ABA (Table III). A 100-ppm ABA solution caused a significant promotion of abscission without marked acceleration of ethylene production, which naturally characterized the abscissing fruits of the control. Moreover, the 100-ppm infused fruits ripened simultaneously with the control fruits, that is, abscission occurred during the preclimacteric phase.

The 1000-ppm solution was apparently supraoptimal and considerably advanced the ripening date. From our work and the results of Cooper and Horanic (1973), it may be claimed that ABA can regulate fruit abscission independently and not necessarily through the acceleration of ripening or ethylene production.

A more detailed elucidation of the role of ABA in the hormonal regulation of the abscission process will be possible after studying the level of endogenous ABA during the abscission process of fruits, and the influence of ethylene in such systems.

Satisfactory penetration and distribution of the plant regulators, which we believe we achieved, help in the understanding of their role in the natural abscission and ripening processes of the fruit. However, this is insufficient for drawing any conclusions about the role of the plant regulators in tree-attached fruits.

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Biosynthesis of Aflatoxin B_1. Conversion of Versicolorin A to Aflatoxin B_1 by *Aspergillus parasiticus*

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Forty-six percent of added [^{14}C]versicolorin A was efficiently converted to aflatoxin B_1 by a resting cell culture of wild-type *Aspergillus parasiticus*. The labeled pigment was isolated from [1-^{14}C]acetate enriched cultures of a mutant of *A. parasiticus* that elaborates several versicolorin pigments, but no aflatoxins. The high level of incorporation into aflatoxin B_1 and the relative specific activity of 0.475 of aflatoxin B_1 isolated from the culture of *A. parasiticus* enriched with [^{14}C]versicolorin A indicate that this C-18 polyketide-derived hydroxyanthraquinone is a precursor to aflatoxin B_1.

Aflatoxins, closely related secondary metabolites produced by certain strains of *Aspergillus flavus* and *A. parasiticus*, are of considerable importance because of their toxicity (Turner, 1971) and their carcinogenicity (Butler, 1969), but until recently little experimental evidence has been accumulated for their biogenesis. Studies on the incorporation of acetate by Hsieh and Mateles (1971) and detailed degradation studies by Biollaz et al. (1968a,b, 1970) indicate that the carbon skeleton of aflatoxin B_1 is derived entirely from acetate. This evidence and the similarity in structure between many anthraquinones, sterigmatocystins, and aflatoxins led to speculation, without experimental evidence, that these compounds share a common pathway (Holker and Underwood, 1964). In the biogenetic scheme hypothesized by Thomas (1965) two hydroxyanthraquinones, averufin (I, Figure 1) and 6-deoxyversicolorin A (III, Figure 1), and sterigmatocystin (IV, Figure 1) are proposed as intermediates in the biosynthesis of aflatoxin B_1 (V, Figure 1).

Versicolorin A (II, Figure 1) has specifically been proposed as such an intermediate by Heathcote et al. (1973). Versicolorin A, sterigmatocystin, and aflatoxins contain a di- or tetrahydrofurobenzofuran group. With one exception (Bassett et al., 1970) the occurrence of this furano group in natural products is peculiar to these or related mold metabolites. *O*-Methylsterigmatocystin (Burkhardt and Forgacs, 1968), aspertoxin (Rodricks et al., 1968), versicolorin C (Heathcote and Dutton, 1969), versicolorin A (Lee et al., 1975), and sterigmatocystin (Schroeder and

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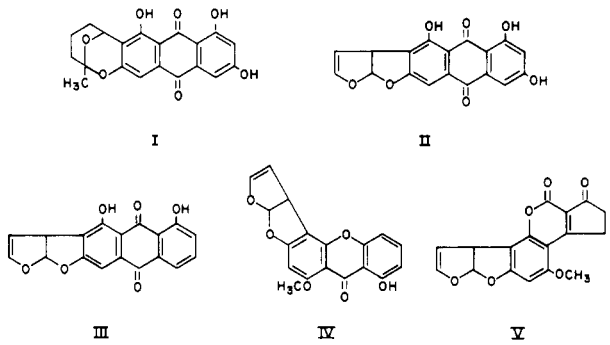

Figure 1. I, averufin, $C_{20}H_{16}O_7$; II, versicolorin A, $C_{18}H_{10}O_7$; III, 6-deoxyversicolorin A, $C_{18}H_{10}O_6$; IV, sterigmatocystin, $C_{18}H_{12}O_6$; V, aflatoxin B_1, $C_{17}H_{12}O_6$.

Kelton, 1975) contain this moiety, and these metabolites have been identified in aflatoxin-producing strains of *A. parasiticus*. It seems logical that mold metabolites containing the di- or tetrahydrofurobenzofuran should be biogenetically linked. However, proof of such a link awaited mutants with enhanced ability to produce some of the proposed intermediates. Such mutants are extremely valuable in synthesizing enough of such intermediates for radioactive incorporation studies. One such mutant which produced large amounts of averufin (Donkersloot et al., 1972) was used in the elegant work reported by Lin et al. (1973) and Lin and Hsieh (1973) on conversion of labeled averufin into aflatoxin B_1 by a resting cell culture of *A. parasiticus*. Another such mutant produced in our laboratory by ultraviolet irradiation of wild-type *A. parasiticus* (Bennett and Goldblatt, 1973) synthesized versicolorin A (Lee et al., 1975). The present report describes the use of this mutant to prepare radioactive versicolorin A, and the subsequent conversion of this labeled pigment into aflatoxin B_1 by resting cell cultures of wild-type *A. parasiticus*.

EXPERIMENTAL PROCEDURES

Strains and Culture Media. The wild-type aflatoxin producing strain of *A. parasiticus* was SU-1 (NRRL A-16, 462). The white spored, versicolorin producing mutant (1-11-105 wh-1) was derived from this parent stock after ultraviolet irradiation (Bennett and Goldblatt, 1973). Stock cultures were maintained on potato dextrose agar (Difco) plus 0.5% yeast extract. Approximately 10^6 conidiospores were used for each initial inoculation.

The growth medium (GM) and replacement medium (RM) were both formulated according to Adye and Mateles (1964). The low sugar replacement medium (LSRM) contained 1.62 g of glucose per liter of medium (Lin et al., 1973).

Preparation of [^{14}C]Versicolorin A. Four 100-ml portions of the GM were inoculated with the mutant strain (1-11-105 wh-1) and incubated 48 h at 30 °C on a rotary shaker. Ball-shaped mycelial clusters approximately 2 mm in diameter were produced by the constant rotary motion of the shaker. These pellets were pooled and collected on cheesecloth and thoroughly washed with RM. Approximately 30 g of wet pellets was added to each of two 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 0.5-ml aliquots were added to each flask at the beginning and at four 1.5-h 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 h. The entire contents of each flask was filtered through cheesecloth to separate mycelia from medium. The medium contained no pigments and was discarded. Pellets were collected on cheesecloth and soaked in acetone and the extract filtered. Two volumes of water were added and the solution was extracted with chloroform. This chloroform extract was evaporated to near dryness and the various pigments were separated by thin-layer chromatography (TLC) on Adsorbosil-1 silica gel coated plates developed in benzene–acetic acid (95:5, v/v). The pigment, R_f 0.32, was eluted from the gel with acetone, and was further purified by rechromatography in chloroform–acetone (9:1, v/v) and in benzene–acetic acid–methanol (90:5:5, v/v/v); yield, 5.2 mg of [^{14}C]versicolorin A. Purity of 97% was established by comparison of fluorescence intensity on TLC of the sample with that of authentic versicolorin A isolated and purified by Lee et al. (1975). Radioactivity of an aliquot of this sample was measured in 15 ml of Eastman I scintillation solvent on a Searle Isocap/300/liquid scintillator system. Disintegrations per minute (dpm) were corrected for quenching, using a standard eight-sample quench curve. The 5.2 mg of [^{14}C]versicolorin A contained 5.5 μCi (0.358 Ci/mol). A stock acetone solution was prepared to contain 1 μCi/ml. All conversion experiments were conducted with aliquots from this solution; no unlabeled carrier was used.

Conversion of [^{14}C]Versicolorin A into [^{14}C]Aflatoxin B_1. Mycelial pellets of the wild-type *A. parasiticus* were prepared by inoculating GM with spores and incubating for 48 h at 30 °C in a rotary shaker. Pellets were collected and prepared in the same manner used for the mutant strain described above. In these experiments, 0.2 ml of acetone containing [^{14}C]versicolorin A was placed in a 50-ml Erlenmeyer flask, 9.8 ml of LSRM was added slowly, and then 1 g (wet weight) of pellets was dropped into each flask. Two levels of [^{14}C]versicolorin A incorporation were used, 0.1 and 0.2 μCi. Controls were run in which sodium [1-^{14}C]acetate was added at two levels, 10 μCi (0.1 ml) and 20 μCi (0.2 ml); all experiments were done in triplicate. Another control for each experiment utilized autoclaved cells to verify the enzymatic activity involved in the conversion. After 20 h incubation at 30 °C on the rotary shaker, the medium was carefully decanted from each flask and extracted with chloroform. Pellets were soaked in acetone, the acetone extract was adjusted to approximately 30% with water, and this aqueous acetone solution was extracted with chloroform. These chloroform extracts were combined with the respective chloroform extracts of the medium and the solvent evaporated. Each sample extract was made to 10-ml volume with chloroform and a 1-ml aliquot was removed for preparative TLC to harvest the labeled aflatoxin B_1. Each sample aliquot was streaked on Adsorbosil-1 silica gel coated plates and developed in chloroform–acetone (9:1, v/v). The B_1 zone (R_f 0.62) was stripped from the plate, eluted with acetone and chloroform, and rechromatographed in benzene–methanol–acetic acid (18:1:1, v/v/v). This B_1 zone, R_f 0.49, contained only a single fluorescent spot when rechromatographed in chloroform–acetone (9:1, v/v). Aflatoxin B_1 zones harvested from the second preparative TLC from each experiment were scraped and eluted from the silica gel with acetone directly into scintillator vials by filtering through loose fritted filters. After the acetone evaporated, 15 ml of scintillating liquid was added and the radioactivity (dpm) was measured. All values of labeled aflatoxins were corrected for quenching and fell within the accepted range, 89 to 91.3%.

A second 1-ml aliquot from each flask was spotted on TLC and aflatoxin B_1 content densitometrically quantitated according to the method of Pons et al. (1968). Micromoles of aflatoxin B_1 reported in Table I were

Table I. Incorporation of [^{14}C]Versicolorin A and Sodium [1-^{14}C]Acetate into Aflatoxin B_1

	Precursor				Product, aflatoxin B_1					
	Amount added			Sp act.,	Amount formed			Sp act.,	Rel	Conversion,[b] %
	μCi	μmol	dpm	Ci/mol	μCi	μmol	dpm	Ci/mol	sp act.[a]	
Versicolorin A	0.1	0.279	222 885	0.358	0.051	0.609	112 617	0.084	0.234	50.5
Versicolorin A	0.2	0.558	445 769	0.358	0.083	0.487	185 090	0.170	0.475	41.5
Acetate	10	0.171	21 635 757	58.5	0.071	0.401	157 220	0.177	0.003	0.73
Acetate	20	0.342	43 271 514	58.5	0.112	0.317	248 310	0.384	0.007	0.57

[a] Specific activity of product/specific activity of precursor. [b] dpm of product/dpm of precursor.

calculated from this quantitation.

Rate of Conversion. Experimental conditions were identical with those of the incorporation studies reported above, except that experiments were run in duplicate and with a new preparation of mycelial pellets from the wild-type *A. parasiticus* incubated for 48 h. Initial flasks were frozen immediately and other flasks were removed from the incubator and frozen after 3, 6, 9, 12, and 20 h. The higher level of [^{14}C]versicolorin A (0.2 μCi) was used so that any nonconverted pigment could be detected.

RESULTS AND DISCUSSION

The conversions of [^{14}C]versicolorin A and of [1-^{14}C]-acetate into aflatoxin B_1 are compared in Table I, which also gives specific activities of the precursors and the product and relative specific activities. There was no incorporation of radioactivity by the autoclaved cells.

At the lower level of [^{14}C]versicolorin A added, conversion of the pigment to aflatoxin B_1 was high (50.5%); none of the added pigment was detected after 20 h of incubation. This limited feeding of precursor probably allowed nearly optimum conversion of pigment precursor to product. At the higher level (0.2 μCi, 0.558 μmol of added pigment), the conversion was lower, 41.5%, and some residual pigment was detected. Since there was more aflatoxin B_1 produced (0.609 μmol) than there was pigment offered (0.279 μmol), particularly at the lower level, some of the glucose contained in the LSRM (approximately 90 μmol) was obviously utilized in the formation of aflatoxin B_1. Specific activities of the aflatoxin B_1 formed after precursor addition at two levels support this observation; at the lower level, specific activity was 0.084 Ci/mol, compared with 0.170 Ci/mol at the higher level. Similar results were obtained in the two control experiments in which [1-^{14}C]acetate was used as a labeled precursor; the specific activity was higher at the higher level of incorporation. Conversion of label was much lower than a similar conversion calculated for the pigment; only 0.73 and 0.57% of the label was found in the aflatoxin B_1 fractions after addition of acetate at the two levels, even though radioactivity added was 100 and 200 times the level of that in the pigment.

Rate of incorporation of [^{14}C]versicolorin A into aflatoxin B_1 is shown in graphic form in Figure 2. After 9 h of incubation, the maximum conversion of [^{14}C]versicolorin A into aflatoxin B_1 had been reached and neither the amount of B_1 nor the conversion of precursor to product increased after that time. At the beginning of incubation, versicolorin A accounted for 98% of the radioactivity, but at 3 h only 16.5% remained in the pigment. Little residual [^{14}C]versicolorin A was detected after 12 h of incubation. The sharp drop in radioactivity of versicolorin A between 0 and 3 h indicates that the initial conversion is rapid. Between 3 and 6 h the rate of conversion is slower. More than one conversion reaction is involved because radioactivity was detected in a new pigment (R_f 0.27 in benzene–acetic acid, 95:5, v/v) after 3, 6, and 9 h of incubation. Incubation for 20 h permitted good comparison

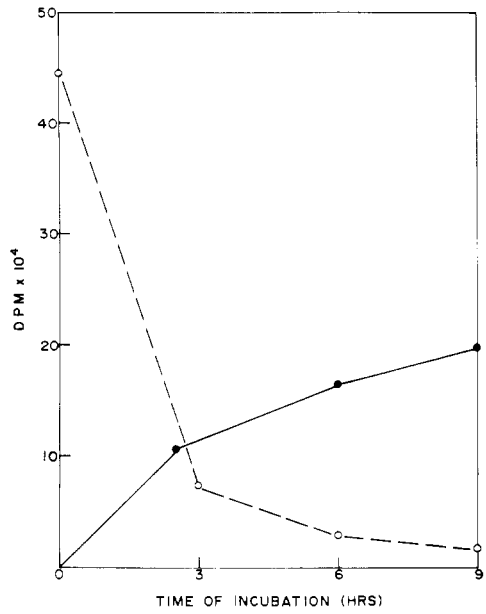

Figure 2. Time course conversion of versicolorin A (○) into aflatoxin B_1 (●).

with earlier conversion experiments at this level of incorporation.

Specific activity, defined as Curies/mole, is a measure of the amount of radioactivity in a compound. Since the sodium acetate used in these studies was not diluted with unlabeled acetate, it consequently had a high specific activity (58.5 Ci/mol). Most of the molecules were labeled. In comparison, the specific activity of the randomly labeled versicolorin A was only 0.358 Ci/mol. Measurements of disintegrations per minute and specific activities on the aflatoxin B_1 formed from the two sources are not as divergent as those for the precursors. This indicates that the label from acetate is not only found in aflatoxin B_1, but also in many other metabolites, whereas nearly half of the label from versicolorin A was detected in aflatoxin B_1.

Relative specific activity (RSA) is a term used to indicate the relationship between precursor and product in isotope labeled compounds (Hsieh et al., 1973). It is the number of labels from the precursor incorporated into the product and is proportional to the concentration of the substrate in question (Hsieh and Mateles, 1971). Our results corroborate this statement; in these incorporation studies, the RSA of B_1 formed is lower (0.234) for the lower level of versicolorin A precursor, 0.1 μCi, and higher (0.475) for the 0.2-μCi level of pigment. This ratio of incorporation could possibly have been higher at a slightly higher level of precursor concentration but due to solubility properties of the pigment in acetone it was not possible to add a higher level of versicolorin A as a precursor without adding a greater amount of acetone. The theoretical maximum RSA for acetate into aflatoxin B_1 is 9, for averufin, 0.9, for sterigmatocystin, 1 (Hsieh et al., 1973), and for versicolorin

A, the RSA should be 0.9 if labeling is the same as that expected from averufin (Lin et al., 1973). In our experiments the quite low RSA for aflatoxin made from acetate (0.007) compared to the relatively high RSA (0.475) for aflatoxin made from the labeled versicolorin A pigment offers additional strong evidence that this pigment is incorporated essentially intact and is not broken down into acetate units before incorporation into aflatoxin B_1.

Hsieh et al. (1973) report 45–58% conversion of sterigmatocystin to aflatoxin B_1 and conclude that sterigmatocystin or a closely related metabolite is an intermediate in the biosynthesis of aflatoxins. The 46% conversion of versicolorin A to aflatoxin B_1 found in our experiments suggests that this pigment is as efficiently converted to aflatoxin B_1 as is sterigmatocystin and offers experimental proof to the theory hypothesized by Heathcote et al. (1973) in which they propose versicolorin A as a precursor to aflatoxin B_1.

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Biosynthesis of Aflatoxin. Conversion of Norsolorinic Acid and Other Hypothetical Intermediates into Aflatoxin B_1

Dennis P. H. Hsieh,* Ming T. Lin,[1] Raymond C. Yao, and Rajendra Singh

Five compounds were separately incubated with resting mycelium of the aflatoxin (AF) producing strain of *Aspergillus parasiticus*, under conditions found optimal for converting averufin (AR) into AFB_1. They were: four tritiated derivatives of C_{18} anthraquinone and anthrone synthesized to simulate possible intermediates in the pathway of AF biosynthesis, and the ring-labeled [^{14}C]norsolorinic acid (NA) produced from [1-^{14}C]acetate by a mutant culture of *A. parasiticus* deficient in AF biosynthesis. Results indicate that only a trace amount of labels from one of the four tritiated compounds was incorporated into AFB_1, whereas over 2% of the label from NA was accountable in the repeatedly purified AFB_1 which possessed a specific activity of 0.19 relative to that of [^{14}C]NA. The efficiency of conversion of NA into AFB_1 as compared to that of AR, an orange pigment (OP), versicolorin A (VA), and sterigmatocystin (ST), along with the structural relationship of NA and AR and the pattern of accumulation of NA by the mutant of *A. parasiticus*, suggest that NA is an intermediate in the pathway of AF biosynthesis one step (or steps) before AR. Experimental evidence is thus provided for the pathway for AFB_1 biosynthesis as 10 acetate → NA → AR → OP → VA → ST → AFB_1.

The remarkable molecular structure of aflatoxins (AF's) and their significance as foodborne carcinogenic mycotoxins have prompted intensive studies on their occurrence, chemistry, and biological effects. Their biosynthesis by *Aspergillus parasiticus* has also been a subject of continued investigation.

Several experimental approaches have been employed by different investigators in the elucidation of the pathways for AF biosynthesis. Based on the structural characteristics of AF's and possible analogy to the synthesis of other fungal secondary metabolites, several plausible pathways were advanced with no experimental evidence (Moody, 1964; Holker and Underwood, 1964; Heathcote et al., 1965; Thomas, 1965). Particularly notable is the

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