# Preparation of $^{14}C$-Labeled Aflatoxins and Incorporation of Unlabeled Aflatoxins in a Blocked Versicolorin-A-Accumulating Mutant of *Aspergillus parasiticus*

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## ABSTRACT

We have compared 2 methods for preparing radiolabeled aflatoxins from [$^{14}C$] acetate for use in biosynthetic studies at the end of the aflatoxin pathway. The Salhab-Edwards method (SE) used a 3-day-old mycelium and a defined medium containing $MnCl_2$ with incubation at 28 C. The Lee-Bennett method (LB) used a 2-day-old mycelium and a defined medium containing low levels of Mn with incubation at 30 C. Generally, the LB method produced lower quantities of aflatoxin but the product had higher specific activities (sp act). The SE method produced 0.157 μmol of aflatoxin $B_1$ and 0.028 μmol of $G_1$ compared to the LB method with 0.058 μmol of aflatoxin $B_1$ and 0.001 μmol of $G_1$. The sp act (in μCi/μmol) for aflatoxin produced by the LB method were: $B_1$ = 1.379; $B_2$ = 0.130; $G_1$ = 5.0 and $G_2$ = 0.063. The sp act of aflatoxin produced by the SE method were: $B_1$ = 0.267; $B_2$ = 0.014; $G_1$ = 0.178; and $G_2$ = 0.133. Unlabeled aflatoxins were presented to resting cell cultures of the versicolorin-A-accumulating mutant of *Aspergillus parasiticus*. No conversion of aflatoxin $B_1$ was noted. However, when aflatoxins $B_2$ or $G_1$ were presented low levels of aflatoxins $B_1$ and $G_2$ were recovered. Aflatoxins $B_2$ and $G_1$ were recovered when aflatoxin $G_2$ was presented. Similar low levels of recovery were obtained in experiments using autoclaved mycelia. Thus we conclude that these minor quantities of aflatoxins recovered were not produced enzymatically.

## INTRODUCTION

Aflatoxins are a family of chemically related compounds produced by 2 species of common molds, *Aspergillus flavus* and *Aspergillus parasiticus*. Contamination of human and animal food supplies by these secondary metabolites constitutes a major world health problem because of their toxicity, carcinogenicity, mutagenicity and teratogenicity (1,2).

Aflatoxins are examples of acetate-derived secondary metabolites called "polyketides." Aflatoxin biosynthesis proceeds through a polyketide pathway in which the general steps are: acetate→anthraquinones→xanthones→aflatoxin $B_1$. There are several good reviews of these early steps in the aflatoxin biosynthetic pathway (2-6).

Most studies on aflatoxin biosynthesis have observed the incorporation of precursors into aflatoxin $B_1$. A precursor-product relationship between aflatoxin $B_1$ and the other major aflatoxins ($B_2$, $G_1$, $G_2$) has been assumed by most workers. According to this view, the G group of aflatoxins and aflatoxin $B_2$ are both produced from aflatoxin $B_1$ but by differing pathways (5). This biosynthetic model is based on meager evidence and numerous assumptions.

In this study, we isolate radiolabeled aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ by 2 methods (7,8) and elucidate the parameters for feeding unlabeled aflatoxins to a blocked versicolorin-A-accumulating mutant. We suggest a possible pathway for the late stages of aflatoxin biosynthesis. A preliminary report of this work has been presented (9).

## EXPERIMENTAL PROCEDURES

### Strains and Culture Media

The wild-type strain was an aflatoxigenic isolate of *A. parasiticus* Speare designated SU-1 (NRRL 5862). The white-spored versicolorin-A-producing mutant, *A. parasiticus* strain *wh-1 ver-1* (ATCC 36,537), was derived from this stock after ultraviolet (UV) irradiation (10). Stock cultures were maintained on potato dextrose agar (Difco) plus 0.5% yeast extract. Ca. $10^6$ conidiospores were used for each initial inoculation.

The growth medium (AM) and the replacement medium (RM) which were used in the Lee-Bennett method (7) and the feeding experiments were formulated according to Adye and Mateles (11). The Salhal-Edwards method (8) used the basal culture medium (BCM) developed by Detroy and Ciegler (12). The replacement basal culture medium (RBCM) contained fresh BCM supplemented with 10 mM $ZnSO_4$.

### Preparation of [$^{14}C$] Aflatoxins

To prepare $^{14}C$-labeled aflatoxins the methods of Lee et al. (7) and Salhab and Edwards (8) were used.

*Salhab-Edwards method (SE).* Five 100-mL portions of BCM in 250 mL cotton-stoppered Erlenmeyer flasks were inoculated with the wild-type strain (SU-1) and incubated for 72 hr at 28 C in the dark on an enclosed rotary shaker set at 100 rpm. Ball-shaped mycelial pellets of ca. 2-mm in diam were produced by the constant rotary motion of the shaker. These pellets were pooled and collected on cheesecloth and thoroughly washed with distilled water. Ca. 10 g of wet pellets were added to each of 5 flasks containing 100 mL of RBCM. Sodium [1-$^{14}C$] acetate, 57-60 Ci/mol (Amersham, Inc.) was dissolved in water (1 mCi/10 mL) and a 0.5-mL aliquot was added to each flask. The flasks were placed back in the rotary shaker and incubated at 28 C for 24 hr.

*Lee-Bennett method (LB).* The LB method was an adaptation of the resting cell culture technique developed by Hsieh and Mateles (13). Five 100-mL portions of AM were inoculated with the wild-type strain, SU-1, and incubated for 48 hr at 30 C on a rotary shaker. The ball-shaped mycelial pellets were pooled and collected on cheesecloth and thoroughly washed with distilled water. Ca. 30 g of wet pellets were added to each of 2 flasks containing 100 mL of RM. Sodium [1-$^{14}C$] acetate, 57-60 Ci/mol (Amersham, Inc.), was dissolved in water (1 mCi/10 mL) and a 0.5-mL aliquot was added to each flask at the beginning and at four 1.5-hr intervals during incubation at 30 C on a rotary shaker. After the last aliquot of [1-$^{14}C$] acetate was added, incubation was continued for an additional 5 hr.

### Feeding Studies Using Unlabeled Aflatoxins

Two 100-mL portions of AM in 250-mL Erlenmeyer flasks were inoculated with $10^6$ spores of the *ver-1* mutant of *A. parasiticus* and incubated in the dark for 48 hr at 30 C on a rotary shaker. Ball-shaped mycelial clusters ca. 2 mm in diam. were produced by the constant rotary motion. These pellets were pooled and collected on cheesecloth and thoroughly washed with distilled water. In these experiments, quantities of unlabeled precursors ranging from 3 to 10 μmol were dissolved in 0.2 mL acetone, and these aliquots were placed in 50-mL Erlenmeyer flasks. RM (9.8 mL) was added slowly, and then 1 g of wet pellets was added to each flask. The flasks were returned to the shaker for time periods ranging from 24 to 96 hr. Autoclaved cells were used as controls to verify the enzymatic conversion. In these controls, the mycelial pellets were autoclaved for 20 min before being added to the flasks containing one of the unlabeled precursors.

### Extraction

After the appropriate time interval in the shaker, the medium was carefully decanted from each flask and extracted with chloroform. Pellets were soaked in acetone, and the acetone extract was adjusted to 30% with water. This acetone solution was then extracted with chloroform. The respective chloroform extracts of the mycelia and medium for each flask were combined and evaporated to dryness under a continuous stream of nitrogen, or allowed to evaporate overnight under the hood.

### Purification of [$^{14}$C] Aflatoxins

All [$^{14}$C] aflatoxins were purified by exhaustive, preparative thin layer chromatography (TLC). The dried samples were resuspended in ca. 1 mL chloroform. Each chloroform aliquot was streaked on Adorbosil-1 silica gel plates. The plates were developed in anhydrous ether/methanol/water (96:3:1, v/v/v) for 45 min. Observation of the plates under UV light revealed 6 respective regions: upper yellow, including solvent front, aflatoxin $B_1$, $B_2$, $G_1$, $G_2$, and lower blue, including the area of plate to which the sample was applied. These regions were stripped from the plates and eluted from the silica with acetone. The samples were evaporated to near dryness and then resuspended in ca. 1 mL of chloroform. Each individual sample was rechromatographed, and each region eluted into the appropriate vial. This process was repeated until only a single band appeared.

### Assays

Extracts were suspended in chloroform to a concentration suitable for aflatoxin assay and the toxins were quantitated using TLC and densitometry as described by Pons et al. (14).

Samples of pure radiolabeled compounds were suspended in 10 mL chloroform. Radioactivity of aliquots of these samples was measured in 15 mL of Eastman I scintillation solvent on a Searle Isocap/300/liquid scintillator system. All values were corrected for quenching using a standard 8-sample quench curve.

## RESULTS

Data on the incorporation of sodium [1-$^{14}$C] acetate into the 4 major aflatoxins using the SE and LB preparation techniques are presented in Table I. Generally, the LB method produced lower quantities of aflatoxin, but the product had higher specific activities (sp act). The SE method produced 0.157 μmol of aflatoxin $B_1$ and 0.028 μmol of $G_1$ compared to the LB method with 0.058 μmol of aflatoxin $B_1$ and 0.001 μmol of $G_1$. The sp act (in μCi/μmol) for aflatoxins produced by the SE method were: $B_1$ = 0.267; $B_2$ = 0.104; $G_1$ = 0.178; $G_2$ = 0.133. The sp act for aflatoxins produced by the LB method were: $B_1$ = 1.379; $B_2$ = 0.130; $G_1$ = 5.0; $G_2$ = 0.063.

Before using the radiolabeled precursors we had produced in the incorporation studies, we isolated the parameters for optimal uptake of these compounds by a blocked versicolorin-A-accumulating mutant of *A. parasiticus*. This mutant produces no aflatoxin because of the block early in the pathway; however, it retains the enzymes for the late stages of the pathway (6). When a single, unlabeled compound is fed to this mutant, recovery of other aflatoxins implies interconversion by the organism. We fed unlabeled $B_1$, $B_2$, $G_1$ and $G_2$ to this blocked versicolorin-A-accumulating mutant, and the results are shown in Table II. When aflatoxin $B_1$ was presented to resting cell cultures of the versicolorin A mutant, only aflatoxin $B_1$ was recovered. However, when aflatoxin $B_2$ or $G_1$ was presented to resting cell cultures, aflatoxins $B_1$ and $G_2$ were recovered in quantities ranging from 0.02 to 0.4% of the quantity fed. Aflatoxins $B_2$ and $G_1$ were recovered in quantities ranging from 0.06 to 0.4% of precursors fed when aflatoxin $G_2$ was presented to resting cell cultures of the versicolorin-A-accumulating mutant.

These levels of incorporation were so low that they implied the absence of enzymatic transformation. Autoclaved mycelia of the versicolorin-A-accumulating mutant were presented with similar levels of unlabeled aflatoxins. The results of these control experiments are shown in Table III. In these control experiments, aflatoxin $B_1$, when presented to the autoclaved mycelia, produced no other aflatoxins; however, when aflatoxin $B_2$ or $G_1$ was presented, aflatoxins $B_1$ and $G_2$ were recovered in quantities

TABLE I

**Incorporation of Sodium [1-$^{14}$C] Acetate into the Aflatoxins Using Two Techniques**

| | | dpm | μCi | μmol | sp act (μCi/μmol) | Rel. sp act[c] |
|---|---|---|---|---|---|---|
| Aflatoxin $B_1$ | S[a] | 93,571 | .042 | .157 | .267 | .005 |
| | L[b] | 178,187 | .080 | .058 | 1.379 | .025 |
| Aflatoxin $B_2$ | S | 11,353 | .005 | .048 | .104 | .002 |
| | L | 22,147 | .010 | .077 | .130 | .002 |
| Aflatoxin $G_1$ | S | 11,087 | .005 | .028 | .178 | .003 |
| | L | 10,332 | .005 | .001 | 5.0 | .087 |
| Aflatoxin $G_2$ | S | 9,002 | .004 | .030 | .133 | .002 |
| | L | 12,914 | .006 | .096 | .063 | .001 |

[a]Method of Salhab et al. (8).

[b]Method of Lee et al. (7).

[c]Specific activity of product/sp act of precursor.

TABLE II

**Recovery of Unlabeled Aflatoxins into Mycelium of Versicolorin-A-Accumulating Mutant of *A. parasiticus* in Replacement Media**

| Aflatoxin fed (μmol) | Time (hr) | Aflatoxin recovered (μmol) | | | |
|---|---|---|---|---|---|
| | | $B_1$ | $B_2$ | $G_1$ | $G_2$ |
| $B_1$ (5) | 24 | 4.0 | ND[a] | ND | ND |
| (10) | 24 | 8.0 | ND | ND | ND |
| (5) | 48 | 0.8 | ND | ND | ND |
| (10) | 48 | 2.1 | ND | ND | ND |
| (3) | 72 | 2.8 | ND | ND | ND |
| (3) | 96 | 1.6 | ND | ND | ND |
| $B_2$ (5) | 24 | .007 | 1.7 | –[b] | ND |
| (5) | 48 | .001 | 1.3 | – | .002 |
| (5) | 72 | .002 | 2.2 | – | .003 |
| (5) | 96 | .005 | 3.3 | – | .001 |
| $G_1$ (5) | 24 | .008 | ND | 3.7 | ND |
| (5) | 48 | .018 | ND | 3.4 | .004 |
| (5) | 72 | .018 | ND | 2.9 | .005 |
| (5) | 96 | .008 | .002 | 3.5 | .003 |
| $G_2$ (5) | 24 | ND | .003 | .02 | 2.4 |
| | 72 | ND | .003 | .02 | 3.0 |

[a]ND = none detected.

[b]Could not detect because quantity of $B_2$ recovered masked $G_1$.

TABLE III

**Recovery of Unlabeled Aflatoxins from Autoclaved Mycelia of a Versicolorin-A-Accumulating Mutant of *A. parasiticus* in Replacement Medium (96 hr)**

| Aflatoxin fed (μmol) | Aflatoxin recovered (μmol) | | | |
|---|---|---|---|---|
| | $B_1$ | $B_2$ | $G_1$ | $G_2$ |
| $B_1$ (3) | 2.4 | ND[a] | ND | ND |
| $B_2$ (5) | .0030 | 2.55 | –[b] | .0005 |
| $G_1$ (5) | .0043 | ND | 4.1 | .0021 |
| $G_2$ (5) | .0027 | .0029 | .0061 | 4.4 |

[a]ND = none detected.

[b]Could not detect because quantity of $B_2$ recovered masked $G_1$.

ranging from 0.01 to 0.09% of the quantity fed. Recovery of aflatoxins $B_1$, $B_2$ and $G_1$ in the range of 0.05-0.1% was noted when aflatoxin $G_2$ was fed. The percentages of aflatoxins recovered in the controls are very similar to those in the feeding experiments, and therefore, we must conclude that these minor quantities of aflatoxins recovered were contaminants of the major aflatoxins fed.

## DISCUSSION

There are a number of hypotheses concerning the final steps of the aflatoxin pathway which involve the interconversions of the 4 major aflatoxins ($B_1$, $B_2$, $G_1$, $G_2$) and aflatoxin $M_1$. Various theories have attempted to resolve this confusion, but no one good explanation exists. Biollaz et al. (15) hypothesized that aflatoxin $B_1$ was the precursor of aflatoxin $G_1$ and $M_1$ based on "paper chemistry" analysis of the structures of these 3 toxins. Experimental support for aflatoxin $B_1$ being the ultimate precursor is derived from 2 sources. Maggon and Venkitasubramanian (16) reported that [$^{14}$C]aflatoxin $B_1$ was converted to aflatoxin $G_1$ by a cell homogenate. Heathcote et al. (17) fed $^{14}$C-labeled aflatoxins to actively growing, as well as resting cell cultures, of *A. flavus*. Their data indicated aflatoxin $B_1$ was converted to aflatoxins $B_2$, $B_{2a}$, $G_1$, $G_2$ and $G_{2a}$. Aflatoxin $B_2$ was converted to aflatoxin $B_1$ but not to aflatoxin $G_2$. Aflatoxin $G_2$ was not converted to any of the other aflatoxins. Moreover, aflatoxin $M_1$ was converted into aflatoxin $B_1$. They proposed a pathway in which the latter stages of aflatoxin biosynthesis were: aflatoxin $M_1$→aflatoxin $B_1$→aflatoxin $G_1$. They did not place aflatoxin $B_2$ into this scheme.

Maggon et al. (5), in their review article, proposed a branching pathway for the interconversion of the 4 major aflatoxins. Aflatoxin $B_1$ was considered the precursor of all the other aflatoxins with the G group of aflatoxins and aflatoxin $B_2$ being produced from aflatoxin $B_1$ by different pathways. They did not consider the placement of aflatoxin $M_1$ into this scheme.

The experiments of Elseworthy et al. (18) do not support aflatoxin $B_1$ as the ultimate precursor. They reported a much higher percentage incorporation of 5-hydroxysterigmatocystins specifically labeled with [$^{14}$C] in the O-methyl group into aflatoxins $B_2$ and $G_2$ than into aflatoxins $B_1$ and $G_1$. From these data, aflatoxins $B_2$ and $G_2$ were proposed as the biogenetic precursors of aflatoxins $B_1$ and $G_1$, respectively.

Other data relevant to the final steps of the aflatoxin biosynthesis pathway come from observations by several investigators of mutant strains of *A. parasiticus* which produced high levels of aflatoxin $B_2$ with little or no aflatoxin $B_1$ production (19-21). The accumulation of aflatoxin $B_2$ in these mutants suggests that aflatoxin $B_1$ is not the precursor of aflatoxin $B_2$.

Our results do not support the theory that aflatoxin $B_1$ is the ultimate precursor of the other major aflatoxins. In earlier work, we observed significant incorporation of sterigmatocystin into the major aflatoxins by the versi-

colorin-A-accumulating mutant (9); however, no such biogenetic relationship was observed among the major aflatoxins when they were presented to this mutant. Therefore, we conclude that the major aflatoxins are produced simultaneously at the end of the aflatoxin pathway, and propose a branching pathway from a common precursor for the production of the aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$.

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REFERENCES

1. "Aflatoxins: Scientific Background, Control, and Implications," edited by L.A. Goldblatt, Academic Press, Inc., New York, NY, 1969.
2. Heathcote, J.G., and J.P. Hibbert, "Aflatoxins: Chemical and Biological Aspects. Developments in Food Science 1," Elsevier Sci. Publ. Co., New York, NY, 1978.
3. Bennett, J.W., and L.S. Lee, J. Food Protection 42:805 (1979).
4. Bennett, J.W., L.S. Lee, S.M. Shoss and G.H. Boudreaux, Appl. Envir. Microbiol. 39:835 (1980).
5. Maggon, K.K., S.K. Gupta and T.A. Venkitasubramanian, Bacteriol. Rev. 41:822 (1977).
6. Singh, R., and D.P.H. Hsieh, Arch. Biochem. Biophys. 178:285 (1977).
7. Lee, L.S., J.W. Bennett, A.F. Cucullu and R.L. Ory, J. Agric. Food Chem. 24:1167 (1976).
8. Salhab, A.S., and G.S. Edwards, J. Label. Compd. Pharm. 15:41 (1978).
9. Floyd, J.C., J.W. Bennett and L.S. Lee, Am. Soc. Microbiol. Abstr. Ann. Meet. 022:191 (1981).
10. Bennett, J.W., and L.A. Goldblatt, Sabouraudia 11:235 (1973).
11. Adye, J., and R.I. Mateles, Biochim. Biophys. Acta 86:418 (1964).
12. Detroy, R.W., and A. Ciegler, J. Gen. Microbiol. 65:259 (1971).
13. Hsieh, D.P.H., and R.I. Mateles, Appl. Microbiol. 22:79 (1971).
14. Pons, W.A., Jr., A.F. Cucullu, A.O. Franz and L.A. Goldblatt, JAOCS 45:694 (1968).
15. Biollaz, M., G. Buchi and G. Milne, J. Am. Chem. Soc. 90:5019 (1968).
16. Maggon, K.K., and T.A. Venkitasubramanian, Experientia 29:1210 (1973).
17. Heathcote, J.G., M.F. Dutton and J.R. Hibbert, Chem. Ind. 1976:207 (1976).
18. Elseworthy, G.C., J.S.E. Holker, J.M. McKeon, J.B. Robinson and L.J. Mulherin, Chem. Comm. 1970:1069 (1970).
19. Papa, K.E., Appl. Envir. Microbiol. 33:206 (1977).
20. Schroeder, H.W., and W.W. Carlton, Appl. Microbiol. 25:146 (1973).
21. van Walbeek, W., P.M. Scott and F.S. Thatcher, Can. J. Microbiol. 14:131 (1968).

# Effect of β-Ionone on *Aspergillus flavus* and *Aspergillus parasiticus* Growth, Sporulation, Morphology and Aflatoxin Production

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## ABSTRACT

The ketone β-ionone is reported to be one of the naturally occurring volatile metabolites of developing corn ears. In testing the effects of volatile compounds on *Aspergillus flavus* and *A. parasiticus*, we found that β-ionone applied to the surface of PDA plates had a striking inhibition of growth and sporulation of these fungi. The colonies were restricted, remained buff-colored and had little or no sporulation. There were major effects on the morphology of the asexual reproductive structures. The conidiophore development was arrested and normal sporulation did not occur. Mycelial transfers from these atypical cultures to potato dextrose agar had normal growth and conidia. Incorporation of β-ionone at levels of 10-1000 μL/L, in liquid media seeded with spore suspensions of *A. parasiticus* (NRRL 2999) severely depressed aflatoxin accumulation in shake culture.

## INTRODUCTION

A possible way to influence aflatoxin contamination of corn is to develop a corn variety that contains chemicals that do not allow growth of the *Aspergillus flavus* group or formation of aflatoxins. Aflatoxin contamination is possible following growth of *A. flavus* in corn, but these characters are not necessarily linked. In screening several volatile compounds reported (1,2) to be present in developing corn, we found that β-ionone had a striking and unexpected effect on growth and sporulation of *A. flavus*. The purpose of these experiments was to document the morphological response to β-ionone and to measure the effect of β-ionone on growth and aflatoxin accumulation in shake culture.

## EXPERIMENTAL PROCEDURES

*Aspergillus flavus* Link (CP-22) isolated from Georgia corn and *Aspergillus parasiticus* Speare (NRRL 2999) were maintained on potato dextrose agar (pda) slants and used in these experiments. The *A. flavus* isolate (CP-22) does not produce aflatoxin and the *A. parasiticus* isolate (NRRL 2999) produces aflatoxins.

Direct contact tests with β-ionone were done using petri dishes (15 × 100 mm) containing 20 mL pda and maintained at 26 C. Varying amounts (1-20 μL) of β-ionone were pipetted directly onto the pda surface just before inoculation. Some of the cultures were placed in plastic bags during incubation. Gross observations, diameter measurements and microscopic observations were periodically taken.

Bioassays of volatile effects of β-ionone were also done using pda in divided plates. These bioassays were conducted using the following procedure: (a) all 4 quadrants were inoculated with stabs of a spore suspension; (b) different concentrations of β-ionone (1-50 μL) were placed in only 1 quadrant; (c) plates were stored in separate plastic bags and incubated at 26 C; (d) gross observations and diameter measurements were recorded after 3, 4 or 5 days of incu-