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# Enzymatic Conversion of Sterigmatocystin into Aflatoxin $B_1$ by Cell-Free Extracts of *Aspergillus parasiticus*

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A cell-free extract, prepared from *Aspergillus parasiticus* ATCC 15517 grown in synthetic medium, was active in converting [$^{14}$C]sterigmatocystin into aflatoxin $B_1$ in the presence of reduced nicotinamide adenine dinucleotide phosphate. The activity was demonstrated by the time course of conversion and the linear dependence of the yield of product on enzyme concentrations. Optimum activity was obtained at pH 7.5 to 7.8 at 27 C. The results confirm sterigmatocystin as a biogenetic precursor of aflatoxin $B_1$. Techniques were developed for enzymatic studies on aflatoxin biosynthesis.

Sterigmatocystin (ST) and aflatoxin $B_1$ (AF) (Fig. 1) are hepatocarcinogenic mycotoxins, both containing a bisfuranomethoxybenzene ring, and have long been considered biogenetically related. ST was proposed as an intermediate in several schemes for AF biosynthesis (2, 5, 10). Their precursor-product relationship has been demonstrated by the conversion of ST into AF by the mycelium of *Aspergillus parasiticus* (6). In this report, we further show that this conversion can be achieved enzymatically, using the cell-free preparations of the biosynthetically active mycelium.

Raj et al. (9) reported the incorporation of radioactivity from labeled acetate, mevalonate, and leucine into AF by the mitochondrial or reconstituted (mitochondrial plus nuclear) homogenate obtained from *A. flavus*. However, the radiochemical purity of the AF recovered by them was not rigorously tested. The simple thin-layer chromatography system used by these authors, in our experience, is not capable of completely isolating AF from other radioactive impurities resulting from the incubation. Consequently, the enzyme activity shown as radioactivity incorporated into AF is questionable.

In the present study, we employed improved analytical techniques to demonstrate the enzyme activity for the conversion of a close precursor (ST) into AF by a cytoplasmic fraction of the mycelium. The techniques thus developed are useful in the further enzymatic study of AF biosynthesis.

## MATERIALS AND METHODS

**Culture techniques and preparation of cell-free extracts.** The AF producer *A. parasiticus* ATCC 15517 was cultivated in a synthetic medium as for the in vivo incorporation of precursors (1, 6). After about 60 h of incubation, when AF production had begun and showed a linear response with time, the mycelial pellets were collected on a cheesecloth. The pellets were washed twice with cold 0.2 M potassium phosphate buffer, pH 7.5. Each 15 g of mycelial pellets was mixed with 20 ml of 0.2 M potassium phosphate buffer, pH 7.5, and 30 g of precooled glass beads (0.45- to 0.50-mm size) (Braun Apparateban Melsungen no. 2886). The mixture was vibrated for 2 min in a Bronwill mechanical cell homogenizer, model MSK 2876 (Bronwill Scientific Inc., Rochester, N.Y.), at 4,000 cycles/min with continuous cooling with liquid $CO_2$. The homogenate was filtered out from glass beads and centrifuged at 20,000 × *g* for 15 min. The resulting supernatant was decanted to yield a "cell-free extract." The protein content in the cell-free extract was determined by the Biuret method (4). The extracts thus obtained varied in their protein content from 7.0 to 12.0 mg/ml.

$^{14}$C-labeled ST was prepared in a culture of *A. versicolor* A-18074 supplemented with [1-$^{14}$C]acetate by the method of Hsieh et al. (6).

**Enzyme assay.** Unless otherwise mentioned, the incubations to study the conversion of ST into AF were carried out in duplicates at 27 C for 1 h. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 0.5 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 10% glycerol, pure [$^{14}$C]ST ($1 \times 10^{-5}$ M; 67,500 dpm) dissolved in *N-N*-dimethylformamide, and enzyme extract to give a protein content of 1.5 mg/ml of incubation mixture. The incubations were done in a total volume of 10 ml in 50-ml baffled flasks by continuous shaking at 100 rpm. In all experiments, reactions were initiated by the addition of enzyme extract and stopped by vigorously shaking the reaction mixture with 10 ml of chloroform.

**Extraction, purification, and measurement of metabolites.** After incubation, the labeled compounds in the reaction mixture were exhaustively extracted with chloroform. The chloroform extracts of each flask were evaporated to dryness under vac-

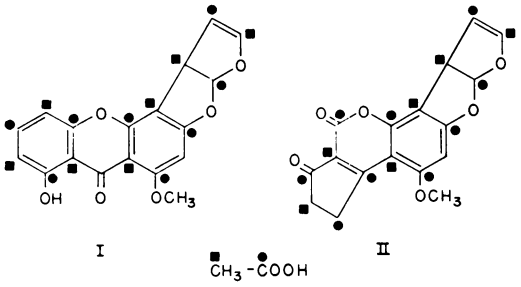


FIG. 1. *Molecular structures of ST (I) and AF (II) with labels derived from acetate.*

uum and redissolved in 0.1 ml of benzene-acetonitrile (98:2). AF was then repeatedly purified by using the following thin-layer chromatography systems: Adsorbosil-1 (Applied Science Laboratories Inc., State College, Pa.) plates developed with chloroform-acetone-isopropanol (85:15:2.5, vol/vol/vol), followed by two-dimensional thin-layer chromatography using precoated Silica Gel-60 plates (EM Laboratories Inc., Elmsford, N.Y.) developed with chloroform-acetone-isopropanol (85:15:2.5, vol/vol/vol) and ethylacetate-isopropanol-water (10:2:1, vol/vol/vol) solvent systems, respectively. AF spots were scraped off into the scintillation vials and counted for radioactivity in a Packard model 2425 Tri-Carb liquid scintillation spectrometer. Preliminary studies had shown that this sequence of thin-layer chromatography purifications would yield AF of constant specific activity.

## RESULTS

The time course of the conversion of ST into AF in the reaction mixture containing the cell-free extract is shown in Fig. 2. The conversion proceeded rapidly with time for a period of 25 to 30 min. After that a decrease in the radioactivity recovered from AF was observed. This decrease in AF in the reaction mixture could either be due to the degradation of the product or its binding to proteins, following the termination of reaction as a result of substrate or cofactor depletion or enzyme inactivation. Similar experiments using boiled cell extract showed no conversion of ST into AF, indicating that the reaction was carried out enzymatically.

Under the same conditions, the conversion of ST into AF was approximately proportional to the extract protein concentration in the reaction mixture, as shown in Fig. 3. The curvilinear response to the enzyme concentration could be a result of reduced oxygen supply or the presence of inhibitors at higher protein concentrations or competition of nonspecific proteins for the substrate. The strong dependence of the conversion on enzyme concentration provides further evidence for the presence of enzyme

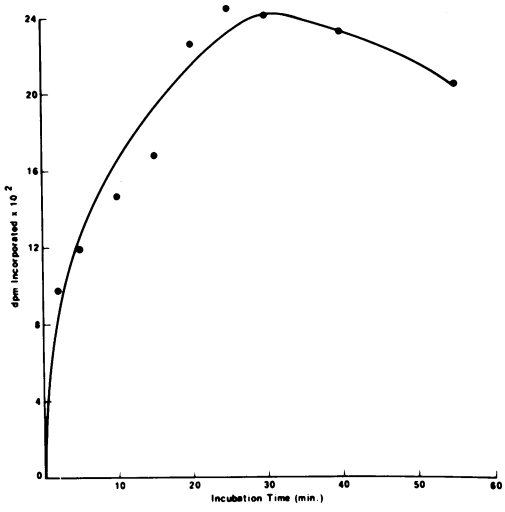


FIG. 2. *Time course of the enzymatic conversion of [$^{14}$C]ST into AF.*

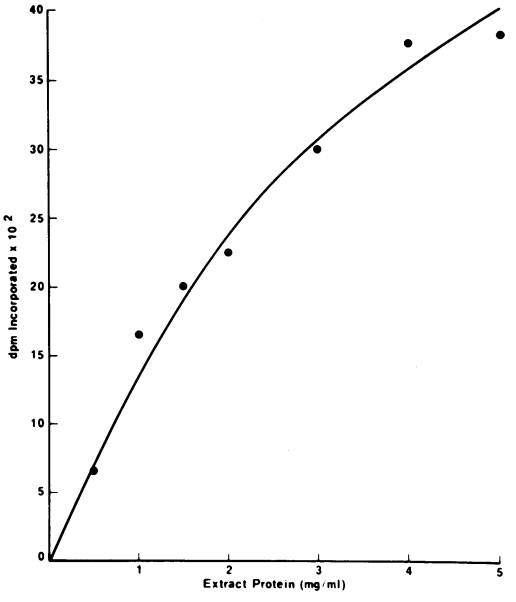


FIG. 3. *Effect of extract protein concentration on the incorporation of [$^{14}$C]ST into AF.*

activity. It also suggests that the termination of reaction in the time course experiment (Fig. 2) was due to the loss of the enzyme activity rather than other factors.

The pH dependence of the reaction was tested over a range of pH 6.0 to 9.0. A distinct optimum was found between pH 7.5 and 7.8. The rapid decrease of enzyme activity at pH above and below the optimum range is evident in Fig. 4. Slightly alkaline pH optimum seems to be a general characteristic of the enzymes involved

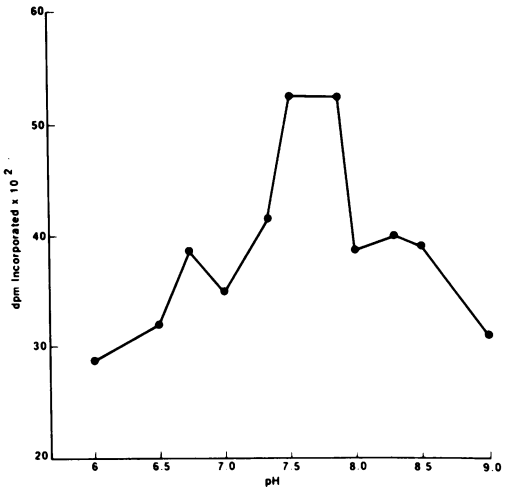


FIG. 4. *Effect of pH on the enzymatic conversion of [$^{14}$C]ST into AF.*

in the biosynthesis of fungal polyketides. For example, Murphy et al. (8) found a pH optimum of 7.5 for the mixed-function oxidase involved in patulin biosynthesis, and a pH of 7.8 was found to be optimum for the biosynthesis of 6-methyl salicylic acid (3).

The activity of the cell-free system was also highly dependent on NADPH. Omission of this cofactor from the incubation mixture or its replacement with NADP resulted in reduction of activity by about 60%. Considering the likely presence of NADPH in the cell-free extract, the residual activity could be attributed to the endogenous cofactor. Thus, one can reasonably conclude that NADPH is required for the activity of the cell-free extract.

## DISCUSSION

The enzyme activity involved in the conversion of ST into AF is demonstrated by the time course of reaction, the dependence of yield of conversion on enzyme concentrations, and the lack of reaction if the cell-free preparation was heated. The sensitive dependence of the extract on NADPH for its activity is evidence that the conversion was indeed catalyzed enzymatically rather than by inadvertently unremoved unbroken cells because the latter would not respond to the enzyme cofactor.

The role of NADPH in the present reaction suggests that an oxidation-reduction reaction was involved in the conversion and that the enzyme catalyzing the reaction is most likely an oxygenase. In fact, it has been proposed by several authors (2, 5, 10) that ST is converted into AF through an oxygenative ring cleavage. The enzyme system catalyzing this type of reaction has been very well established in patulin biosynthesis (8). The presence of enzyme activity in the postmitochondrial fraction of the mycelium is consistent with the prediction of the extramitochondrial synthesis of AF as advanced by Hsieh and Mateles (7).

The enzymatic conversion of ST into AF thus confirms the biogenetic relationship of these two compounds. Further studies are in progress to characterize these enzyme systems and to convert other precursors into AF. The biochemical techniques developed in this investigation should hopefully facilitate enzymatic studies on aflatoxin biosynthesis and fungal secondary metabolism, which have lagged considerably behind the main stream of experimental biochemistry.

## ACKNOWLEDGMENTS

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