The Correlation of Aflatoxin and Norsolorinic Acid Production

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ABSTRACT

Production of aflatoxins and norsolorinic acid by a mutant strain of *Aspergillus parasiticus* follows a similar course. Both substances are completely or partially inhibited when the mutant is grown in a chemically defined medium while illuminated continuously, at 37 C, in medium lacking zinc, and in the presence of para-aminobenzoic acid. Higher yields of both compounds are obtained when the mold is grown in an enriched medium.

INTRODUCTION

Mutants which produce little or no aflatoxin have been isolated from a strain of *Aspergillus parasiticus* Speare which originally produced high levels of B and G aflatoxins, following UV light irradiation. Many of these mutant strains are also mutant for mycelial pigmentation (1).

One brown spored mutant strain with low aflatoxigenicity produced large amounts of an orange-red pigment which has been identified as norsolorinic acid (2). While culturing the mold for experiments on the isolation and identification of norsolorinic acid, it was observed that conditions which had been reported in the literature as suitable for obtaining optimal aflatoxin yields in liquid culture (3) were also suitable for obtaining high yields of the pigment. A preliminary report of this work has been presented (4).

This report is an extension of those observations. The mutant strain has been cultured in different controlled environments and the amounts of aflatoxins and norsolorinic acid produced were measured.

EXPERIMENTAL PROCEDURES

Culture Conditions

Stock cultures of the mutant strain of *A. parasiticus* (NRRL A-17,996) used in these investigations were maintained on potato dextrose agar (Difco) supplemented with 0.5% yeast extract. Dense spore suspensions (2-3 ml/flask) from stock cultures were used to inoculate each 250 ml of liquid medium cotained in liter Erlenmeyer flasks.

The chemically defined medium devised by Adye and Mateles (AM) (5), or an AM medium modified as stated, was used in all experiments except one. In that experiment a 2% yeast extract, 20% sucrose medium (YES) was used (6). Para-aminobenzoic acid (PABA) was added to AM medium in a concentration of 2 g/liter; for zinc free medium the $ZnSO_4 \cdot 7H_2O$ was omitted from AM medium. Unless otherwise stated, flasks were incubated for seven days at 30 C in the dark. All experiments were done in stationary culture.

Four flasks were used for each treatment. To minimize possible variations in the microenvironment, at the end of the incubation period the contents of two flasks were pooled, resulting in two pairs. The contents of one pair of flasks were used to measure aflatoxin production in the culture filtrate and the mycelium; the contents of the other pair of flasks were used to measure aflatoxin production in the culture filtrate, dry weight of the mycelium, and norsolorinic acid production. Each experiment was replicated twice. Aflatoxin, norsolorinic acid and dry weights are expressed in units per 500 ml of medium (the contents of two flasks). For the experiment on aflatoxin and norsolorinic acid production over eight days of incubation, aflatoxin assays were made for the culture filtrate but not the mycelium.

Extractions and Assays

The culture filtrates were separated from the pigmented mycelia by filtering through a double layer of cheesecloth. The mycelial mats were dried for three to four days in a vacuum oven at room temperature over a drying agent. Before extraction, the dried mats were soaked for several hours in aqueous acetone and then the mixture was blended at high speed in a Waring Blendor with acetone. The mycelial slurry in acetone was filtered through filter paper with suction. The blending-filtering process was repeated until the mycelial powder was colorless. The acetone extract was filtered through Na_2SO_4 to remove water and then evaporated to dryness on a steam bath. The extracts were suspended in chloroform for aflatoxin assays or in methanol for norsolorinic acid assays.

[1]So. Utiliz. Res. Dev. Div., ARS, USDA.

TABLE I

Aflatoxin and Norsolorinic Acid Production Over Eight Days of Incubation in Chemically Defined Medium (AM) at 30 C in the Dark

Days of incubation	Mycelial dry weight, g/500 ml	Norsolorinic acid, mg/500 ml	Aflatoxins in culture filtrate, μg/500 ml		
			B	G	Total
1	0.02	ND[a]	Trace[b]	Trace	Trace
2	1.6	2.6	84	34	118
3	3.4	21.7	494	264	758
4	4.4	41.6	895	486	1381
5	5.1	59.4	941	470	1411
6	6.7	75.6	1176	746	1922
7	7.2	82.5	1736	555	2291
8	7.6	83.5	1634	334	1968

[a]None detected.
[b]Less than 1 μg.

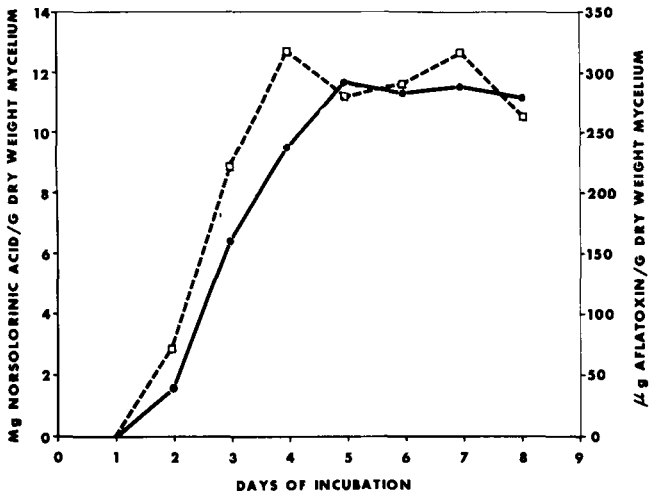

FIG. 1. Aflatoxin and norsolorinic acid production per unit dry weight of mycelium, o——o milligram of norsolorinic acid per gram dry weight of mycelium; □——□ μg aflatoxin per gram dry weight of mycelium.

Aflatoxins were extracted from the culture filtrates by partitioning into chloroform in a separatory funnel. Extracts were suspended in chloroform to a concentration suitable for aflatoxin assay, and the toxins measured using thin layer chromatography and densitometry as described by Pons et al. (7). Since norsolorinic acid absorbs at 314 mμ (2), the amount of crude acid produced was estimated from absorption of methanolic solutions at this wave length using a Beckman DK-2A recording spectrophotometer. Extracts from cultures grown in YES medium and in defined medium plus PABA contained some interfering pigments and norsolorinic acid was estimated from the shoulder at 314 mμ in these samples.

RESULTS

Under the disparate conditions studied, production of aflatoxins and norsolorinic acid was inhibited or enhanced concomitantly, although the degree of inhibition or enhancement was not the same for each compound.

Data on the production of aflatoxins and norsolorinic acid each day for eight days of incubation are presented in Table I. Synthesis of both compounds is easily detected on the second day. Maximum aflatoxin production is reached on the seventh day; norsolorinic acid production is highest on the eighth day. These data on aflatoxins and norsolorinic acid, expressed per unit of dry weight of mycelium, are plotted in Figure 1. The production of the two compounds follows a similar pattern. Furthermore, between days four and eight both reach a fairly constant level with aflatoxins representing about 0.03% and nor-

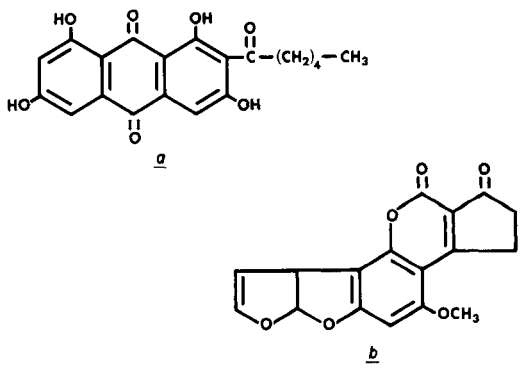

FIG. 2. Structures for norsolorinic acid (a) and aflatoxin B_1 (b).

TABLE II

Aflatoxin, Norsolorinic Acid and Mycelial Dry Weight Production After Seven Days of Incubation Under Six Different Conditions

Conditions	Mycelial dry weight, g/500 ml	Norsolorinic acid, mg/500 ml	Aflatoxin μg/500 ml							
			Aflatoxin B			Aflatoxin G			Total aflatoxin	
			Culture filtrate	Mycelium	Total B	Culture filtrate	Mycelium	Total G	Culture filtrate	Mycelium	Total aflatoxin
Control[a]	6.3	75.0	1405	2120	3525	452	192	644	1857	2312	4169
Continuous diffused light[b]	6.0	56.2	549	272	821	66	NDe	66	615	272	887
37 C[c]	5.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PABA[a]	4.7	20.0	ND	40	40	45	4	49	45	44	89
-Zinc[a]	0.6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
YES medium[d]	24.9	221.0	4753	4197	8950	4013	1622	5635	8766	5819	14,585

[a]Grown in chemically defined medium by Adye and Mateles (AM) (5), at 30 C in dark.
[b]Grown in AM medium at 30 C.
[c]Grown in AM medium in dark.
[d]Grown at 30 C in dark.
[e]None detected.

solorinic acid about 1.0% of the mycelial mass.

Results reported in Table II show that a reduction or loss of pigmentation was noted when the mutant mold was grown under four different regimes reported to inhibit aflatoxin production: continuous illumination (8), zinc deprivation (9), the presence of PABA (10) and high temperature (3). Conversely, higher production of norsolorinic acid accompanied greater aflatoxin production when the mutant was grown in the enriched medium developed by Diener et al. (6). Growth in this YES medium led to a 4-fold increase in mycelial weight, a 3.5-fold increase in aflatoxins and a 3-fold increase in norsolorinic acid production.

In the AM medium 37 C is inhibitory to aflatoxin production while on more complex substrates (3) a higher temperature is required for complete inhibition. Incubation at 37 C and in zinc deficient medium completely inhibited aflatoxin and norsolorinic acid production. Dry weights, recorded in Table II, indicate growth was only moderately depressed at 37 C, but was severely inhibited in zincless medium (Table II, column 2).

In the presence of continuous illumination, only about 20% of the amount of aflatoxins were produced as compared to that produced by the controls, which were grown in the dark. Pigment production and mycelial growth are not as adversely affected by light as is aflatoxin production: about 75% the amount of norsolorinic acid is produced and the amount of mycelial growth was almost equal to that of the dark controls.

When PABA is added to the medium, aflatoxin synthesis is depressed 50-fold and norsolorinic acid production is depressed 4-fold. The dry mycelial mass is 75% that of the controls. No aflatoxin B, only G, was detected in the culture filtrate, while in the mycelium aflatoxin B content was 10X that of G.

The effects of zinc deprivation and growth in YES medium are strongly correlated with differences in dry mycelial weights, while the inhibitory effects of light, 37 C, and PABA are not accompanied by a severe inhibition of growth. Inhibition of a specific compound under certain conditions, despite good growth, is an attribute of secondary metabolites.

It has been theorized that aflatoxins and anthraquinone compounds such as norsolorinic acid are secondary metabolites derived via the condensation of acetyl-CoA units (11,12) as illustrated in Figure 2. Thus, it is likely that the correlation between the production of the two compounds is due to the fact that they share a common precursor pool. This correlation is useful in studies with this mutant because norsolorinic acid as a bright orange-red pigment is much easier to detect than aflatoxin. Its presence or absence and intensity can be used as an indicator of possible aflatoxin production.

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