32	4	54	11	32	6	19	4	32	3
Peanuts	19	Trace	71	17	38	10	46	10	2	0.7	5	0.8
Peanuts + methionine	32	Trace	64	11	47	14	40	6	5	ND[b]	11	ND
Soybeans	0.8	0.2	16	2	0.8	ND	2	ND	≪0.03	<0.007	<0.02	<0.003
Soybeans + methionine	0.08	0.05	0.6	ND	1	0.3	2	0.2	≪0.03	<0.007	<0.02	<0.003
Corn	24	6	20	3	19	5	21	2	2	1	2	0.5
Corn + methionine	24	6	20	3	38	12	16	2	2	1	2	0.5
Wheat	25	4	36	7	9	2	7	1	2	0.5	4	0.6
Wheat + methionine	11	4	40	7	8	2	8	1	4	0.5	4	0.6
Rice	42	17	40	8	127	20	33	5	5	1	4	0.6
Rice + methionine	63	17	53	6	76	20	27	3	9	2	11	1

[a] As determined by thin-layer chromatography, and expressed as micrograms of aflatoxin produced per gram of substrate.

[b] None detected.

tations that contain many kinds of molds. Poor koji starters obtained from some Oriental sources can be expected to be contaminated with *A. flavus*. Rice, an ideal substrate for aflatoxin production, is used to produce koji.

The agricultural commodities sorghum, peanuts, corn, wheat, and rice not only supported growth of *A. flavus* strains, but made possible production of appreciable amounts of aflatoxin. It would not be surprising to discover aflatoxin in these commodities in some samples, especially after long storage, under conditions conducive to mold growth. It might be predicted that soybeans would contain very little of the toxin because they are such a poor substrate, even though they support the growth of *A. flavus*. Recently, however, Hodges et al. (22) reported the isolation of aflatoxin from *Penicillium puberulum*. It is possible that molds other than *A. flavus* might produce aflatoxin when grown on soy-

TABLE 6. *Production of aflatoxin by strains of Aspergillus flavus on shaken substrates*[a]

Substrate	NRRL 3000				NRRL 2999				NRRL A-11,613			
	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
Sorghum	76	17	80	12	85	22	80	8	84	67	80	25
Sorghum + methionine	76	17	106	17	109	25	64	11	76	25	213	25
Peanuts	48	12	106	12	109	28	128	17	152	40	256	40
Peanuts + methionine	42	11	92	14	168	44	160	25	169	44	182	28
Soybeans	13	3	69	12	22	4	35	4	8	4	96	1
Soybeans + methionine	11	2	96	25	22	4	34	7	22	15	320	60
Corn	285	75	214	33	283	15	12	7	164	33	321	33
Corn + methionine	326	66	240	30	253	37	106	7	326	55	321	30
Wheat	190	50	365	50	305	80	427	57	336	89	916	143
Wheat + methionine	190	50	320	57	760	182	640	111	380	89	800	111
Rice	124	33	80	12	380	34	80	13	108	29	71	14
Rice + methionine	190	50	71	11	169	40	46	8	253	100	213	25

[a] As determined by thin-layer chromatography, and expressed as micrograms of aflatoxin produced per gram of substrate.

beans. Numerous problems remain unanswered, and among these are the metabolic pathways by which fungi form aflatoxin, the distribution of aflatoxin in foods and feeds, and practical methods of detoxification of materials containing aflatoxin.

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