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Synthesis and Degradation of Aflatoxins by *Aspergillus parasiticus*. I. Synthesis of Aflatoxin B_1 by Young Mycelium and Its Subsequent Degradation in Aging Mycelium

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Abstract

Aflatoxin production and degradation were examined in three isolates of *A. parasiticus*. Maximum yields were present after incubation for 14 days and these declined gradually as the culture aged. Young mycelia (4 days old) synthesized the greatest amounts of aflatoxin, but aging mycelia (14 days old) were mainly responsible for degradation. Addition of cycloheximide to young cultures and removal of mycelia from aging cultures both prevented further aflatoxin degradation. Intramycelial substances released from fragmented or homogenized mycelium were capable of degrading aflatoxins, and their concentration increased as the mycelium aged. When ^{14}C-labelled aflatoxin was added to a 2-day-old culture and further incubated, 75% of the radioactivity at 12 days was intramycelial, but at 20 days, most radioactivity was in the filtrate.

Extra keywords: Aspergillus flavus

Introduction

Aflatoxins are secondary metabolites synthesized by most isolates of *Aspergillus flavus and A. parasiticus* (Codner *et al.* 1963; Shotwell *et al.* 1966). Numerous investigators (Ashworth *et al.* 1965; Arseculeratne and Bandunatha 1972; Pons *et al.* 1972; Alderman and March 1974; Schultz and Luedecke 1977) have reported that toxigenic cultures commonly produce maximum amounts of aflatoxins when incubated for 5–14 days; continued incubation beyond this period was invariably accompanied by a decline in the amount of aflatoxin present.

Fungal metabolites may lower the pH of the medium and the subsequent acidic condition could reduce aflatoxin levels (Ciegler *et al.* 1977). Alternatively, aflatoxins could be utilized as a nutrient substrate by the fungus (Schroeder 1966), or mycelial lysis followed by extramycelial degradation could be responsible for decline in aflatoxin levels (Ciegler *et al.* 1966). The purpose of the work reported here was to test the validity of these proposed hypotheses.

Materials and Methods

Cultures and Media

Isolates of *Aspergillus flavus* 5A were obtained from the Department of Microbiology, University of New England, and isolates *A. parasiticus* NRRL 2999 and NRRL 3000 were obtained from the Northern Regional Research Laboratory, Peoria, Ill., U.S.A. Cultures were maintained on potato–dextrose–agar slopes, and when required a solution of Tween 80 in sterile distilled water (1 : 10000) was added to cultures 1–3 weeks old and the spore suspension used to inoculate liquid media. The following media, adjusted to pH 6·0 with 1 M HCl were used: Czapek–Dox; Czapek–Dox–Corn extract (8%); Czapek–Dox–peptone (2%); peptone (1%)–malt extract (1%)–glucose (3%) (PMEG); PMEG–ferric ammonium citrate (0·05%); yeast extract (2%)–sucrose (20%) (YES).

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Experimental

Maximum aflatoxin production of the isolates listed was determined by growth in 10 flasks (500 ml) of each of the six media (100 ml) mentioned above. Incubation was at 28°C on an orbital shaker (200 rpm) for periods ranging from 20 to 24 days. Aflatoxin levels were determined at 2–4-day intervals by the methods of Nabney and Nesbitt (1965) and Pons and Goldblatt (1969). Duplicate samples (25 ml) were ground with glass beads and extracted (× 3) with equal volumes of chloroform. Extracts were combined, filtered through anhydrous sodium sulfate, evaporated to dryness in a Buchi rotary evaporator under reduced pressure at 30°C and redissolved in 1 ml of chloroform. Extracts were chromatographed by thin-layer techniques in chloroform–acetone (9 : 1 v/v) for 50 min and then dried. The blue and green fluorescent bands seen under ultraviolet light with approximate R_F values of 0·56 (B_1), 0·53 (B_2), 0·48 (G_1) and 0·46 (G_2) (Asao *et al.* 1963) were cut out and eluted with methanol. The presence of two characteristic peaks at 264–265 nm and 362–365 nm respectively was accepted as confirmation of aflatoxins. Quantitative estimates of aflatoxin were calculated from optical density readings taken at 363 nm (Asao *et al.* (1965) and Nabney and Nesbitt (1965)).

To examine the effect of nutrient availability on aflatoxin synthesis, cultures of *A. parasiticus* NRRL 2999 grown in YES medium were processed in two ways. The 12-day-old cultures were pooled, supplemented with an equal volume of YES medium, dispersed in 100-ml fractions and further incubated for 8 days. The filtrate from 14-day-old cultures was sterilized by passage through a 0·45 μm membrane filter, divided into 100-ml fractions in 500-ml flasks, and then each flask was inoculated with a spore suspension of the same fungal isolate and incubated for 8 days. Aflatoxin levels were determined at 2-day intervals.

The synthesis of aflatoxin by mycelia of *A. parasiticus* NRRL 2999 of different ages was examined by growing the fungus in YES medium and at 2-day intervals wet mycelium was harvested, washed and 1 g suspended in each of 8 flasks containing 10 ml of replacement medium. The medium contained glucose (3%), sodium acetate (0·5%) and inorganic ions, but the respiring mycelium was prevented from growing by the absence of a nitrogen source (Hsieh and Mateles 1970, 1971). Flasks were incubated at 28°C on an orbital shaker for 40 h.

Degradation of aflatoxin in culture filtrates was examined by removing mycelia from 14-, 16- and 18-day-old cultures, the culture filtrates sterilized by passage through a 0·45 μm membrane filter and the sterile filtrates reincubated. Aflatoxin levels were determined at 2-day intervals for up to 6 days.

Degradation of aflatoxin by fragmented or killed mycelium was examined by taking 100-ml fractions from a 14-day-old culture of *A. parasiticus* NRRL 2999 and treating as follows: mycelium in the culture was aseptically fragmented in a blender and then incubated at 28°C mixed with 10 ml ether oxide or autoclaved to kill the mycelium and then incubated at 28°C, and stored at 0°C. Aflatoxin levels were determined at 2-day intervals for up to 6 days.

To determine if mycelial extracts could degrade aflatoxin, *A. parasiticus* NRRL 2999 was grown in YES medium for 8–18 days and on each sampling day, 1 g of mycelium harvested, homogenized in 2 ml of 0·067 M phosphate buffer (pH 6·5), centrifuged at 3000 rpm for 15 min and the supernatant mixed with a solution of aflatoxin B_1 in phosphate buffer to give a final concentration of approximately 50 μg/ml, the mixture sterilized by passage through a 0·22 μm membrane filter and then incubated at 28°C for 72 h.

Degradation of ^{14}C-labelled aflatoxin was examined by mixing a cold sterile solution of ^{14}C-labelled aflatoxin B_1 in 0·067 M phosphate buffer (pH 6·5) (Hsieh and Mateles 1970, 1971) with a 2-day-old culture of *A. parasiticus* previously grown in YES medium, and further incubating at 28°C. The mixture contained 1·50 μg/ml aflatoxin B_1 with a radioactivity of 142 368 cpm. At days 12 and 20, mycelia and filtrates were separated and radioactive materials extracted with 10% methanol in chloroform and autoradiographs obtained from each extract.

To examine the effect of cycloheximide on aflatoxin degradation, an alcoholic solution of cycloheximide was added to 8-, 12- and 16-day-old cultures of *A. parasiticus* previously grown in YES, to give a final concentration of 150 μg/ml. Aflatoxin levels were determined at 2-day intervals for up to 12 days.

Results

Maximum Aflatoxin Production

All three isolates produced their highest levels of aflatoxin when grown in YES medium at 28°C for 14 days (Fig. 1). *A. parasiticus* NRRL 2999 averaged 216 μg/ml, a desirable concentration for enhancement of the fluorescent bands and measurable optical densities.

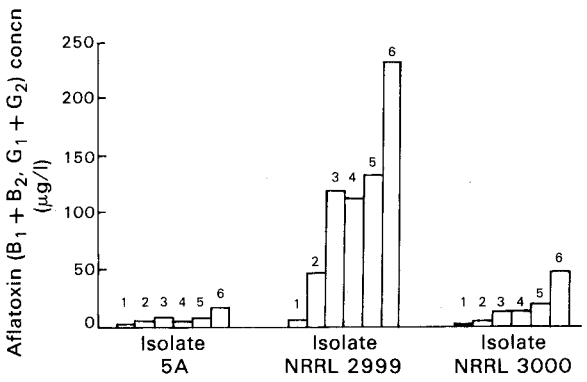
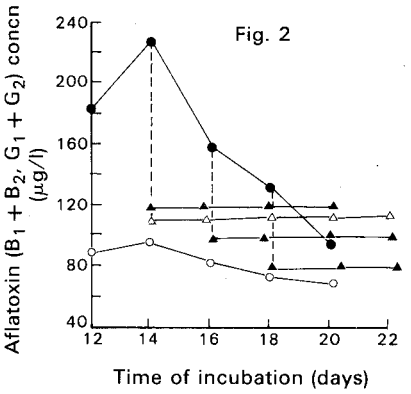


Fig. 1. Aflatoxin production by isolates of *A. flavus* and *A. parasiticus* grown in different liquid media for 14 days. 1, Czapek-Dox; 2, Czapek-Dox-corn extract; 3, Czapek-Dox-peptone; 4, PMEG; 5, PMEG-ferric ammonium citrate; 6, YES.

Effect of Nutrient Availability on Aflatoxin Synthesis

When a 14-day-old sterile filtrate of *A. parasiticus* was inoculated with a spore suspension of the same fungus, the aflatoxin concentration remained at 117–120 μg/ml during a further 8 days of incubation (Fig. 2), showing that the sterile filtrate was an unsuitable medium for fungal growth and subsequent aflatoxin production.



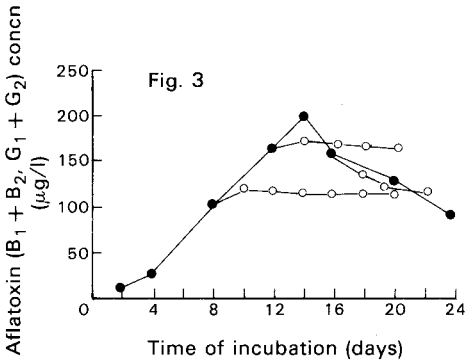


Fig. 2. Aflatoxin levels of *A. parasiticus* cultures and sterile culture filtrates either seeded with a spore suspension or supplemented with a nutrient solution. ● Unsupplemented culture. ○ Culture supplemented with YES medium at day 12. ▲ Sterile culture filtrates. △ Sterile culture filtrate seeded with a spore suspension of *A. parasiticus* at day 14.

Fig. 3. Aflatoxin levels in *A. parasiticus* cultures with and without cycloheximide. ● Without cycloheximide. ○ Cycloheximide added to 8-, 12- and 16-day-old cultures.

Eight days after adding the spore suspension, only 0·5 g dry weight of mycelium per 100 ml culture was produced and by 16 days 1 g of mycelium compared with 3·2 g for the unsupplemented culture after 14 days of incubation.

Addition of fresh YES medium to a 12-day-old culture of *A. parasiticus* did not prevent the decline in aflatoxin levels (Fig. 2). Two days after adding fresh YES medium, aflatoxin levels increased from 92 to 97 μg/ml but then decreased to 71 μg/ml by day 20. Without supplementation aflatoxin levels increased in the culture until day 14 and thereafter declined steadily (Figs 2 and 3).

Synthesis of Aflatoxin by Mycelia of Different Ages

When washed 2-day-old mycelium was suspended in replacement medium supplemented with sodium acetate, the aflatoxin level increased from 0 to 188 μg after 40 h incubation; with 4-day-old mycelium the level increased from 7 to 573 μg (Table 1). Washed older mycelia, especially 16-, 18- and 20-day-old mycelia, contained considerably less aflatoxins after 40 h incubation.

Table 1. Maximum levels of aflatoxins B_1 and G_1 obtained when mycelia of different ages were washed and placed in a replacement medium (devoid of a nitrogen source) and incubated for a further 40 h at 28°C

Age of mycelium (days)	Aflatoxin levels at 0 h		Aflatoxin levels at 40h		Aflatoxins $B_1 + G_1$ (μg)
	B_1 (μg)	G_1 (μg)	B_1 (μg)	G_1 (μg)	
2	0	0	97	91	188
4	4	3	298	282	573
6	4	3	225	200	418
8	4	3	136	123	252
10	3	2	46	37	78
12	4	3	40	37	70
14	4	3	26	23	42
16	3	2	13	11	19
18	4	3	8	6	7
20	4	3	5	4	2

Degradation of Aflatoxin by Culture Filtrates

When mycelia were removed from 14-, 16- and 18-day-old cultures, aflatoxin levels remained unchanged, even after a further 6 days incubation (Fig. 2).

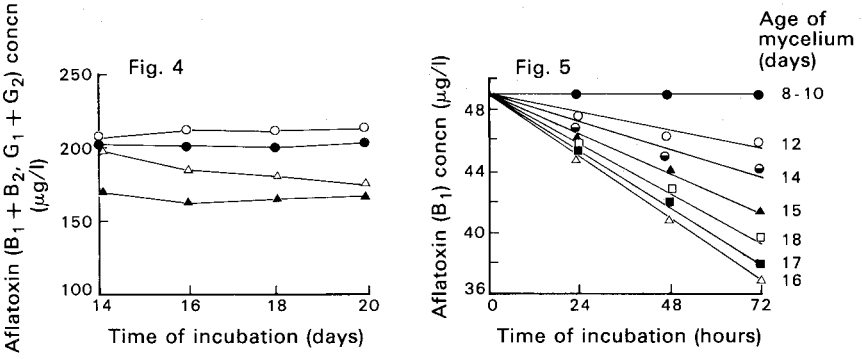


Fig. 4. Aflatoxin levels of a 14-day-old culture of *A. parasiticus* subjected to different treatments. ● Autoclaved culture. △ Mycelium fragmented. ▲ Ether-treated culture. ○ Stored at 0°C.

Fig. 5. Degradation of aflatoxin B_1 by mycelial extracts of *A. parasiticus* previously grown in YES medium for 8–18 days.

Degradation of Aflatoxin by Fragmented or Killed Mycelium

Fragmented 14-day-old mycelium of *A. parasiticus* degraded a small amount of aflatoxin (Fig. 4). The aflatoxin level decreased from 195 to 173 μg/ml after 6 days of incubation.

Dead mycelium (autoclaved or ether-treated) and chilled mycelium were unable to degrade aflatoxins.

Degradation of Aflatoxin by Mycelial Extracts

Mycelial extracts from 12–18-day-old mycelia degraded aflatoxin B_1, the most rapid being the extract from 16-day-old mycelia where aflatoxin B_1 declined from 49 to 37 μg/ml during 72 h of incubation (Fig. 5). No aflatoxin degradation was recorded with extracts from 8- and 10-day-old mycelia.

Table 2. Distribution of radioactivity (%) in cultures of *A. parasiticus*

^{14}C-labelled aflatoxin was added to 2-day-old cultures and the radioactivity of aflatoxins and their degradation products measured in the mycelia and filtrates after a further 12 and 20 days of incubation

R_F value[A]	Percentage radioactivity in lipophilic phase of: 12-day-old culture[B]		20-day-old culture[C]	
	Mycelium	Filtrate	Mycelium	Filtrate
—	65·4 (8·3)[D]	17·5 (7·3)	31·2 (12·5)	33·9 (20·6)
0·60			0·7	0·9
0·56 (B_1)	1·5	0·9		
0·53 (B_2)	1·2		0·1	0·1
0·48 (G_1)	1·6			
0·46 (G_2)	1·1		0·1	0·1
0·34	55·5	15·7	6·2	5·8
0·24	1·8	0·5	0·1	0·1
0·18	0·9	0·2	17·0	21·3
0·10–0	1·7	0·2	7·0	5·6

[A] Aflatoxins B_1, B_2, G_1 and G_2 were identified by comparing their R_F values and fluorescence with that of standard aflatoxins B_1, B_2, G_1 and G_2.

[B] 1·5% loss of radioactivity.

[C] 2·0% loss of radioactivity.

[D] Values in parentheses are the percentage radioactivity in the aqueous phase, making total percentage radioactivities 73·7 (mycelium) and 24·8 (filtrate) for the 12-day-old cultures, and 43·7 (mycelium) and 54·5 (filtrate) for the 20-day-old cultures.

Degradation of ^{14}C-labelled Aflatoxin

When radioactive ^{14}C-labelled aflatoxin was added to a 2-day-old culture of *A. parasiticus* and further incubated for 12 days, approximately 74% of the radioactivity appeared in the mycelium and only 25% in the filtrate (Table 2). After 20 days of incubation, radioactivity in the mycelium had decreased to 44% and conversely, increased to 54% in the filtrate. The main radioactive compound in the 12-day-old mycelium (radioactivity 55%) was non-fluorescent with an R_F value of 0·34, whereas in the 20-day-old filtrate the main radioactive compound (radioactivity 21%) was non-fluorescent with an R_F value of 0·18.

Effect of Cycloheximide on Aflatoxin Degradation

Addition of cycloheximide to 8- and 12-day-old cultures, but not to the 16-day-old culture, prevented the subsequent decline in aflatoxin levels (Fig. 3).

Discussion

All three isolates produced their highest levels of aflatoxin when grown in YES medium at 28°C for 14 days. Continued incubation resulted in a gradual decline in aflatoxin levels.

It has been suggested that reduction in aflatoxin levels after 14 days incubation may be due to exhaustion of nutrients and the subsequent utilization of aflatoxin by the fungus itself (Hayes *et al.* 1966; Schroeder 1966). This explanation appears unlikely as the addition of fresh nutrients to aging cultures did not prevent aflatoxin degradation. This finding is in agreement with that of Ciegler *et al.* (1966).

When mycelium of different ages was suspended in a replacement medium containing the supposed precursor acetate, most aflatoxin was synthesized by 4-day-old mycelium. Older mycelium failed to synthesize much aflatoxin, mycelium 16, 18 and 20 days old synthesizing 19, 7 and 2 μg, respectively, relative to younger mycelium (see Table 1). This suggests that the high concentration of aflatoxin found in a 14-day-old culture was the result of aflatoxins synthesized mainly when the mycelium was much younger and retained within the mycelium.

By removing 14-, 16- and 18-day-old mycelia from culture media, loss of aflatoxins was precluded, which suggests that mycelium was, at least in part, responsible for degradation of aflatoxins as the culture aged. Intramycelial substances released from fragmented or homogenized mycelium were capable of degrading aflatoxins and their concentration increased as the mycelium aged. These findings are consistent with those of Doyle and Marth (1978*a*, 1978*b*).

Ciegler *et al.* (1966) reported that loss of aflatoxins following peak yields could be prevented by reducing the incubation temperature to below 20°C and decreasing the speed of the agitator. These conditions were thought to reduce mycelial lysis and therefore delay the degradation of aflatoxins. An alternative hypothesis would be that low temperatures inhibit activity of these 'aflatoxin-degrading substances' and this is supported by the unchanged levels of aflatoxins after keeping a 14-day-old culture at 0°C for up to 6 days.

When ^{14}C-labelled aflatoxin B_1 was added to a 2-day-old culture and further incubated for 12 days, most of the radioactive materials, including new metabolites, were intramycelial, suggesting that aflatoxin B_1 was first absorbed into the mycelium and then degraded. This finding is in agreement with that of Searcy (1968).

The effect of cycloheximide on subsequent aflatoxin degradation depended on the period at which cycloheximide was added to the culture. The earlier the addition, the more cycloheximide prevented aflatoxin decline. Thus when cycloheximide was added to an 8-day-old culture, there was no decline in subsequent levels of aflatoxin, but when added to an older culture (16 days old) cycloheximide appeared not to inhibit aflatoxin-degrading substances already synthesized and consequently there was a decline in aflatoxin levels.

We postulate that aflatoxin-degrading substances are synthesized in young mycelia at a time when synthesis of aflatoxins is greater than degradation and consequently levels of aflatoxins increase. Ultimately, as the mycelium ages, synthesis of aflatoxins decline and degradation increases, resulting in a reduction in aflatoxin levels.

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