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Biosynthesis of Versicolorin A

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The incorporation of various potential intermediates into versicolorin A by a versicolorin A-accumulating mutant of *Aspergillus parasiticus* was studied. Both whole mycelium and cell-free extracts of this mutant were able to convert ^{14}C-labeled versiconal hemiacetal acetate to versicolorin A. By the use of a labeled double substrate technique it was shown that two other compounds, versicolorin A hemiacetal and its acetate derivative, were also converted to versicolorin A. It is concluded that one or both of these compounds are intermediates in the biosynthesis of versicolorin A and therefore may possibly be involved in the biogenesis of the aflatoxins.

The role of versicolorin A (VA) as a key intermediate in the biosynthesis of aflatoxin B_1 (compound III) is now generally accepted. There is a sufficient body of evidence to support this conclusion, including the conversion of VA to aflatoxin B_1 by whole mycelium (8), protoplasts (3), and nuclear magnetic resonance studies of ^{13}C-enriched material (6). The accumulation of VA in the mutant *Aspergillus parasiticus* (1-11-105 Wh1) is also suggestive of its role in aflatoxin biosynthesis (9).

However, one result which is not in agreement with this large volume of evidence is that obtained by utilizing cell-free preparations derived from protoplasts of *Aspergillus flavus* (1). Although the closely related anthraquinone versiconal hemiacetal acetate (VHA) was converted to aflatoxin B_1 (16% conversion), no incorporation of VA was observed. Thus, this particular area in aflatoxin B_1 biosynthesis is rather obscure, although it has been demonstrated that VHA is a precursor of VA (14). The work reported here investigates the role of VHA and attempts to identify other related intermediates involved in the biosynthesis of VA.

MATERIALS AND METHODS

Preparation of labeled metabolites. Labeled precursors were prepared as per the reference cited. Purity of the material was ensured by repeated preparative chromatography, which involved the use of two different solvent systems. This was followed by a recrystallization step. The amount of material present was determined by weighing, and this was checked from the extinction coefficient at a suitable absorbance maximum. The activity of the material was found by dissolving a known quantity in scintillation fluid in a scintillation vial and counting as outlined below. Identity of the material was established by comparison with authentic material both by mass spectrometry and thin-layer chromatography.

VHA (I). [^3H]- and [^{14}C]VHA were prepared from sodium [^3H]- and [^{14}C]acetate utilizing a culture of *A. flavus* (N1) treated with dichlorvos (15). The solvent systems used were toluene-ethyl acetate-acetone-acetic acid (50:35:15:2) and chloroform-acetone (9:1) on Silica Gel G60 layers. R_f values were 0.73 and 0.22, respectively; λ_{max} was 450 nm; Σ was 7,400.

VA (II). [^3H]- and [^{14}C]VA were prepared from sodium [^3H]- and [^{14}C]acetate with *A. parasiticus* (1-11-105 Wh1) (8). The solvent systems used were toluene-ethyl acetate (7:1) and chloroform-acetone, both with Silica Gel G60 layers and R_f values of 0.70 in both cases; λ_{max} was 453 nm; Σ was 8.166.

VC. ^{14}C-labeled versicolorin C (VC) was conveniently prepared by acid hydrolysis of [^{14}C]VHA (12). [^{14}C]VHA was treated with an excess of 2 M sulfuric acid containing acetone (20%, vol/vol). The mixture was refluxed for 8 h, cooled, and extracted with portions of ethyl acetate until no further pigment passed into the organic layer. The ethyl acetate was removed under reduced pressure, and the [^{14}C]VC was separated chromatographically by three runs in the toluene-ethyl acetate system (R_f, 0.68) and one run in the chloroform-acetone system (R_f, 0.64). The product was recrystallized from acetone; λ_{max} was 456; Σ was 6,100.

VAOH (IVA). ^3H- and ^{14}C-labeled versicolorin A hemiacetal (VAOH) were prepared from [^3H]- and [^{14}C]VA by treating with an excess of cold 2 M sulfuric acid containing acetone (20%, vol/vol) with stirring for 18 h. The reaction mixture was extracted with ethyl acetate and separated until chromatographically pure by three runs in the toluene-ethyl acetate system (R_f, 0.60) and one run in the chloroform-acetone system (R_f, 0.45). The chromatographic properties of this derivative were exactly the same as those of a nonactive sample prepared in the same manner, which had UV and mass spectral properties identical to those reported by Chen et al. for this compound (2); λ_{max} was 456; Σ was 6,100.

VAAC (IVB). ^3H- and ^{14}C-labeled versicolorin A hemiacetal acetate (VAAC) were prepared from [^3H]- and [^{14}C]VA by adding excess glacial acetic acid and a few drops of thionyl chloride and allowing to stand at room temperature for 24 h. The reaction mixture was extracted with ethyl acetate and separated chromatographically until pure by the same

systems as used for VAOH, with R_f values of 0.65 and 0.49 for the toluene-ethyl acetate and chloroform-acetone systems, respectively; λ_{max} was 459; Σ was 7,100. The reaction for the preparation of this derivative is analogous to that for preparing a similar acetoxy derivative of aflatoxin B_1 (5). The mass spectrum of this compound was consistent with its proposed structure (IVB).

Whole-cell experiments. *A. parasiticus* (1-11-105 Wh1) mycelium, incubated in Reddy synthetic low-salts medium (11) for 48 h, was filtered and suspended in replacement medium (70 ml) (7). Labeled substrate was added as an acetone solution (0.4 ml) and incubation was continued. At suitable intervals samples were removed and assayed.

Cell-free extracts. Protoplasts of *A. parasiticus* (1-11-105 Wh1) were prepared as previously described for *A. flavus* N1 (3). Protoplast suspensions in 0.2 M phosphate buffer containing 0.4 M $MgSO_4$, pH 5.8, were pelleted by centrifugation (500 × *g* for 10 min) and lysed by suspending the pellet in 5 ml of lysing buffer (0.1 M phosphate buffer, pH 8.0, at 4°C). This suspension was then homogenized in a hand-held ground glass homogenizer (3 min at 4°C). The lysate was centrifuged (10,000 × *g* for 30 min), and the supernatant was utilized as a cell-free extract. Protein was measured by the biuret method (protein concentration of 3 mg/ml). Cofactors were added to give a final concentration of flavin adenine dinucleotide (10^{-6} M), EDTA (10^{-3} M), dithiothreitol (10^{-3} M), NADPH (1 μmol/4 ml), NADH (1 μmol/4 ml) and substrate (1 μmol/4 ml). Radioactive substrates were added as solutions in *N,N*-dimethyl formamide. Cell-free extracts were incubated in standard Warburg flasks with KOH papers as a CO_2 trap at 30°C with constant shaking. Samples were removed at zero time; after 1 h of incubation, metabolites were extracted. Zero time counts (disintegrations per minute) of all metabolites were subtracted from 1-h counts to give actual counts recorded.

Assay of metabolites. The metabolites were extracted from samples (15 ml) of whole cultures by filtering off the mycelium and washing it in sequence with acetone (5 ml), chloroform (20 ml), and ethyl acetate (5 ml). The washings were shaken with the filtrate, and the organic layer was separated and dried over anhydrous sodium sulfate. The filtrate was extracted with a portion of ethyl acetate (10 ml), which was also dried and added to the washings. The solvent was removed under reduced pressure, and the extract was chromatographed on Silica Gel G60 with toluene-ethyl acetate-acetic acid (60:30:1) as the solvent system. R_f values for this system were: VA, 0.72; VC, 0.70; VHA, 0.52; VAOH, 0.55; and VAAC, 0.61. The thin-layer chromatograph plate was subjected to autoradiography (Kodirex X-ray plate), and the active spots were identified by developing the plate after 10 days of exposure. The silica containing the active metabolites was scraped from the plate, and the compound was washed off with acetone. The compounds were rechromatographed until a single band was observed with two to three runs of the toluene-ethyl acetate-acetic acid solvent system and one run with chloroform-acetone-acetic acid (85:15:1). The R_f values in the latter system were: VA, 0.72; VC, 0.70; VHA, 0.28; VAOH, 0.48; and VAAC, 0.55. Samples of the whole-cell cultures were taken at zero time and treated as above to act as controls for activity appearing in the various fractions which was not due to metabolic activity. The absorption spectra of the metabolites were recorded on a Pye Unicam SP 1800. The activities of the metabolites were determined on a Packard Tricarb 330 liquid scintillation counter; counts were corrected for background, efficiency, and quenching.

Samples (2 ml) from the cell-free systems were extracted with ethyl acetate (2 × 4 ml); the combined extract was dried over anhydrous sodium sulfate and then treated in a manner similar to that described above for the whole cell extracts. When ^{14}C and ^{3}H were counted in the presence of each other, background, efficiency, and quenching were estimated with an external standard.

Effect of dichlorvos on cell-free extracts. (i) Esterase activity. The esterase activity of cell-free extracts was determined by the method of Rahim and Sih (10). The assay was repeated in the presence of various concentrations of dichlorvos (5 to 50 μg/ml); the results are expressed as micromoles of *p*-nitrophenol released per milligram per minute.

(ii) Oxygen uptake. Cell-free extracts (4 ml) containing cofactors were placed in a Warburg flask containing KOH paper as a CO_2 trap. A solution of dichlorvos (0.2 ml) was placed in the side arm, giving a final concentration of 20 μg/ml when added to the main chamber. The flask was equilibrated at 25°C, and oxygen uptake was followed for 1 h. The dichlorvos solution was then added, and oxygen uptake was followed for another hour.

(iii) Inhibition of conversion of substrates. A cell-free extract containing the substrate under test was set up in a Warburg flask as described above, except that the mixture contained 20 μg of dichlorvos per ml. This mixture was incubated for 1 h, and the metabolites were extracted and counted as described above.

Incubation of VC with alcohol dehydrogenase. A solution of alcohol dehydrogenase (Sigma Chemical Co.) was prepared in phosphate buffer (0.1 M, pH 6.0) together with NAD^+ (0.2 μmol/ml). VC was added in *N,N*-dimethyl formamide (0.1 ml) to give a final concentration of 0.5 mg/ml. The mixture (4 ml) was incubated at 25°C for 10 h and then extracted and examined for various metabolites.

RESULTS AND DISCUSSION

The conversion of [^{14}C]VHA to VA by whole cells (Table 1) and cell-free extracts (Table 2) of *A. parasiticus* confirms the results of other workers (13) (Fig. 1). It is evident from the structures of these compounds that the conversion is not a single step, as treatment of VHA with dilute acid (12) has been shown to yield VC; thus, an oxidative step is required to produce VA. As the conversion rates of [^{14}C]VC and [^{14}C]VHA by cell-free preparations were similar, it is possible that VC is a metabolic intermediate between VHA and VA and that the step VC to VA is rate limiting. A possible mechanism for

TABLE 1. *Incorporation of ^{14}C-labeled VHA, VAOH, and VAAC into VA by whole mycelium of the VA-accumulating mutant A. parasiticus (1-11-105 Wh1)*

^{14}C-labeled precursor[a]	Incubation period (h)	Isolated VA	
		Radioactivity (dpm)	Incorporation efficiency (%)[b]
VHA	6	585	11.7
	24	755	15.1
	48	705	14.1
VAOH	48	950	19.0
VAAC	48	940	18.8

[a] Concentration and activity of ^{14}C-labeled precursors: VHA, 4.08 μmol, 5,000 dpm; VAOH, 3.4 μmol, 5,000 dpm; VAAC, 1.3 μmol, 5,000 dpm.

[b] (Microcuries of product)/(microcuries of precursor) × 100.

TABLE 2. *Incorporation of ^{14}C-labeled VHA, VC, VAOH, and [^{3}H]VAAC into VA by a cell-free extract derived from the VA-accumulating mutant A. parasiticus (1-11-105 Wh1)*

Labeled precursor[a]	Precursor activity (added dpm)	Isolated VA[b]	
		Radioactivity (dpm)	Incorporation efficiency (%)[c]
[^{14}C]VHA	5,500	340	6
[^{14}C]VC	4,500	270	6
[^{14}C]VAOH[d]	5,000	765	15
[^{3}H]VAAC[d]	8,500	1,310	13
[^{14}C]VHA[e]	5,500	0	0
[^{14}C]VAOH[e]	7,800	1,248	16
[^{3}H]VAAC[e]	6,900	1,242	18

[a] Concentration of labeled precursor added, 1 μmol/4 ml.

[b] Incubation period, 1 h in each case.

[c] (Microcuries of product)/(microcuries of precursor) × 100.

[d] Labeled substrates added in the presence of each other.

[e] Dichlorvos (20 μg/ml) added to the incubation mixture.

such a reaction is that the ring open form of VC behaves like an alcohol and is converted to an aldehyde by the action of an alcohol dehydrogenase "type" enzyme. An alternative possibility is that the terminal section of the bisdihydrofuran ring system is hydroxylated in some manner by means of an oxygenase. To verify which mechanism occurs, if either, several experiments were carried out.

When VC was incubated with alcohol dehydrogenase there was no observable action; however, this may be due to other factors, such as enzyme specificity. Experiments have shown that dichlorvos inhibits aryl esterase activity (Table 3) which may suggest that the inhibitor blocks VA biosynthesis by preventing hydrolysis of the ester VHA. However, dichlorvos also prevents oxygen uptake by cell-free extracts, the rate being reduced from 0.75 to 0.15 μl of O_2/min, suggesting the inhibition of an oxygenase. Thus, it is possible that an oxygenase which may be involved in the conversion of VHA to VA is being inhibited.

A possible scheme is suggested in Fig. 2. An oxygenase introduces an hydroxyl adjacent to the ester oxygen atom, causing the formation of an unstable acylal intermediate (V) which can either eliminate water to form VAAC (IVB) or eliminate acetic acid to form VAOH (IVA). These could then eliminate acetic acid or water, respectively, to produce VA (II). This system may be regarded as a metabolic grid if the oxygenase is relatively specific and causes, by an

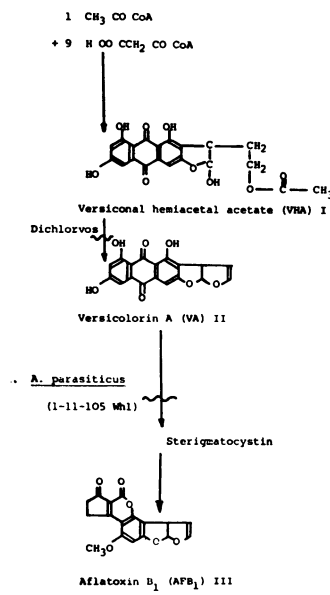

FIG. 1. *Partial scheme for the biosynthesis of aflatoxin B_1 (after Singh and Hsieh [13]).*

TABLE 3. *Effect of increasing concentrations of dichlorvos on the aryl esterase activity of cell-free extracts derived from A. parasiticus (1-11-105 Wh1)*[a]

Concn of dichlorvos added (μg ml^{-1})	*p*-Nitrophenol liberated (μmol mg of protein^{-1} min^{-1})
0.0 (control)	0.024
5.0	0.020
10.0	0.012
20.0	0.003
50.0	0.001

[a] Measured by the method of Rahim and Sih ().

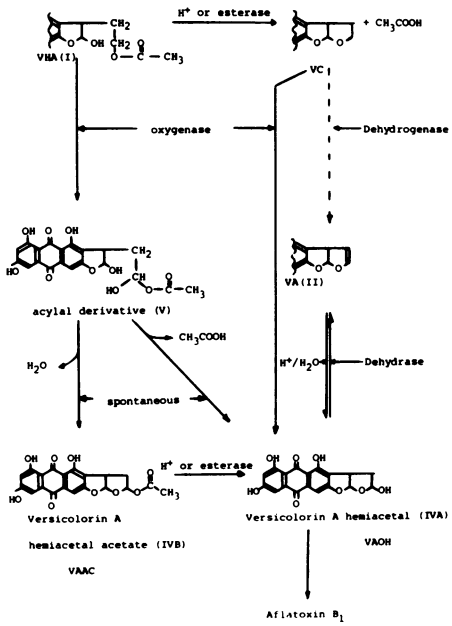

FIG. 2. *Proposed pathway for the conversion of VHA to VA.*

analogous reaction, the conversion of VC to VAOH, while esterase activity causes the hydrolysis of VAAC to VAOH.

For investigation of this possibility, both [^{3}H]VAAC and [^{14}C]VAOH were added to a cell-free extract, in which they were rapidly converted to VA at similar rates (Table 2); comparable results were also found for this conversion in whole mycelium (Table 1). The inability of dichlorvos to inhibit the conversion of [^{3}H]-VAAC and [^{14}C]VAOH to VA in cell-free extracts (Table 2) supports the view that both of these compounds can act as precursors to VA and that they are closer in this capacity than VHA or VC, although this does not necessarily mean that they are intermediary between VHA and VA. The lack of incorporation of VA into aflatoxins found in previous work with cell-free extracts (1) may have been due to difference in water solubility of VHA and VA. However, a scheme is suggested in Fig. 2 whereby VA becomes a side shunt metabolite and VAOH (or VAAC) becomes a direct precursor of VA and is intermediate in aflatoxin biosynthesis. However, VA is metabolically related in such a way as to account for the ^{13}C studies and may enter the mainstream again by addition to water to give VAOH, a process which readily occurs at the acid pH normally found in fungal cultures. Thus, the lack of incorporation of VA into aflatoxins in cell-free preparations (1) may be explained by their alkaline pH. Evidence for VAOH as an intermediate is supported by the observation that it accumulates in cultures of *A. parasiticus* (1-11-105 Wh1) even though the culture pH may be alkaline; hence, it probably arises enzymatically (4).

To clarify the proposed metabolic scheme outlined in Fig. 2, work in this laboratory is currently proceeding with cell-free extracts of an aflatoxin-producing strain of *A. flavus*. It is intended that study of the enzymes involved in VHA conversion will clarify the oxygenase or dehydrogenase (14) theory.

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