Production of Sterigmatocystin by Some Species of the Genus Aspergillus and Its Toxicity to Chicken Embryos

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Sterigmatocystin was produced by 59% of Aspergillus flavus cultures and by 16% of A. parasiticus cultures. All sterigmatocystin-producing cultures of the A. flavus group also simultaneously produced aflatoxin or O-methylsterigmatocystin. Sterigmatocystin was produced by A. chevalieri, A. ruber, and A. amstelodami, species not previously reported to produce the compound. In 5-day-old chicken embryos, the no-effect level of toxicity of sterigmatocystin was between 1 and 2 µg/egg; the mean lethal dose was 5 to 7 µg; and 90 to 100% of the embryos were killed with 10 µg. Teratogenic effects and weight reduction were generally associated with nonlethal doses.

Sterigmatocystin, a metabolite of Aspergillus versicolor, consists of a xanthone nucleus attached to a bifuran structure and bears a close structural relationship to aflatoxin B1. The compound was isolated and its structure was characterized in a series of studies by several workers (1–4). Dickens et al. (6) demonstrated that the compound was carcinogenic but that aflatoxin was 250 times as effective in inducing tumors. A mean lethal dose (LD50) (intraperitoneal) of 60 mg/kg reported in rats (7) compared with an LD50 of 6 mg/kg for aflatoxin B1 (9) further shows the substance to be much less toxic than aflatoxin. In the standard duckling test, aflatoxin is 125 times as effective in initiating bile duct hyperplasia (10). However, sterigmatocystin is produced in larger quantities than aflatoxin, up to 1.2 g/kg of substrate (7), by fungi of such widely divergent phylogenetic relationship as the genera Aspergillus, Penicillium, and Bipolaris and therefore must be considered potentially hazardous to humans and other animals. Our study reports observations on the production of sterigmatocystin by common storage fungi and some aspects of its toxicity.

MATERIALS AND METHODS

The fungi tested for sterigmatocystin production were isolated over a 2-year period from sources ranging from soil to crops such as peanuts, rice, cottonseed, and pecans. These fungi were cultured on a variety of substrates, and due to differences in growth among the isolates the incubation period varied from 7 to 21 days. Isolates were grown on autoclaved cracked yellow field corn, milled rice, slurries of ground peanut or cottonseed, and YES medium of Davis et al. (5). All cultures were incubated at 25 C. The detection of sterigmatocystin was usually secondary to the detection of aflatoxins; therefore cultures were extracted by the method of Pons and Goldblatt (11), which was originally devised for the detection and quantification of the aflatoxins.

The identity and quantity of sterigmatocystin in the extracts were determined by thin-layer chromatography (TLC). TLC plates of silica gel, 0.25 mm thick, were spotted with both the extract and a benzene solution of authentic sterigmatocystin and developed with an acetone-chloroform (15:85, vol/vol) solvent system in unequilibrated chambers. Sterigmatocystin was identified by its Rf and brick-red fluorescence under long-wave ultraviolet light. Its identity was confirmed by the preparation and TLC of the acetate and hemiacetal derivatives. Fluorescence was enhanced by spraying plates with KOH or AlCl3 solutions by the method of Stack and Rodricks (12). In those extracts that contained very small amounts of the compounds or also contained interfering or masking compounds with a similar Rf, preparatory TLC was used to clean up and concentrate the sterigmatocystin before confirmatory chemical tests were made.

To prepare pure crystalline sterigmatocystin the extract was applied to a butt tube containing silica gel. Sterigmatocystin was eluted with diethyl ether, leaving the bulk of the impurities on the silica gel. The ether extract was then adsorbed on silica gel (Merck; <0.08 mm) and placed on a column (15 by 34 cm). The column was developed with 0.5% methanol in methylene chloride. Fractions were monitored by TLC, and those containing sterigmatocystin were bulked, evaporated to dryness, and redissolved in a minimum amount of methylene chloride. An equal volume of hexane was added, and the solution was then dried on a steam bath until crystals started to form. It was then removed from the steam bath, and crystallization was allowed to proceed. After several recrystallizations, chromatographically pure sterigmatocystin crystals were obtained.

For the toxicity tests, 5-day-old embryos weighing between 52 and 63 g were used. White Leghorn eggs
Chicken embryo assay of toxicity of sterigmatocystin. In three tests, 50% of 5-day-old chicken embryos were killed with 5- to 7-µg doses of sterigmatocystin (Table 2). Injections of 10 µg or more usually killed 90 to 100% of the embryos. The weights of the surviving embryos were reduced, often by as much as 50%, at the LD₅₀ and higher injection rates. Teratogenic effects were also noted but they did not appear consistently and were sometimes associated with low dosage. The most common deformity was twisted feet. There was no difference between injections into the air cell or into the yolk. The no-effect level was between 1 and 2 µg/egg. At 2 µg/egg, one embryo died (of 10) and one was stunted. At 1 µg/egg no apparent effect was observed.

**DISCUSSION**

Hsieh et al. (8) showed that resting cells of A. parasiticus efficiently converted sterigmatocystin to aflatoxin B₁, and concluded that sterigmatocystin was a precursor of aflatoxin. Our data (Table 1) showed less accumulation of sterigmatocystin by A. parasiticus than by A. flavus. The amount of sterigmatocystin detected in any A. parasiticus culture did not exceed 12

| Test no. | No. of replications | Dose range (µg/embryo) | LD₅₀ (µg/embryo) |
|----------|---------------------|------------------------|------------------|
| 1        | 12                  | 1.14–22.25             | 7                |
| 2        | 20                  | 1.25–30                | 5                |
| 3        | 10                  | 1–10                   | 5–6              |
| 1        | 10                  | 1.25–20                | 5                |

* Inoculated into air cell.
* Inoculated into yolk sac.

**Table 2. Toxicity of sterigmatocystin in chick embryos**

| **Aspergillus spp.** | **No. of isolates tested** | **No. of isolates producing aflatoxins** | **No. of isolates producing sterigmatocystin** | **Sterigmatocystin produced (µg/flask)** |
|----------------------|----------------------------|----------------------------------------|-----------------------------------------------|------------------------------------------|
| A. flavus            | 96                        | 86                                    | 57                                            | Trace                                     |
| A. parasiticus       | 127                       | 127                                   | 20                                            | Trace                                    |
| A. versicolor        | 3                         | 0                                     | 3                                             | 2,500                                    |
| A. nidulans          | 2                         | 0                                     | 2                                             | 600                                      |
| A. rugulosus         | 8                         | 0                                     | 8                                             | 30                                       |
| A. chevalieri        | 8                         | 0                                     | 8                                             | — b                                     |
| A. ruber             | 1                         | 0                                     | 1                                             | —                                        |
| A. amstelodami       | 1                         | 0                                     | 1                                             | —                                        |

a Difference in yield includes variance due to different media and incubation periods.

b Yield small and not quantified.

c Yield on basis of µg/kg of cracked corn medium.
µg compared to 381 µg for A. flavus. Similarly, a greater percentage of A. flavus cultures contained sterigmatocystin. Apparently A. flavus strains generally do not convert sterigmatocystin to aflatoxin as efficiently as A. parasiticus. Since we found no cultures of either species that accumulated sterigmatocystin without a concurrent accumulation of aflatoxin, the enzyme system that converts sterigmatocystin to aflatoxin appears to be common to these species.

In contrast, aflatoxin production by such heavy producers of sterigmatocystin as A. versicolor, A. nidulans, and A. rugulosus has neither been reported elsewhere nor observed in this study. Therefore the required enzymes must be almost totally absent in these species. In a possible exception, we have observed in this laboratory aflatoxin production by a strain of A. rugulosus, but the phenomenon was not repeated consistently, so the possibility of undetected contamination cannot be completely ruled out.

We have also observed production of small amounts of aflatoxin by cultures of the A. glaucus group. With these species, the ability to synthesize aflatoxin also appears to be transient and is usually observed in cultures newly isolated from natural sources. Production of sterigmatocystin was also inconsistent but was not necessarily associated with fresh isolates from nature. Apparently, therefore, the possibility of natural contamination of agricultural products with either aflatoxin or its precursor, sterigmatocystin, is not limited to products infested by a relatively few fungal species.

The chicken embryo was suggested as a bioassay organism for detection of aflatoxin by Verrett et al. (13) in 1964. They reported an LD₉₀ for aflatoxin B₁ of 0.025 µg/egg when injected into the air cell before incubation and reported that resistance to the toxin increased rapidly with age of the embryo. By using 5-day-old embryos, nonfertile eggs and nondeveloping embryos can be rejected before inoculation; thus one source of error can be eliminated. Although the LD₉₀ is higher at 5 days than earlier, the order of sensitivity is still very high.

Our data also show that the acute toxicity (calculated at the all-killed level) of sterigmatocystin for the chick embryo is 1/16 that of aflatoxin B₁, slightly greater than that reported for rats (7). This suggests that toxicity for some organisms is greater than indicated by the standard duckling test (10) and by its ability to produce carcinoma (6). The 5-day-old chick embryo is a useful bioassay to confirm toxicity when the presence of sterigmatocystin is suspected.

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