CONVERSION OF STERIGMATOCYSTIN TO AFLATOXIN B_1 BY ASPERGILLUS PARASITICUS

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Received April 20, 1973

SUMMARY: ^{14}C-Sterigmatocystin isolated from cultures of Aspergillus versicolor supplemented with (1-^{14}C)acetate was shown to be efficiently converted to aflatoxin B_1 by the resting mycelium of A. parasiticus. The experimental results may indicate a biosynthetic pathway leading from 5-hydroxysterigmatocystin to sterigmatocystin and then to aflatoxin B_1.

Sterigmatocystin (ST) and aflatoxin B_1 (AF) (Fig. 1) are carcinogenic mycotoxins produced by Aspergillus versicolor and certain strains of A. flavus and A. parasiticus, respectively. These toxins have been subjects of vigorous investigations due to the widespread contamination of human foodstuffs and animal feeds.

Since both compounds contain a bifuranomethoxybenzene ring, they have long been considered biogenetically related, and ST has been proposed as an intermediate in several hypothetical schemes for the biosynthesis of AF (1,2,3). These hypotheses are supported by the similarity in the label distribution in the molecules when the compounds are biosynthesized from (^{14}C or ^{13}C)acetate (4,5), as well as by the isolation of O-methylsterigmatocystin (6) and aspertoxin (7) from aflatoxin-producing strains of A. flavus. However, direct experimental evidence that ST can be converted to AF has not been provided. Holker and Underwood (1) mentioned that Townsend (in an unpublished experiment) failed to incorporate [^{14}C] ST into AF using cultures of A. flavus or A. parasiticus, but no experimental details were given. It is not known whether the negative result was due

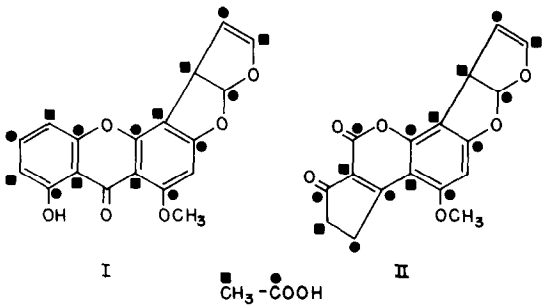

Fig. 1. Molecular structures of sterigmatocystin (I) and aflatoxin B_1 (II) with labels derived from acetate.

to methodological reasons, such as the low substrate solubility, or to the short active phase of the cultures.

In the present study we used the technique developed for incorporation of [^{14}C] averufin into AF (8) to demonstrate that [^{14}C] ST can indeed be converted to AF by the mycelium of A. parasiticus.

[^{14}C] Sterigmatocystin was prepared in a culture of A. versicolor A-18074 supplemented with [1-^{14}C] acetate. The fungus was cultured in 150 ml of a broth containing 15% sucrose and 2% yeast extract as standing cultures. After incubation for 4 days at 30°C, the broth was replaced with a nitrogen-free, resting cell medium (9), and 2 mCi of [1-^{14}C] acetate (57 Ci/mol) were added to the resting cell culture at each of four 12 hr intervals. After incubation, the mycelial mat was collected, and ST exhaustively extracted with acetone. Sterigmatocystin in the crude extract was purified by using three tlc systems in succession: Adsorbosil-1 (Applied Science Lab., State College, Pa.) plates developed respectively with benzene:acetone (96:4, all ratios by volume), chloroform:acetone:n-hexane (85:15:20, CAH), and benzene: petroleum ether:acetone (90:8:2). The tlc purified [^{14}C] ST was then mixed with an appropriate amount of unlabeled authentic ST (Makor Chemicals, Jerusalem, Israel) and was repeatedly crystallized from benzene to a constant specific activity (0.039 Ci/mol). The [^{14}C] ST so purified appeared as a single compact spot on the chromatogram and the corresponding autoradiograph.

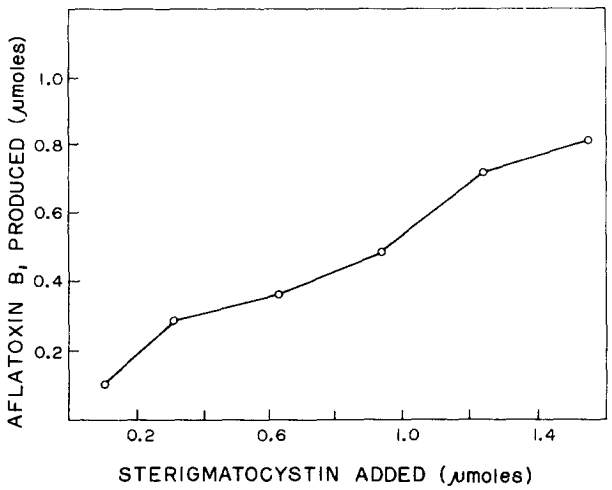

Fig. 2. Conversion of sterigmatocystin to aflatoxin B_1 in 10 ml resting cell cultures of Aspergillus parasiticus ATCC 15517 incubated 20 hr.

To determine whether ST can be converted to AF, pure [^{14}C] ST (0.65 μmol) dissolved in 0.2 ml of acetone was placed in each of three 50 ml baffled flasks. To these, 9.8 ml of the resting cell medium containing 90 μmol of glucose, were added slowly with shaking. Immediately after this, one gram of aflatoxin-synthesizing A. parasiticus ATCC 15517 (10) cells which had been grown for 48 hr in an ammonium-glucose medium (9) was added to each flask, and the flasks shaken (150 rpm) for 20 hr at 30°C. A set of control flasks containing autoclaved cells was used to demonstrate the enzymatic activity involved in the conversion. Another set of parallel flasks each containing 0.2 ml of acetone and 30 μmol of [1-^{14}C] acetate (0.17 Ci/mol), instead of [^{14}C] ST, was used to check the de novo synthetic activity of the cells.

After incubation, the labeled compounds in the cells and broths were exhaustively extracted with acetone and chloroform, respectively. The acetone and chloroform extracts of each flask were pooled, evaporated to dryness under vacuum, and redissolved in 0.2 ml of chloroform. Aflatoxin B_1 and residual ST were then separated and purified using, in succession, the following tlc systems: Biosil-A precoated plates (Quantum Ind., Fairfield,

N.J.) developed respectively with benzene:acetic acid: methanol (90:5:5), CAH, and chloroform:methanol (98:2). The R_f values of AF in these tlc systems were 0.58, 0.52 and 0.54 respectively, whereas those of ST were 0.81, 0.91, and 0.96, respectively. The radioactivity retained in the purified AF from triplicate flasks to which [^{14}C] ST had been added was 10,620 dpm (17% of the added), 13,620 dpm (22%), and 10,530 dpm (17%). No radioactivity was detected in the AF isolated from control flasks containing autoclaved cells. The inability of autoclaved cells to utilize ST was indicated by the fact that about 90-98% of the added radioactivity was recovered in the residual ST. The actual percent conversion of ST to AF must be much higher than the percent radioactivity retained, considering losses of materials during the lengthy extraction and purification procedures. In another experiment, in which percent conversion was calculated from the radioactivity recovered in the AF spots isolated on the two-dimensional tlc plates, values as high as 45-58% were obtained. The de novo synthesis of aflatoxin in the cells used was evident by the incorporation of [1-^{14}C] acetate into AF (0.2-0.3% conversion in triplicate flasks) in the parallel flasks. The active conversion of ST to AF was also indicated by the proportional increase in yield of AF with increase in ST added to the resting cell cultures of A. parasiticus (Fig. 2). We anticipated that there might be permeability problems with the uptake of an insoluble and apolar compound such as sterigmatocystin, but the resting cell system apparently had little difficulty with this compound. This acetone containing system thus provides a useful means for testing other possible intermediates.

The terms, relative specific activity (RSA) and relative isotope content (RIC) of the biosynthetic product have been used to indicate the precursor-product relationship in incorporation studies with isotope labeled compounds (9,11). By definition, the terms refer to the number of lables in the precursor incorporated into a molecule of product. Therefore, based upon the findings that each molecule of AF and ST contains

9 carboxyl-carbons of acetate (3,4,5), the theoretical maximal RSA values with respect to acetate and ST are 9 and 1. The experimental values under the conditions used were 0.32 and 0.33, respectively, despite the fact that the acetate concentration in the medium was 46 times higher than that of ST. It has been shown that the RSA of the product is proportional to the concentration of the substrate in question (11). When averufin was used as substrate at 1.5 µmol per 10 ml, the RSA value was 0.65 (8); if we assume that ST is incorporated as efficiently as is averufin, then, by calculation, a RSA value as high as 0.76 could be accounted for by ST at the same concentration. In practice, only a concentration of 0.65 µmol per 10 ml was used due to the solubility limitation. The high RSA of AF derived from ST indicates that the substrate did not undergo major degradation before the labels were incorporated into the product, and that ST, or a closely related metabolite, is an intermediate in biosynthesis of aflatoxin. Evidence from studies with purified enzymes is needed to determine whether ST is actually on the direct pathway.

Elsworthy et al. (12) have reported incorporation of 5-hydroxydihydrosterigmatocystin, labeled with ^{14}C in the O-methyl group, into aflatoxin B_2 and G_2, implicating 5-hydroxysterigmatocystin as a biogenetic precursor of aflatoxin B_1. Isolation of 5-methoxysterigmatocystin from a mutant of A. versicolor (13), a known producer of ST, has indicated that 5-hydroxysterigmatocystin is probably a precursor of ST. Therefore, the conversion of ST to AF may indicate a biosynthetic pathway in A. parasiticus leading from 5-hydroxysterigmatocystin to sterigmatocystin and then to aflatoxin B_1.

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14. We thank D.I. Fennell for the culture A. versicolor A-18074. This study is supported by PHS grants ES 00612 and ES 00125