Step of Dichlorvos Inhibition in the Pathway of Aflatoxin Biosynthesis

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Dichlorvos (dimethyl 2, 2-dichlorovinyl phosphate) inhibits the biosynthesis of aflatoxin by Aspergillus parasiticus. Cultures treated with dichlorvos excrete an orange pigment which can be converted into aflatoxin B₁ by the untreated mycelia. The orange pigment was partially identified as an acetyl derivative of versiconol-type compound. In the presence of dichlorvos, sterigmatocystin is converted into aflatoxin B₁ without being interfered, but averufin is converted into the orange pigment instead of aflatoxin B₁. Therefore, dichlorvos appears to block an enzymatic step in the aflatoxin biosynthetic pathway, which lies beyond averufin but at before sterigmatocystin, at the formation of the orange pigment.

Dichlorvos (dimethyl 2, 2-dichlorovinyl phosphate), an organophosphate insecticide, was recently found to inhibit aflatoxin (AF) biosynthesis both in synthetic media (8) and in rice, corn, wheat, and peanut samples inoculated with AF producers (14). Hsieh (8) reported that dichlorvos at 10 ppm inhibited over 90% of AF biosynthesis by Aspergillus parasiticus ATCC 15517 grown on different liquid media. Complete inhibition of toxin production occurred with 20 ppm dichlorvos. The treated cultures, however, excreted relatively large quantities of an orange pigment (OP) which is possibly the same pigment recently isolated by Schroeder et al. (17) from cultures of other AF-producing strains treated with dichlorvos. The pigment was probably an acetyl derivative of versiconol-type compound (D. P. H. Hsieh, R. C. Yao, and C. A. Reece, Abstr. Pacific Conf. on Chemistry and Spectroscopy, 1973).

Previously our incorporation studies (9, 12) indicated that sterigmatocystin (ST) and averufin (AR) can be converted into AF B₁ by A. parasiticus and are probably intermediates in AF biosynthesis. We also found that AR can be converted into ST by a ST-producing strain of A. versicolor. Therefore, experimental support is provided for the biosynthetic scheme for AF B₁ (III) involving AR (I) and ST (II) as proposed by Thomas (18) (Fig. 1). Of particular interest in the scheme is the mechanism of derivation of the bisfuran ring system in the ST and AF molecules from the C₄ side chain in the AR molecule. The biogenetic origin of this unusual structure has been a subject of controversy in the study of Aspergillus bisfuranoid biogenesis (2, 6, 7, 13, 18).

In the present study, we show that the aforementioned OP can be converted into AF B₁ by A. parasiticus and hence is probably an intermediate in AF biosynthesis, and that dichlorvos inhibits an enzymatic step in the biosynthetic pathway between AR and ST, a step involved in the development of the bisfuran ring system in the molecules of ST and AF B₁ from the C₄ side chain of AR.

MATERIALS AND METHODS

Organisms. The fungi A. parasiticus ATCC 15517 and its nitrosoguanidine-derived, averufin-producing mutant, A. parasiticus ATCC 24651 (4), and A. versicolor ATCC 18643, a sterigmatocystin producer, were used in this study. Conidia were harvested from well-sporulated cultures on mycological agar (Difco) and were stored in 0.01% aqueous sodium lauryl sulfate solution.

Chemicals and media. Technical-grade dichlorvos (Shell Chemical Co., N.Y.), dissolved in acetone, was used in the inhibition studies. Two chemically defined liquid media were used to cultivate the organisms in submerged culture in shaken flasks or in surface culture under static condition. The minimum mineral (MM) medium of Adye and Mateles (1) containing ammonium and 50 g of glucose per liter was used for the production of AF, AR, and OP. Sterigmatocystin was produced by A. versicolor in the low-salt (LS) medium of Reddy et al. (16) containing asparagine. The nitrogen-free resting cell (RC) medium of Hsieh and Mateles (11) containing various amounts of glucose was used for incorporation studies and accumulation of OP. All the chemicals used were of reagent grade, and solvents were doubly distilled.

Cultural conditions. Wild-type A. parasiticus was...
DICHLORVOS INHIBITION IN AF BIOSYNTHESIS

![Chemical structure](image)

**FIG. 1.** Biosynthetic pathway from averufin (I) to sterigmatocystin (II) and aflatoxin B₁ (III) (15). Symbol: ●, labeling pattern derived from [1-¹⁴C]acetate (2, 7).

grown in 100 ml of MM medium in 500-ml indented flasks inoculated with 10⁷ conidia. Incubation was done in a gyratory water bath shaker at 30 C, shaking at 100 rpm for the first day and 200 rpm thereafter. The effects of dichlorvos on AF biosynthesis was brought about by incubating 1.5 g of 48-h-old mycelial pellets in 50-ml flasks containing 10 ml of MM or RC medium, to which 0.1 ml of acetone solution contain-
spectrum as the authentic sample obtained from Donkersloot (4) and gave only a single spot on thin-layer chromatogram and autoradiograph. AR was quantitated by measuring its absorbance at 453 nm (ε 10,500; reference 4).

The labeled ST was purified twice on ChromAR Sheet 500 and Adsorbil-1 plates developed in benzene:acetone and chloroform:acetone:n-hexane, respectively. The zone containing ST was collected and the ST was eluted with acetone. The eluate was concentrated and mixed with an appropriate amount of unlabeled ST (Makor Chemical, Jerusalem, Israel), and was then repeatedly crystallized in benzene to a constant specific activity. The purity of the ST was verified by both ultraviolet spectrum and autoradiography. The concentration of ST was determined by measuring the absorbance at 325 nm (ε 13,100; reference 3).

The OP and AF in the acetone extract were separated and purified chromatographically with aluminum oxide column impregnated with 6% oxalic acid. Aflatoxin B1 was eluted with 35% chloroform in benzene and OP with 20% acetone in chloroform. The pigment thus obtained was further purified by thin-layer chromatography (Adsorbil-1) plates developed with benzene:ethylacetate:isopropanol:water (25:10:2:1) and then crystallized in 20% acetone in chloroform and quantitated by measuring its absorbance in methanol at 312 nm (ε 17,100; Hsieh, Yao, and Reece, Abstr. Pacific Conf. on Chemistry and Spectroscopy, 1973).

Radioactivity measurement. Radioactive samples were pipetted or scraped off the thin-layer chromatography plates into glass vials containing 15 ml of toluene base scintillation fluid (12). The radioactivity was counted in a Packard model 2425 Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Counting efficiency, as determined by the automatic external standardization method, was in the range of 84 to 88% for all samples measured.

Autoradiogram was prepared by covering a thin-layer chromatography plate with a No-Screen medical X-ray film (Eastman Kodak Co., Rochester, N.Y.) and storing the plates in the dark for 2 to 7 days. The developed autoradiograms were superimposed on the thin-layer chromatography plates to examine the coincidence of spots.

RESULTS AND DISCUSSION

Inhibition of aflatoxins biosynthesis by dichlorvos and accumulation of the orange pigment. The effects of dichlorvos on AF biosynthesis of the 48-h mycelia of A. parasiticus in MM and RC media are indicated in Fig. 2. In both media, AF production was terminated and OP began to accumulate in the mycelia and excrete into the media upon addition of 20 ppm dichlorvos. Although AF production was inhibited to the same extent, the yield of OP in RC medium was three times that in MM medium despite the higher glucose concentration in the latter (36 versus 50 g/liter). The lower yield of OP in MM medium, which contained nitrogen, might have been due to the induction of OP metabolizing enzymes, whereas, in RC medium, no such enzymes were synthesized due to the absence of nitrogen source.

When the pregrown cells, further incubated with 20 ppm dichlorvos in MM medium for 24 h, were thoroughly washed with sterile water and reincubated in dichlorvos-free MM and RC media, both containing 50 g of glucose/liter, the relative production of AF B1 and OP was as shown in Fig. 3. In MM medium where there is ample supply of nitrogen, AF synthesis resumed and increased appreciably, with OP disappear-

![Fig. 2. Inhibition of aflatoxin biosynthesis and the accumulation of an orange pigment in minimum mineral (MM) and resting cell (RC) media by A. parasiticus treated with 20 ppm dichlorvos at 48 h of growth. Symbols: O, aflatoxin B1 in dichlorvos-free cultures; ●, aflatoxin B1 in dichlorvos-treated cultures; ▲, orange pigment in dichlorvos-treated cultures.](image)

![Fig. 3. Effect of removal of dichlorvos on aflatoxin biosynthesis. Cells previously treated with 20 ppm dichlorvos in minimum mineral (MM) medium for 24 h were washed and resuspended in dichlorvos-free, fresh MM, and resting cell (RC) medium. Symbols: ■, aflatoxin B1; ▲, orange pigment in MM medium; O, aflatoxin B1; ▲, orange pigment in RC medium.](image)
ing from the medium after a slight initial increase. Apparently the repair-synthesis of the dichlorvos-susceptible enzyme in the presence of nitrogen was responsible for the resumption of AF synthesis. In RC medium where no external nitrogen supply was available, AF synthesis resumed at a much slower pace, indicating that dichlorvos inhibition was slowly reversible. The resumption of the enzyme activity became more clear as incubation continued when the cellular level of OP decreased with a faster increase in AF formation.

**Identification of the orange pigment.** The orange pigment described above was produced using the resting cell culture of *A. parasiticus* supplemented with different amounts of dichlorvos. The effect of dichlorvos concentration on the yield of labeled OP is shown in Fig. 4. The highest incorporation efficiency concurred with 10 ppm dichlorvos. Above that level, the incorporation percentage decreased significantly. The lower yield of OP at higher concentrations of the inhibitor indicated an effect of dichlorvos on another enzyme involved in the earlier portion of the biosynthetic pathway.

We previously reported that the unknown OP gave orange needle-like crystals with a melting point of 219 to 225°C (decomposition) (Hsieh, Yao, and Reece, Abstr. Pacific Conf. on Chemistry and Spectroscopy, 1973). Its ultraviolet spectrum (Beckman DK-2A) showed λ\text{max} 224, 225 (sh), 265, 295, 323 and 480 nm (λ\text{max} 23,800, 13,000, 14,000, 23,000, 11,300, and 7,250 respectively). The ultraviolet spectrum of OP closely resembled that of versicolor (λ\text{max} 224, 255, 265, 295, 322, 460 nm) previously reported by Hatsuda et al. (5). The infrared spectrum (Perkin-Elmer 337) showed bands at 1,675 and 1,625 cm\(^{-1}\), indicating the presence of a non-chelated and chelated anthraquinone carbonyl groups. The mass spectrum (CEC model 21-110 with electron multiplier detector) revealed principal ions at \(m/e\) of 340, 322, 312, 311, and 297. Other significant ions at 382, 354, and 325 were also noted. At the same time, low-mass ions at 42, 45, and 60 revealed the presence of acetyl group. Based on the physical properties of OP and how it was being produced from cultures of AF-producing strains, there is good reason to believe that OP isolated in this study is probably the same pigment recently isolated by Schroeder et al. (17). Our data indicated that OP is probably an acetyl derivative of a versicolor-type compound. Analytic information obtained thus far is not sufficient to allow an unequivocal identification of OP. Further studies are in progress for its structural elucidation.

**Conversion of orange pigment into aflatoxin B\(_1\).** The incorporation of [\(^{14}\)C]OP and [\(^{1-14}\)C]acetate into AF B\(_1\) by wild-type *A. parasiticus* is indicated in Table 1. After repeated thin-layer chromatography purification, AF B\(_1\) retained 13.7% of the radioactivity from the [\(^{14}\)C]OP, but less than 1% of the activity from [\(^{1-14}\)C]acetate, despite the fact that the concentration of OP (0.06 mM) used in the experiment was much lower than that of acetate (8.0 mM). The efficient incorporation of radio-

![Graph](image)

**Fig. 4.** Effect of dichlorvos concentration on the efficiency of [\(^{1-14}\)C]acetate incorporation into the orange pigment. Cells were incubated in nitrogen-free resting cell medium containing 18 g of glucose per liter.

| \(^{14}\)C-labeled precursor | Net radioactivity (\(10^4\) dpm) | Sp act of precursor (\(10^4\) dpm/\(\mu\)mol) | Aflatoxin B\(_1\)* |
|-----------------------------|-------------------------------|-----------------|-----------------|
| Orange pigment              | 0.586                         | 2.03            | 0.486           |
| Sodium acetate              | 88.5                          | 1.1             | 0.404           |

* Portions of 1.5-g mycelial pellets grown in minimum mineral medium for 48 h were washed and resuspended in 10 ml of resting cell medium containing 300 \(\mu\)mol of glucose and 0.1 ml of \(^{14}\)C-labeled precursor in methanol. Results were the mean of triplicate samples.

* Aflatoxin B\(_1\) was purified in three different thin-layer chromatography systems.
activity from OP over that from acetate, a known precursor of AF (10), in the nitrogen-free resting cell medium indicates that OP was converted into AF B₁ without undergoing major degradation and is probably an intermediate in AF biosynthesis.

Conversion of sterigmatocystin and averufin by dichlorvos-treated cultures of A. parasiticus. Lin et al. (12) and Hsieh et al. (9) have shown that AR and ST could both be converted into AF B₁ by the resting mycelia of wild-type A. parasiticus. The results in Table 2 indicate the effect of 10 ppm of dichlorvos on the incorporation of labels from acetate, AR, and ST into AF B₁, in the resting cell culture. The incorporation efficiency for [1-¹⁴C]acetate into AF B₁ by the untreated culture indicates that the mycelia used had normal AF synthetic activity. Incorporation of [¹⁴C]AR into AF B₁ was completely inhibited by dichlorvos, but incorporation of [¹⁴C]ST was unaffected, indicating that the enzyme system responsible for converting AR into ST was the site of dichlorvos action. In the presence of dichlorvos, the biosynthetic pathway terminates at the formation of OP, as indicated by the incorporation into OP of ¹⁴C from both sodium acetate and AR. Therefore, OP is an intermediate in the conversion of AR to ST, and its structure should give useful information as to the mechanism of the development of the bisfuran ring structure in AF B₁.

Thus, dichlorvos at low concentrations inhibits a specific enzymatic step in AF biosynthesis and affects the accumulation of a biosynthetic intermediate of AF. The step of inhibition resides beyond AR and preceding ST on the AF biosynthetic scheme. Our experimental results also revealed the involvement of another C₂₀ compound, i.e. OP, in addition to AR (C₁₈H₁₈O₇), in the biosynthesis of AF.

The experimental evidence accumulated thus far does not seem to provide a definite answer to the pending question of how the bisfuran structure was developed in the biosynthesis of ST and AF. Based on the labeling pattern of AF B₁ derived from [1-¹⁴C]acetate and [2-¹⁴C]acetate and the uniform label density throughout the molecule (Fig. 1), Biollaz et al. (2) advanced a hypothetical pathway involving a single C₁₄-polyketomethylene compound as intermediate; the bisfuran ring structure was proposed to be derived from endoperoxidation of a terminal benzene ring in a hypothetical polyhydroxynaphthacene or polyhydroxybenzanthracene intermediate. This scheme does not accommodate AR as an intermediate, which has been experimentally shown to be convertible in vivo into AF B₁ (9). Therefore, the C₁₄-polyketide theory is highly questionable. Recently, Thomas (18) postulated that AR is an intermediate in the biosynthesis of ST and AF and that the C₄-bisfuran structure is derived from the C₆ side chain of AR through an acetylated furan structure as intermediate. Unfortunately, this acetylated furan structure was not found in our OP or the recently found versicolace acetate (17).

Alternatively, it has been proposed that ST and AF are synthesized in vivo from a fusion of two preformed polyketide units, namely C₄ and C₁₄ moieties (6, 7). The “two-unit theory” was supported by the difference found in the labeling density of the bisfuran and the xanthone moiety of the [¹⁴C]ST molecule synthesized from [¹⁴C]acetate (7), and also by the efficient incorporation of acetoacetate into AF B₁ (6).

Table 2. Incorporation of ¹⁴C-labeled sodium acetate, averufin, and sterigmatocystin into aflatoxin B₁ by A. parasiticus treated with 10 ppm dichlorvos

| Precursor* | Amount (μmol) | Radioactivity (10⁴ dpm) | Incorporation efficiency (%) | Radioactivity (10⁴ dpm) | Incorporation efficiency (%) |
|-----------|---------------|-------------------------|-----------------------------|-------------------------|-----------------------------|
| Sodium acetate | 0.392 | 8.28 | 0.94 | NR | 1.18 |
| Sodium acetate + dichlorvos | 0.024 | NR | 10.4 | 9.4 |
| Averufin | 0.640 | 10.38 | 49.4 | NR | 79.4 |
| Averufin + dichlorvos | 0.029 | NR | 16.68 | NR | 79.4 |
| Sterigmatocystin | 0.651 | 16.25 | 65.0 | NR | 79.4 |
| Sterigmatocystin + dichlorvos | 0.528 | 17.87 | 71.5 | NR | 79.4 |

* A. parasiticus grown for 48 h in minimum mineral medium was transferred to 10 ml of resting cell medium containing 300 μmol of glucose. Results were the mean of duplicate samples.

* Amount of ¹⁴C-labeled precursors added: sodium acetate (1.1 x 10⁴ dpm/μmol), 80 μmol; averufin (4.8 x 10⁴ dpm/μmol), 0.438 μmol; sterigmatocystin (5.9 x 10⁴ dpm/μmol), 0.423 μmol.

* NR, No radioactivity.
The substitution of the C$_4$ side chain of AR with an acetoacetate-like C$_4$ unit to form a versiconol-like structure would provide an easy explanation of how the bisfuran ring system in the molecules of ST and AF is developed. More experimental work needs to be done to further understand the origin and mechanism of formation of the fungal bisfuranoid structure.

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