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 B_1. 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 (LD_{50}) (intraperitoneal) of 60 mg/kg reported in rats (7) compared with an LD_{50} of 6 mg/kg for aflatoxin B_1 (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 phylogenic 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 R_f 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 $AlCl_3$ 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 R_f, 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

from a local hatchery were incubated for 5 days and prepared for inoculation by swabbing the shell over the air cell with 1% iodine dissolved in ethanol, and a hole was punched through the shell with an egg punch. From 10 to 20 embryos were used for each treatment. For inoculation into the yolk sac, a no. 22 needle was inserted about 30 mm straight down into the egg yolk. For air cell inoculation, no. 22 needles were cut to about 5 mm in length and filed smooth. The needle was placed into the hole, and the inoculum was dropped onto the membrane. The holes were covered with tape. The mycotoxins were dissolved and diluted in methanol to give the desired amount per 0.02 ml of inoculum. Comprehensive tests of the carrier, methanol, did not show any deleterious effect at these levels. Volume of inoculum was 0.02 ml in all tests. The eggs were candled daily, and deaths were recorded. At 18 days of age the surviving embryos were refrigerated. The embryos were then separated from the rest of the egg, examined for deformities, blotted, and weighed.

RESULTS

Production of sterigmatocystin. Sterigmatocystin was produced by 59% of the *A. flavus* cultures but by only 16% of *A. parasiticus* cultures (Table 1). Most isolates of these two species also produced aflatoxins. There was no indication of a correlation between the amounts of aflatoxin and sterigmatocystin produced, but *A. flavus* isolates that produced neither aflatoxin nor O-methylsterigmatocystin did not produce sterigmatocystin. *A. versicolor*, *A. nidulans*, and *A. rugulosus* produced the largest quantities of sterigmatocystin. The species of the *A. glaucus* group (*A. chevalieri*, *A. ruber*, and *A. amstelodami*) that produced small quantities of sterigmatocystin in our tests have not previously been reported as producers. The quantity produced by all but the one isolate of *A. amstelodami* was quite low, but the identity of the toxin was chemically confirmed (12) in each case.

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 LD_{50} 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 B_1 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

TABLE 2. *Toxicity of sterigmatocystin in chick embryos*

Test no.	No. of replications	Dose range (μg/embryo)	LD_{50} (μg/embryo)
1[a]	12	1.14–22.25	7
2	20	1.25–30	5
3	10	1–10	5–6
1[b]	10	1.25–20	5

[a] Inoculated into air cell.
[b] Inoculated into yolk sac.

TABLE 1. *Production of sterigmatocystin by species of the genus Aspergillus*

Aspergillus spp.	No. of isolates tested	No. of isolates producing aflatoxins	No. of isolates producing sterigmatocystin	Sterigmatocystin produced (μg/flask)[a]	
				Minimum	Maximum
A. flavus	96	86	57	Trace	381
A. parasiticus	127	127	20	Trace	12
A. versicolor	3	0	3	2,500	16,000
A. nidulans	2	0	2	600	4,100
A. rugulosus	8	0	8	30	7,900
A. chevalieri	8	0	8	—[b]	—
A. ruber	1	0	1	—	—
A. amstelodami	1	0	1	—	1,375[c]

[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 LD_{50} for aflatoxin B_1 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 LD_{50} 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 B_1, 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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