Inhibition of Aflatoxin Biosynthesis of Dichlorvos

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Dichlorvos (dimethyl 2,2-dichlorovinyl phosphate) was found to possess a strong inhibitory effect on aflatoxin biosynthesis by *Aspergillus parasiticus* ATCC 15517. At 10 ppm it inhibited 90% of aflatoxin production in three types of liquid media without affecting the fungal growth. When the actively synthesizing cultures were supplemented with 10 ppm of dichlorvos and [1-^{14}C]acetate, a reduced amount of aflatoxin B_1 was produced which contained almost no label from the acetate, suggesting that dichlorvos inhibits an early step in the secondary metabolic pathway for aflatoxin biosynthesis. Experiments with other organophosphorus insecticides showed that dichlorvos was particularly inhibitive to the biosynthesis of aflatoxins.

Aflatoxins are a mixture of toxic metabolites produced by certain strains of the fungus *Aspergillus flavus* or *A. parasiticus*, which frequently infest animal feeds and human foodstuffs (Goldblatt, 1969). The major toxin, aflatoxin B_1, is the most potent hepatocarcinogen ever known for the rat (Newberne and Butler, 1969). Due to the ubiquitous nature of the mold and the potent toxicity and carcinogenicity of aflatoxins, they have become a serious threat to food safety and public health.

Considerable research effort has been directed toward inhibition and prevention of aflatoxin production by chemical treatment. Using a basal medium containing glucose, ammonia, and inorganic salts, Lee *et al.* (1966) ascertained the inhibitory effect of barium. At the 1-ppm level, barium completely inhibited aflatoxin production, with no effect on mycelial growth. Inhibition of aflatoxin production in a yeast extract–sucrose medium by *p*-aminobenzoic acid, potassium sulfite, and potassium fluoride was observed by Davis and Diener (1967). Over 1000 ppm of these inhibitory chemicals was needed to reduce 50% of toxic production, but mycelial growth was again not affected. Recently, dimethyl sulfoxide was also found to possess the similar inhibitory activity at a relatively high concentration (5000 ppm) (Bean *et al.*, 1971).

Dichlorvos (dimethyl 2,2-dichlorovinyl phosphate), an organophosphorus insecticide, was recently found to inhibit aflatoxin production in rice, corn, wheat, and peanut samples that had been inoculated with *Aspergillus flavus* NRRL 2999, a known aflatoxin producer (Rao and Harein, 1972). Only 20 ppm of the chemical was enough to effect a complete inhibition. The present report describes the inhibition of aflatoxin production by dichlorvos in the defined liquid media under conditions used for the study of aflatoxin biosynthesis.

EXPERIMENTAL SECTION

Organism. The fungus *Aspergillus parasiticus* ATCC 15517 was used in this study. The conidia were obtained from well-sporulated cultures on Mycological agar (Difco) and were suspended in 0.01% aqueous sodium lauryl sulfate solution.

Media and Cultures. Three liquid media supporting aflatoxin yields at different levels were used. The minimum mineral medium (MM) of Adye and Mateles (1964) was used for submerged cultures in shaken flasks. MM supplemented with 0.1% of yeast extract (YEM) and the yeast extract–sucrose medium (YES) of Davis and Diener (1967) were used for surface cultures in standing flasks.

Each 100 ml of medium contained in a 500-ml Erlenmeyer flask was inoculated with approximately 10^5 conidia and was incubated at 30° with or without shaking.

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Incorporation of [1-^{14}C]acetate into aflatoxins was carried out by feeding 100 μmol of [1-^{14}C]acetate (0.1 mCi/mmol) into each 20 ml of resting cell culture contained in a 50-ml baffled flask. The resting cell cultures of *A. parasiticus* were prepared from the mycelial pellets of 48 hr which had been cultivated in MM, as described by Hsieh and Mateles (1971).

Any departures from these culture conditions are indicated in the Results Section.

Organophosphates. In addition to dichlorvos (Shell Chem. Co., N. Y.), the following commonly used organophosphorus insecticides were tested for their effect on aflatoxin production: parathion (Niagara Chem. Co.), malathion (Stauffer Chem. Co.), guthion (Chemagro Corp.), diazinon (City Chem. Corp.), phosdrin (Shell Chem. Co.), ethion (Niagara Chem. Co.), trithion (Stauffer Chem. Co.), atrazine (Geigy Chem. Co.), and triguvon (Chemagro Corp.).

All chemicals, including dichlorvos, were of technical grade. Appropriate amounts of the chemicals were dissolved in 1 ml of absolute ethanol and added to the culture medium. Slow-release dichlorvos pellets (Shell Chem. Co.), prepared to support 10 ppm of dichlorvos for 7 days in a 100-ml medium, were used as such.

Analysis. Each 5-ml sample of the fermented broth was extracted with 10 ml of chloroform, twice per sample. Chloroform was evaporated from the combined extracts and aflatoxins were redissolved in 1 ml of benzene–acetonitrile (98:2) and purified using the improved tlc system: Adsorbosil-1 (Applied Science Lab., Inc., State College, Pa.) plates developed with chloroform–acetone–water (88:12:1.5) (Stubblefield *et al.*, 1969).

Quantification of aflatoxins was made directly on the tlc plates using a Schoeffel Model SD 3000 recording spectrodensitometer operated in the transmission mode. Adsorbent in the aflatoxin spots was scraped into scintillation vials for radioactivity measurements using a Packard Tri-Carb Model 2425 liquid scintillation spectrometer.

RESULTS AND DISCUSSION

Aflatoxin production in the various media was markedly reduced by the presence of a small amount of dichlorvos in the media, but the fungal growth was not affected (Table I). Over 90% inhibition was effected by 10 ppm of dichlorvos added as ethanol solution. The slow-release dichlorvos pellet prepared to maintain the same level (10 ppm) of the chemical appears to be less effective. The inhibitory effect was somewhat inversely related with the concentration of yeast extract in the medium, which is know to enhance the aflatoxin production by the mold (Davis and Diener, 1967). Thus, the effect of dichlorvos decreased in the order of MM > YEM > YES.

On the tlc plate, the extract from the treated cultures was seen to contain a new metabolite with an R_f value lower than that of aflatoxin B_2, fluorescent in yellowish

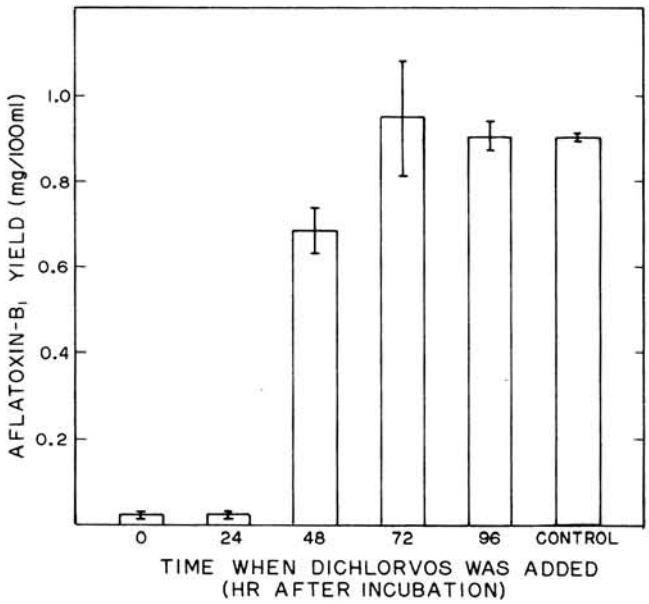

Figure 1. Inhibition of aflatoxin biosynthesis by dichlorvos added to the producing cultures of *A. parasiticus* at different times of incubation.

color under the long wavelength ultraviolet light. Further characterization of this new metabolite is presently in progress.

To examine the mechanism of the inhibition of dichlorvos, 10 ppm of dichlorvos was added to the cultures of *A. parasiticus* growing in YEM medium at different times of incubation. Only additions prior to the initiation of toxin production, which occurs at about 40 hr after incubation, stopped the toxin synthesis effectively (Figure 1). Addition at 48 hr when the idiophase had just begun, yet was not completed, did not suppress the production significantly. The ineffective inhibition in the idiophase suggests that dichlorvos inhibited an early step in the secondary metabolic pathway, allowing the intermediates following that step to be converted to aflatoxin B_1 without interruption.

When the resting cell cultures of *A. parasiticus* supplemented with 10 ppm of dichlorvos were used to incorporate [1-^{14}C]acetate into aflatoxin, very little radioactivity was recovered in aflatoxin as compared to the control (Figure 2). Since the incorporation of [1-^{14}C]acetate into aflatoxin represents a *de novo* synthesis, any interruption in the pathway would result in failure to incorporate ^{14}C into aflatoxin by the culture. However, the inhibition on the aflatoxin yields of the treated cultures was not as drastic as the inhibition on the label incorporation. In the cultures containing dichlorvos, aflatoxin was produced at a slow but constant rate. This discrepancy in the inhibition between aflatoxin production and label incorporation from [1-^{14}C]acetate lends support to the earlier hypothesis that dichlorvos inhibits an early step in the biosynthetic pathway. Thus, interruption of the *de novo* synthesis resulted in the poor incorporation of acetate into aflatoxin, but continuing conversion of the biosynthetic intermediates following the blocked step sustains the slow but steady production of aflatoxin.

The small amount of radioactivity found in the aflatoxin isolated from the treated cultures might have been due to the incorporation of [1-^{14}C]acetate during the short period of time before the responsible enzyme was completely inhibited or due to the incomplete separation of aflatoxin from certain radioactive impurity by merely a single tlc purification. But in any event, a significant shutdown of the *de novo* synthesis is clearly demonstrated.

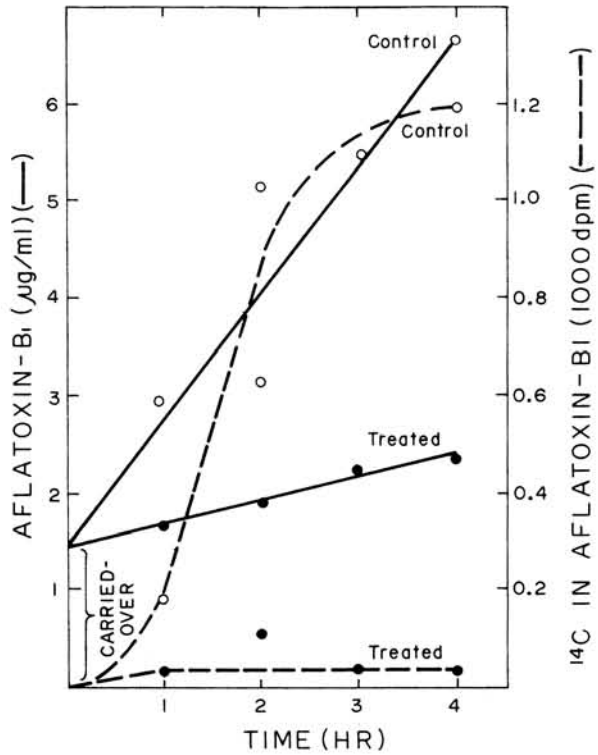

Figure 2. Effect of dichlorvos (10 ppm) on the production of aflatoxin B_1 and the incorporation of label from [1-^{14}C]acetate into aflatoxin B_1 by the resting cell cultures of *A. parasiticus*. Control: O——O; treated: ●——●.

A number of organophosphorus insecticides were subject to a similar experiment to examine whether the effect of dichlorvos on aflatoxin biosynthesis is a general property of organophosphates, mechanistically analogous to the inhibition of acetylcholinesterase. In this experiment, *A. parasiticus* was cultured in a series of 50-ml conical flasks, each containing 10 ml of YEM, at 30° without shaking. Each insecticide chemical was added to the culture as ethanol solution to make the final concentration of each chemical and ethanol 10 ppm and 1%, respectively. Cultures supplemented with 1% ethanol only were used as control. The experimental result (Table II) shows that,

Table I. Effect of Dichlorvos on Mycelial Growth and Aflatoxin Production in Liquid Media

Medium[a]	Dichlorvos, ppm	Aflatoxin B_1, mg	Dry cell mass, g	Relative specific productivity, %
MM	0	0.790	1.42	100
	10	0.002	1.40	0.3
	20	ND[c]	1.41	
	Pellet[b]	0.030	1.06	5.0
YEM	0	1.080	1.00	100
	10	0.075	0.99	7.0
	20	ND	0.94	
	Pellet	0.310	0.82	35.0
YES	0	16.2	4.65	100
	10	0.55	4.54	3.5
	20	0.21	4.39	1.4

[a] One-hundred milliliters of each medium were used. MM = minimum mineral medium incubated for 3 days; YEM = MM plus 0.1% yeast extract incubated for 5 days; YES = 20% sucrose and 2% yeast extract incubated for 5 days. [b] Slow-release pellet of dichlorvos prepared to support 10 ppm of dichlorvos for 7 days in 100 ml of medium. [c] ND = not detectable.

Table II. Effect of Some Organophosphorus Insecticides on Aflatoxin Biosynthesis in YEM Medium[a]

Chemical added[b]	Aflatoxin B_1, μg	Dry cell mass, mg	Relative specific productivity, %
Dichlorvos	75	164	13
Parathion	325	159	59
Malathion	350	161	62
Guthion	325	142	66
Diazinon	390	148	76
Atrazine	395	144	79
Ethion	416	147	81
Trition	455	158	83
Phosdrin	465	157	85
Triguvon	545	165	95
Control	540	155	100

[a] Production in 10 ml of medium in 5 days. [b] Each in a 10 ppm concentration.

while not all the organophosphates are inhibitory to aflatoxin biosynthesis, dichlorvos is much more potent than the others. Therefore it is unlikely that the mechanism of inhibition of aflatoxin biosynthesis is similar to that of acetylcholinesterase inhibition.

It was noted that the yield of aflatoxin B_1 in the 100-ml YEM medium (10.8 mg/l., Table I) was considerably higher than the yield in the 10-ml medium (7.5 mg/l., Table II), but the susceptibility to the inhibition of dichlorvos was much higher in the 100-ml medium than in the 10-ml medium. One explanation of this difference may be that in the smaller volume of medium more acids were produced (as shown by the lower pH of the broth) because of a better oxygen transfer, which enhanced conversion of aflatoxin B_1 to its hemiacetal, aflatoxin B_{2a}, and also antagonized the action of dichlorvos.

Dichlorvos has been found also to strongly inhibit biosynthesis of Zearalenone by *Fusarium roseum* 'Graminearum' (Walf *et al.*, 1972). Since Zearalenone, like aflatoxins, is biosynthesized *via* the acetate–polymalonate metabolic system (Mirocha, 1973), dichlorvos may very well be an inhibitor of a key enzyme in the general polyketide pathways and inhibit synthesis of many fungal secondary metabolites. Dichlorvos is widely used as a household insecticide and its residues have been found in stored grains (Rowlands, 1971). As the residual dichlorvos did inhibit aflatoxin production in peanut, rice, and other grains (Rao and Harein, 1972), its role in the control of mycotoxin problems deserves continued attention and investigation.

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Gas–Liquid Chromatographic Detection of Actively Metabolizing *Aspergillus parasiticus* in Peanut Stocks

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A method incorporating air sweeping, trapping, and gas–liquid chromatography of headspace gas over the heated trapping agent was devised for detecting a major volatile metabolite elaborated by *Aspergillus parasiticus* cultured on wet peanuts. The metabolite was identified as acetone by mass spectral analysis. Weight dilutions of the wet moldy peanuts with uninoculated sound peanuts gave a 40-fold increase in the acetone peak for 1:70 dilution and a 4.5-fold increase for a 1:1400 dilution as compared to the control samples. Drying the wet moldy peanuts to a 6% moisture level resulted in a marked decrease of the acetone metabolite, indicating that the technique is applicable only to actively metabolizing mold.

Sporadic and nonselective invasion of many agricultural products, including peanuts, by mycotoxin elaborating strains of *Aspergillus flavus* and *Aspergillus parasiticus* during unfavorable conditions of harvesting and storage is now recognized as a serious agricultural problem (Wogan, 1964). Accordingly, the peanut industry adopted a code of good practice which calls for an extensive monitoring system of peanut stocks using microscopic detection of *A. flavus* and aflatoxin assay to assure that all peanuts entering the edible trade are wholesome (Marketing Agreement for Peanuts No. 146, 1968). However, evidence that aflatoxin in peanut stocks is associated with only a small proportion of the kernels (Cucullu *et al.*, 1966) imposes formidable problems of adequate sampling of raw materials. If the presence of mold-contaminated kernels could be de-

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