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Identification of *O*-Methylsterigmatocystin as an Aflatoxin B_1 and G_1 Precursor in *Aspergillus parasiticus*

DEEPAK BHATNAGAR,* SUSAN P. McCORMICK, LOUISE S. LEE, AND ROBERT A. HILL†

Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, New Orleans, Louisiana 70179

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An isolate of *Aspergillus parasiticus* CP461 (SRRC 2043) produced no detectable aflatoxins, but accumulated *O*-methylsterigmatocystin (OMST). When sterigmatocystin (ST) was fed to this isolate in a low-sugar medium, there was an increase in the accumulation of OMST, without aflatoxin synthesis. When radiolabeled [^{14}C]OMST was fed to resting mycelia of a non-aflatoxin-, non-ST-, and non-OMST-producing mutant of *A. parasiticus* AVN-1 (SRRC 163), ^{14}C-labeled aflatoxins B_1 and G_1 were produced; 10 nmol of OMST produced 7.8 nmol of B_1 and 1.0 nmol of G_1, while 10 nmol of ST produced 6.4 nmol of B_1 and 0.6 nmol of G_1. A time course study of aflatoxin synthesis in ST feeding experiments with AVN-1 revealed that OMST is synthesized by the mold during the onset of aflatoxin synthesis. The total amount of aflatoxins recovered from OMST feeding experiments was higher than from experiments in which ST was fed to the resting mycelia. These results sugget that OMST is a true metabolite in the aflatoxin biosynthetic pathway between sterigmatocystin and aflatoxins B_1 and G_1 and is not a shunt metabolite, as thought previously.

Aflatoxins are toxic secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus* (4, 10, 13, 30, 41). The mode of action, metabolism, and biosynthesis of these toxins have been extensively studied (5, 8, 18, 19, 29, 41, 45). Mutants of *A. parasiticus* impaired in aflatoxin biosynthesis have been useful in studying the aflatoxin pathway (30, 31). By using blocked mutants, metabolic inhibitors, and radioactive labeling with resting cells and cell-free extracts of *Aspergillus* spp., some of the major steps of the pathway have been established (1, 2, 7, 15–17, 20, 21, 25–28, 33, 35–37, 39, 42, 46). Some of the details of this pathway are still unclear and in other parts there is conflicting evidence (2, 16, 17, 47, 48). The generally accepted steps in the biosynthetic pathway of aflatoxins are the following: acetate → norsolorinic acid → averantin → averufin → versiconal hemiacetal acetate → versicolorin A → sterigmatocystin (ST) → aflatoxin B_1.

O-Methylsterigmatocystin (OMST) is a fungal secondary metabolite (Fig. 1) that has been isolated from aflatoxin-producing strains of *A. flavus* (9), as well as from *Chaetomium* spp., *Fusarium* spp. (34, 43), and *Monocillium* sp. (3). Cell-free studies with *A. parasiticus* (24) have shown that ST can be converted to both OMST and aflatoxin B_1; the report, however, suggested that OMST is a side shunt metabolite of the aflatoxin biosynthetic pathway.

In this study we have characterized for the first time an isolate of *A. parasiticus* that does not produce aflatoxins (CP461; 14) and have demonstrated that it naturally accumulates OMST. Cell cultures of this isolate (CP461) and another non-aflatoxin-producing mutant strain of *A. parasiticus* (AVN-1) were used to characterize the role of OMST in the aflatoxin biosynthetic pathway. Some preliminary results of this study have been reported earlier (D. Bhatnagar, L. S. Lee, and R. Hill, Plant Physiol. Suppl. **80**:18, 1986).

* Corresponding author.

† Present address: Ruakura Soil and Plant Research Station, Ministry of Agriculture and Fisheries, Hamilton, New Zealand.

MATERIALS AND METHODS

Strains and growth conditions. The wild-type strain used in this study was an aflatoxigenic isolate of *A. parasiticus* designated SU-1 (NRRL 5862). An *A. parasiticus* (SRRC 163) blocked mutant strain (AVN-1) which accumulates averantin (ATCC 56774) was provided by Joan Bennett, Tulane University, New Orleans, La.; this strain was isolated from a versicolorin A-accumulating strain (SRRC 164) of *A. parasiticus* (ATCC 36537) as described by Bennett et al. (7). An isolate of *A. parasiticus* which does not produce aflatoxins was originally designated as isolate CP461 by Dorner et al. (14); this isolate was deposited in the Southern Regional Research Center (SRRC) collection and identified as SRRC 2043.

The growth medium (GM), the replacement medium (RM), and the low-sugar replacement medium (LSRM) used for incubating fungal cultures were made by the method of Adye and Mateles (1). Conidia were produced on potato dextrose agar slants. A 0.1-ml portion of the culture spore suspension (10^8 spores per ml) was transferred to 100 ml of GM in 250-ml flasks, and the flasks were incubated on a shaker incubator (Lab-Line Instruments, Inc.) at 150 rpm and 30°C. For large-scale production of metabolites, cultures were incubated in 1 liter of GM in 2.8-liter Fernbach flasks for 7 days. Mycelia were harvested from the growth medium by vacuum filtration and washed with sterile distilled water, RM, or LSRM.

Conversion of secondary metabolites to aflatoxins. For feeding experiments in biosynthetic studies, the incubation medium was prepared by careful addition of 9.9 ml of LSRM to 0.1 ml of the metabolite solution in acetone in 50-ml Erlenmeyer flasks according to established techniques (6, 21, 28). Three-day-old mycelia were harvested by vacuum filtration and washed extensively with LSRM. One gram (wet weight) of washed mycelia was added to each flask containing the incubation medium. The contents were then incubated for 20 h at 30°C with constant shaking at 150 rpm. The OMST and ST used in some of the feeding studies were purchased from Sigma Chemical Co.

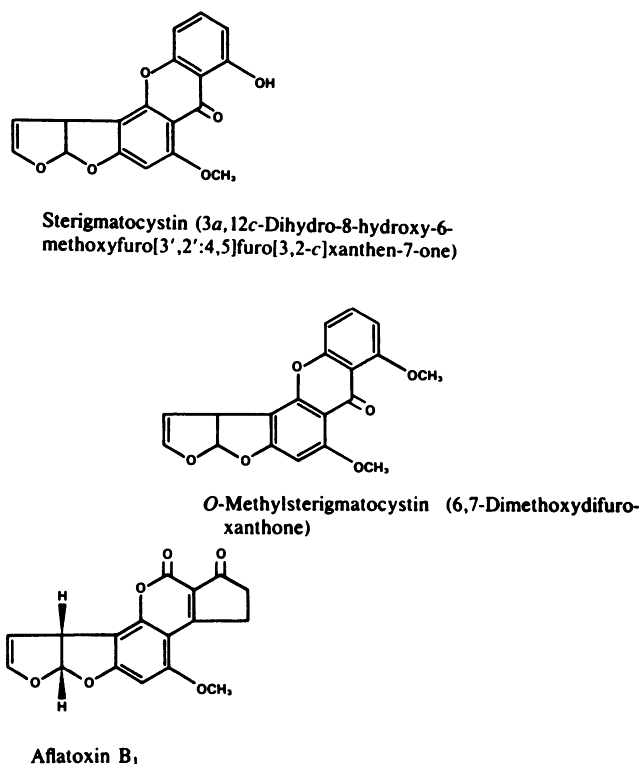

FIG. 1. Structure of aflatoxin B_1 and selected precursors (11).

Extractions. After the desired length of incubation, the mycelia were extracted by standard procedures as described earlier (17, 26). The metabolites were purified primarily by preparative thin-layer chromatography (TLC) with ether-methanol-water (96:3:1, vol/vol/vol) as the developing solvent. Further purification of the secondary metabolites was carried out by Sephadex LH-20 column chromatography (column size, 3.5 by 15 cm) with methanol or acetone as the developing solvents.

Analysis of metabolites. Identities of the metabolites were established by chromatographic behavior on TLC and by mass spectrometry with a Finnigan 4000 spectrometer, using analytical standards of ST, OMST, and aflatoxins.

Aflatoxins, ST, and OMST were separated and identified by one-dimensional TLC in the ether-methanol-water (96:3:1, vol/vol/vol) solvent system. After development, a preliminary visual estimate of the metabolite was made by inspection under long-wavelength UV light. This estimation was used to appropriately dilute or concentrate the extract samples to desired volumes for further analysis. Specific volumes of test extracts (containing 10 to 20 ng of either aflatoxins or metabolites) were spotted on prescored 250-μm-thick Silica Gel 60 plates (20 by 20 cm; EM Science/Merck & Co., Inc., 5763-7) to obtain optimum fluorescence for densitometric readings. ST was detected by spraying the plates with 20% (vol/vol) aluminum chloride in ethanol and heating for a few minutes in an oven at 120°C (44). Plates were scanned for fluorescent materials (Shimadzu dual-wavelength TLC scanner, CS-930), with excitation wavelength of 310 nm for OMST and 360 nm for aflatoxins and ST. The quantities of aflatoxins, ST, and OMST were calculated by comparisons with areas of peaks of standards run on the same plate. Quantities of ST and OMST solutions were verified spectrophotometrically (Shimadzu UV-visible dual-beam spectrophotometer, UV-160), using extinction coefficients of ε_{235} = 24,500 or ε_{249} = 27,500 M^{-1} cm^{-1} for ST in ethanol and ε_{236} = 40,700 or ε_{310} = 16,500 M^{-1} cm^{-1} for OMST in methanol (11).

Preparation of [^{14}C]OMST. Four 100-ml portions of GM were inoculated with *A. parasiticus* isolate CP461 and incubated for 48 h at 30°C on a rotary shaker. Ball-shaped mycelial clusters were produced. These pellets were pooled and harvested over Miracloth (Calbiochem-Behring) by vacuum filtration and thoroughly washed with RM. Approximately 30 g of wet pellets was added to each of two Erlenmeyer flasks containing 100 ml of RM. Sodium [1-^{14}C]acetate, 59 mCi/mmol (Moravek Biochemicals, Inc.), without any unlabeled acetate, was dissolved in water (1 mCi/10 ml), and 0.5-ml samples were added to each flask at the beginning and at four 1.5-h intervals during incubation at 30°C on a rotary shaker (150 rpm) as described earlier (23, 26). After the last sample of [1-^{14}C]acetate was added, the incubation was continued for an additional 5 h. The metabolites were extracted from the pellets and radiolabeled OMST was purified (see Table 3). Purified OMST was dissolved in 5 ml of Kodak I (Eastman Kodak Co.) scintillation solvent, and the radioactivity was determined in a Beckman LS 1800 liquid scintillation counter. Disintegrations per minute were corrected for quenching, using a standard quench curve. The concentration of [^{14}C]OMST was determined by both TLC and spectrophometric analyses.

Radiolabel incorporation into aflatoxins. Radiolabel incorporation experiments (feeding studies) were conducted with samples of [^{14}C]OMST; no unlabeled carrier was used. After appropriate incubation, the metabolites were extracted and separated on TLC in ether-methanol-water (96:3:1,vol/vol/vol). The bands of interest were scraped off, eluted with the solvent, and rechromatographed in the same solvent. Densitometric scans of single fluorescent bands were made to determine the concentration of aflatoxins and metabolites; specific zones of the TLC plate were stripped directly into scintillation vials, and the radioactivity in each band was determined.

Autoradiograms of TLC plates were prepared by covering the plate with X-ray film (Kodak XAR-5) without an intensifying screen, and plates were stored in darkness for 1 to 5 days. After being developed and fixed, the autoradiograms were superimposed on TLC plates and examined under long-wavelength UV light to visualize the bands on the TLC plates that contain radioactivity.

RESULTS

Characterization of *A. parasiticus* isolate CP461. The cultures of *A. parasiticus* (CP461) were grown for 2 to 7 days on GM and extracted, and metabolite production was examined by TLC in the ether-methanol-water solvent (Table 1). The extracts contained no detectable fluorescence at the R_f values for aflatoxin B_1 (0.37), B_2 (0.33), G_1 (0.28), G_2 (0.24), B_{2a} (0.21), or M (0.18). A pale-blue fluorescent component with a somewhat higher R_f than that of aflatoxin B_1 was detected; this compound was chromatographed in various solvent systems (Table 1) that distinctly separated ST, OMST, and aflatoxins B_1 and G_1. The metabolite from isolate CP461 was observed to be chromatographically identical to OMST. The most appropriate developing media for resolving ST, OMST, and the aflatoxins (B_1 and G_1) were ether-methanol-water (96:3:1, vol/vol/vol) and toluene-ethyl acetate-acetic acid (50:30:4, vol/vol/vol). Since the putative

TABLE 1. TLC separation of ST, OMST, aflatoxins B_1 and G_1, and the metabolite from *A. parasiticus* (CP461)

Solvent system	Ratio (vol/vol)	R_f, 10^2					Developing time (min)[a]
		ST	OMST	B_1	G_1	CP461 product	
Ether-methanol-water	96:3:1	97	44	37	28	44	45
Toluene-ethyl acetate-acetic acid	50:30:4	75	43	35	24	43	45
Toluene-ethyl acetate-acetone	60:25:15	86	29	41	30	29	45
Chloroform-acetone	10:0.5	74	24	22	11	24	40
Chloroform-methanol	10:0.5	93	69	63	55	69	40
Carbon tetrachloride-methanol	10:2	81	42	39		41	45

[a] The TLC plates were spotted with approximately 50 ng of various compounds and developed for a distance of nearly 14 cm.

OMST exhibited mobility significantly higher than B_1 in the ether-methanol-water and toluene-ethyl acetate-acetic acid solvent systems, it could not have been confused with aflatoxin B_1, as had been suggested earlier by Jeenah and Dutton (24). Also, when sprayed with aluminium chloride, the apparent OMST spot yielded the bright yellow-green fluorescence characteristic of OMST and xanthones but not aflatoxins (24, 38). The purity of OMST and aflatoxins B_1 and G_1 obtained in these studies was independently verified by repeated TLC in different solvent systems and by comparison of their mass spectra with known standards and published data (11).

The metabolite of isolate CP461, on spectral analysis in methanol, exhibited two prominent characteristic absorbance peaks at 241 and 318 nm that were identical to peaks exhibited by an OMST standard. Spectral results were similar to reports by Cole and Cox (11) and Davies et al. (12) of sharp absorbance peaks at 236 and 310 nm for OMST. Mass spectra of the isolated metabolite exhibited a molecular ion at m/z 338 and major fragments at m/z 323 and 309, almost identical to that observed for a commercial standard of OMST (Table 2) and that described by Cox and Cole (11). The m/z 323 and 309 fragments are most likely due to losses of the C-19 methyl group and the C-13 carbonyl (Fig. 1), respectively, determined from a comparison with the mass spectrum of ST reported by Cole and Cox (11).

Feeding ST, a known aflatoxin precursor, to resting 3-day-old cultures of CP461 resulted in the production of only OMST without the production of aflatoxins. Incubation for 24 h with increasing concentrations of ST in LSRM produced higher concentrations of OMST by the mycelia of isolate CP461 relative to controls (Fig. 2). From the data presented in Fig. 2 for the correspondence of ST feeding and OMST accumulation (correlation coefficient = 0.99), it was determined (from the slope of the least-squares analyses) that >86% of ST was converted to OMST. In all cases, total loss of added ST was observed after the 24-h incubation with the mycelium. That this reaction (ST → OMST) was enzymatic was indicated from the observation that incubation of autoclaved or boiled 3-day-old mycelia from CP461 cultures with ST produced no detectable conversion of ST to OMST, and essentially all of the substrate ST fed to the inactivated mycelia was recovered from the incubation medium.

Incorporation of ^{14}C label into OMST. To verify the presence of enzymes for OMST synthesis within the aflatoxin biosynthetic pathway, *A. parasiticus* isolate CP461 cultures were used to synthesize ^{14}C-labeled OMST from [1-^{14}C]sodium acetate. The autoradiograph of the developed TLC plate containing the purified [^{14}C]OMST indicated a concentration of all detectable radioactivity within the absorbant zone containing the OMST. With additional TLC developing systems (toluene-ethyl acetate-acetic acid and chloroform-methanol [Table 1]), it was observed that the purified [^{14}C]OMST had a constant specific activity. These results indicate that all of the observed ^{14}C radiolabel was contained in [^{14}C]OMST.

The typical yield of OMST was between 0.05 and 0.06 mg per g of mycelial wet weight. Radiolabel from [1-^{14}C]acetate was incorporated into OMST with about 1.5% efficiency, resulting in [^{14}C]OMST with specific activity of nearly 1.35 Ci/mol (Table 3). All acetate disappeared from the medium during the 24 h of incubation in RM with the fungal mycelia.

TABLE 2. Mass spectral data of OMST standard and the product isolated from *A. parasiticus* (CP461)

m/z	Relative intensity (%)	
	OMST	CP461 product
338	100	100
323	29.90	31.61
309	53.06	48.23
292	18.16	17.88
279	13.34	13.11
265	20.52	19.48
249	25.52	21.66
221	10.05	8.80

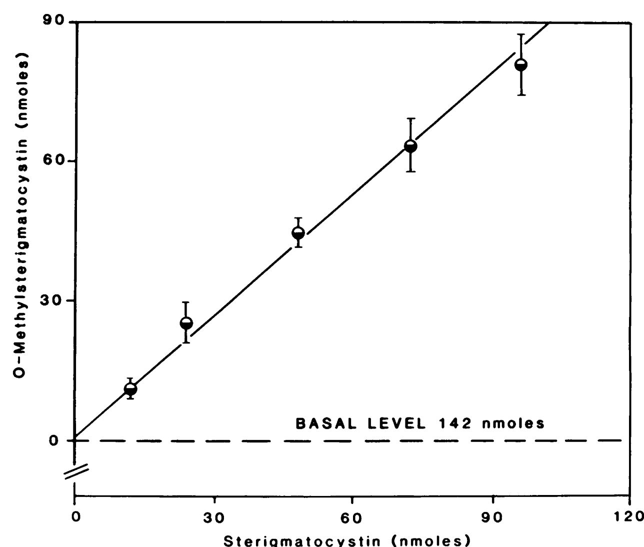

FIG. 2. Conversion of ST to OMST by *A. parasiticus* (CP461). A 1.0-g (wet weight) amount of 3-day-old mycelia was incubated with various concentrations of ST in low-sugar resting medium. The products were extracted after 24 h and assayed on TLC as described in Materials and Methods.

TABLE 3. ^{14}C-labeled OMST produced by *A. parasiticus* (CP461) from sodium [1-^{14}C]acetate

Expt[a]	OMST recovered (mg)	Radioactivity in OMST (μCi)[b]	Sp act of OMST (Ci/mol)	Incorporation efficiency (%)[c]
1	3.44	14.1	1.38	1.41
2	3.91	15.6	1.34	1.56

[a] The [1-^{14}C]acetate (59 Ci/mol) was added to 70 g (wet weight) of 2- to 3-day-old fungal cultures in several portions as described in Materials and Methods.

[b] Data were corrected for recovery (90 to 95%) of added radioactivity.

[c] Percentage of added radioactivity (1 mCi) recovered in purified, ^{14}C-labeled OMST.

TABLE 4. Incorporation of [^{14}C]OMST and [1-^{14}C]acetate into aflatoxin B_1

Precursor added	Fungal strain used[a]	Radio-activity added (μCi)	Sp act of precursor (Ci/mol)	Product of aflatoxin B_1 (μCi)[b,c]	Sp act of aflatoxin B_1 (Ci/mol)[c]
[^{14}C]OMST	AVN-1	1.41	1.38	1.02 ± 0.14	1.28 ± 0.10
[^{14}C]OMST	SU-1	1.41	1.38	0.91	1.18
[^{14}C]acetate	SU-1	333	59	1.46 ± 0.18	

[a] The description of the fungal strains and the conditions used for the incorporation studies are given in Materials and Methods.

[b] Data were corrected for recovery (90 to 95%) of added radioactivity.

[c] Values represent the mean and standard error of three experiments.

Conversion of OMST and ST to aflatoxins. To study the role of OMST in the aflatoxin biosynthetic pathway, [^{14}C]OMST was fed to the *A. parasiticus* mutant AVN-1. The AVN-1 mutant is blocked in the aflatoxin pathway before ST synthesis. Nearly 70% of the radiolabel from OMST was incorporated into aflatoxin B_1 by AVN-1 or the parent strain SU-1 (Table 4). In contrast, <0.5% of the ^{14}C label was incorporated from [1-^{14}C]acetate into aflatoxin B_1 by the aflatoxin-producing *A. parasiticus* (SU-1). The specific activity of aflatoxin B_1 obtained in feeding experiments was 93% of that of the OMST precursor (Table 4).

Both ST and OMST were fed to cultures of AVN-1 in LSRM, and the aflatoxins produced after a 24-h incubation were extracted and quantitated by TLC. A 10-nmol amount of OMST produced 7.8 ± 0.5 nmol of B_1 and 1.0 ± 0.1 nmol of G_1, whereas 10 nmol of ST produced 6.4 ± 0.6 nmol of B_1 and 0.6 ± 0.1 nmol of G_1.

TLC of the post-incubation media and mycelial extracts (with spots enhanced with $AlCl_3$) showed no detectable enhancement of fluorescence at the R_f values for ST or OMST, indicating that the total amount of precursors fed to the resting cultures was utilized. No detectable radioactivity was observed in the ST region on autoradiograms of TLC plates from the [^{14}C]OMST feeding experiments. Therefore, OMST was not being converted to ST from the pH change (5.5 to 2.5) during the incubation, prior to uptake of OMST by the mycelium. In controls, the inability of autoclaved cells to utilize ST or OMST was demonstrated by the presence of 90 to 95% of the test precursors (ST or OMST) in the incubation medium at the end of the 24 h of incubation.

The time course of conversion of ST and OMST into aflatoxin B_1 by resting cultures of AVN-1 was elucidated (Fig. 3). In ST feeding experiments (Fig. 3A) detectable levels of OMST were produced during the early phase of ST conversion to aflatoxin B_1 (0 to 30 min). However, during the stages of rapid, linear conversion of the precursor (1 to 4 h), no OMST production was detected. The ST was totally converted to aflatoxin B_1 in nearly 6 h, whereas the complete conversion of OMST to B_1 took just 4 h (Fig. 3B). The OMST-to-B_1 reaction did not exhibit the slow, gradual conversion kinetics in the early stages of the process (0 to 30 min) similar to the apparent "lag" phase observed during the conversion of ST to B_1. In the OMST feeding studies no detectable ST formation was observed.

DISCUSSION

In this study the major metabolite of the nonaflatoxigenic *A. parasiticus* isolate (CP461; 14) was identified as OMST. This isolate did not produce any detectable aflatoxins even

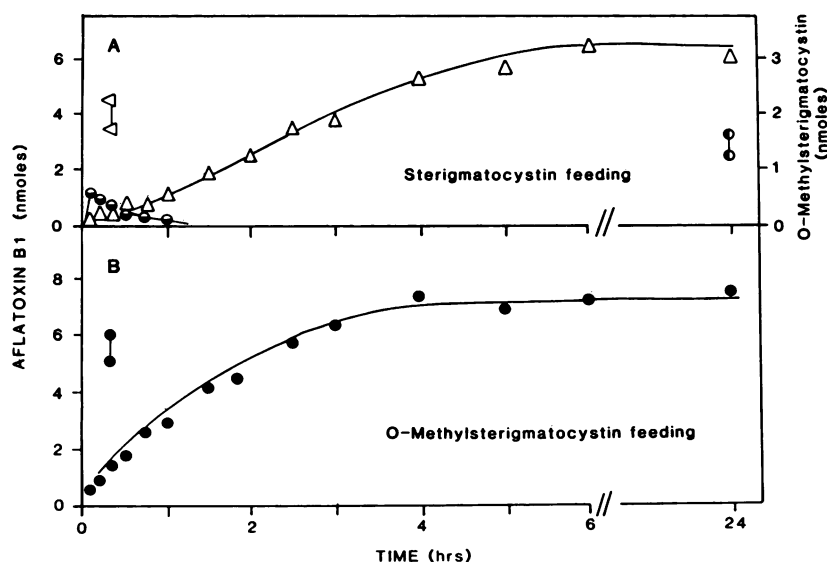

FIG. 3. Time course of conversion of OMST (●) and ST (△) to aflatoxin B_1. A 1.0-g (wet weight) amount of 3-day-old mycelia was incubated with 10 nmol (in 10 μl of acetone) of ST (A) or OMST (B) in low-sugar resting medium. The aflatoxins and OMST (from ST feeding, ○) produced were extracted at various intervals and assayed by TLC as described in Materials and Methods. No aflatoxins were observed in control experiments with autoclaved cells during the 24-h incubation. The standard deviation between replicates (3) was never >8%.

when relatively high proportions of ST, a known precursor of aflatoxins (21, 24, 37, 40), were fed to resting cultures. However, an active conversion of ST to OMST was discovered in these cultures.

Jeenah and Dutton (24) have reported that cell-free cultures of *A. parasiticus* can convert ST to OMST. However, OMST has been considered to be a side shunt metabolite in the aflatoxin biosynthetic pathway (24, 38). Dutton et al. (17) have suggested that there may be an intermediate between ST and aflatoxin B_1. From the observation presented here, OMST appears to be the aforesaid intermediate between ST and aflatoxin B_1 in the aflatoxin biosynthetic pathway. The results supporting this hypothesis are as follows. (i) ST is directly converted to OMST in vivo by the nonaflatoxigenic *A. parasiticus* isolate (CP461). (ii) [1-^{14}C]acetate was incorporated into OMST by the fungus, and since acetate is an established precursor of aflatoxins (22) and ST (23), it is reasonable to believe that these metabolites share the same biosynthetic pathway and are subject to similar metabolic regulations. (iii) Incorporation efficiency of [1-^{14}C]acetate into OMST (1.5% CP461) is close to that obtained for aflatoxin B_1 (0.44%, AVN-1) from the same amount of starting radioactivity. (iv) The efficiency of conversion of OMST to aflatoxins is higher in the AVN-1 mutant than that obtained from ST (Fig. 3). (v) The total amount of aflatoxins recovered from OMST feedings of the AVN-1 isolate was higher than that from feeding ST (Fig. 3), differences which cannot be accounted for by differences in recovery of metabolites from the extraction procedure (90 to 95% in either case). (vi) OMST is more rapidly converted to aflatoxin B_1 than is ST, and the conversion of OMST to B_1 does not exhibit the lag phase observed in the first 30 min of the ST-to-B_1 reaction, at which time ST is apparently being converted to OMST as well. The differences in polarity of ST and OMST affecting their differential uptake by the mycelia cannot explain these putative differences in reaction rates since both OMST and ST are taken up by the mycelia in <5 min and converted to aflatoxins (Fig. 3). (vii) Most important, the specific activity of aflatoxin B_1 product from [^{14}C]OMST feeding experiments is nearly identical to that of the parent precursor [^{14}C]OMST (Table 4), demonstrating that there was no pooling or dilution of the precursors in the synthesis of [^{14}C]aflatoxin B_1.

The results of the current study demonstrate that OMST is probably a required intermediate in aflatoxin biosynthesis by *A. parasiticus*. OMST fulfills two of the postulated requirements (28) for an intermediate; i.e., it is synthesized by an isolate (CP461 in this case) blocked in aflatoxin synthesis, and it is converted into aflatoxin by parent strain. Other preliminary evidence, using the fungal cell-free system, which supports this hypothesis has been recently reported from our laboratory (A. R. Lax, T. E. Cleveland, D. Bhatnagar, and L. S. Lee, Plant Physiol. Suppl. **80**:19, 1986; T. E. Cleveland, A. R. Lax, L. S. Lee, and D. Bhatnagar, Plant Physiol. Suppl. **80**:18, 1986). Detailed reports of these preliminary studies will be published elsewhere. Studies with purified enzymes are needed to further characterize the aflatoxin biosynthetic pathway in explicit detail. The data presented here, however, suggest that the latter stages of aflatoxin biosynthesis include the following steps: sterigmatocystin $\rightarrow$ OMST $\rightarrow$ aflatoxins B_1 and G_1.

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