Distribution of Sterigmatocystin and Fungal Mycelium in Individual Brown Rice Kernels Naturally Infected by *Aspergillus versicolor*

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Cereal Chem. 61(1):48-52

ABSTRACT

The distribution of fungal mycelium and the mycotoxin sterigmatocystin in individual brown rice kernels naturally infected by *Aspergillus versicolor* was studied with scanning electron and fluorescence microscopy. Mycotoxin content in the milled rice or rice bran at various milling stages was determined by fluorodensitometry. Scanning electron microscopy showed that invading mycelia were often in the adjacent germ, aleurone layer, or starchy endosperm. The fungus usually occurred around germ as an entangled mycelial mass, but it was rarely found in the major part of the endosperm unless the grain was cracked. Characteristic yellow fluorescence caused by the mycotoxin was found in the germ, aleurone layer, and starchy endosperm adjacent to them, and conspicuously around the germ. Concentrations of mycotoxin in brown rice were 3.8–4.3 ppm. Mycotoxin content in milled rice decreased gradually from 71.6 to 7.7%, depending on the milling yields from 97.7 to 56.4%. The major portion of mycotoxin produced in brown rice could be removed at the minimum milling yield (56.4%).

Cereal grains are often infected with various fungi and occasionally contaminated with mycotoxins produced by them (Hesseltine 1974). Among these grains, rice is the most important food source for Japan, and it can be an excellent substrate for production of mycotoxins, such as aflatoxin or sterigmatocystin, by toxigenic fungi (Schroeder 1968). Many reports have been published concerning the mycoflora and mycotoxins in stored rice (Kurata et al 1968, Miyaki et al 1970, Manabe et al 1973, Tsuruta and Manabe 1974). Some of the toxins were detected in brown rice naturally infected by toxigenic molds in Japan (Manabe and Tsuruta 1975, Sugimoto et al 1977).

It is important to know the distribution of the internal fungal mycelium and associated mycotoxin in individual rice grains infected by toxigenic molds. Rice is generally consumed in the form of a milled rice after the removal of the caryopsis coat, germ, and aleurone. Few papers have been reported on fungal and toxin distribution in moldy rice grains, however.

This article deals with the distribution of internal fungal mycelium and sterigmatocystin, a hepathocarcinogenic mycotoxin, in rice kernels naturally infected by *Aspergillus versicolor* (Vuill) Tiraboshi. A combined electron and fluorescence microscopy study was used. The content of the mycotoxin in the milled rice and the bran fraction was also measured.

MATERIALS AND METHODS

The fungus-infected brown rice, which had been stored in a warehouse for two to three years after harvest, was found in 1975 as described previously (Manabe and Tsuruta 1975). Since 1975, it has been preserved in our laboratory at −20°C to prevent further fungal growth.

Scanning Electron Microscopy

The brown or milled rice grains were dehydrated with a graded series of ethanol solutions in the usual manner (Matsumoto and Azuma 1979). Then, they were sliced transversely or longitudinally with a razor blade. The sliced grains were mounted on stubs and coated with gold. Those specimens were observed with a Hitachi S-450 scanning electron microscope. The sliced grains of healthy rice grain were observed without dehydration.

Fluorescence Microscopy

The surface of the sliced grains was sprayed with 20% ethanolic solution of aluminum chloride and heated for 15 min at 80°C to enhance the fluorescence due to sterigmatocystin. Then, the specimens were examined to locate fluorescence from the mycotoxin. We used an Olympus Vanox photomicroscope equipped with a reflected light fluorescence attachment, a 200-W super-pressure mercury burner, and two excitation/barrier fluorescence filter combinations having excitation maxima at 360 nm (UG-1) and over 500 nm (Y 495).

Milling, Extraction, and Determination of Sterigmatocystin

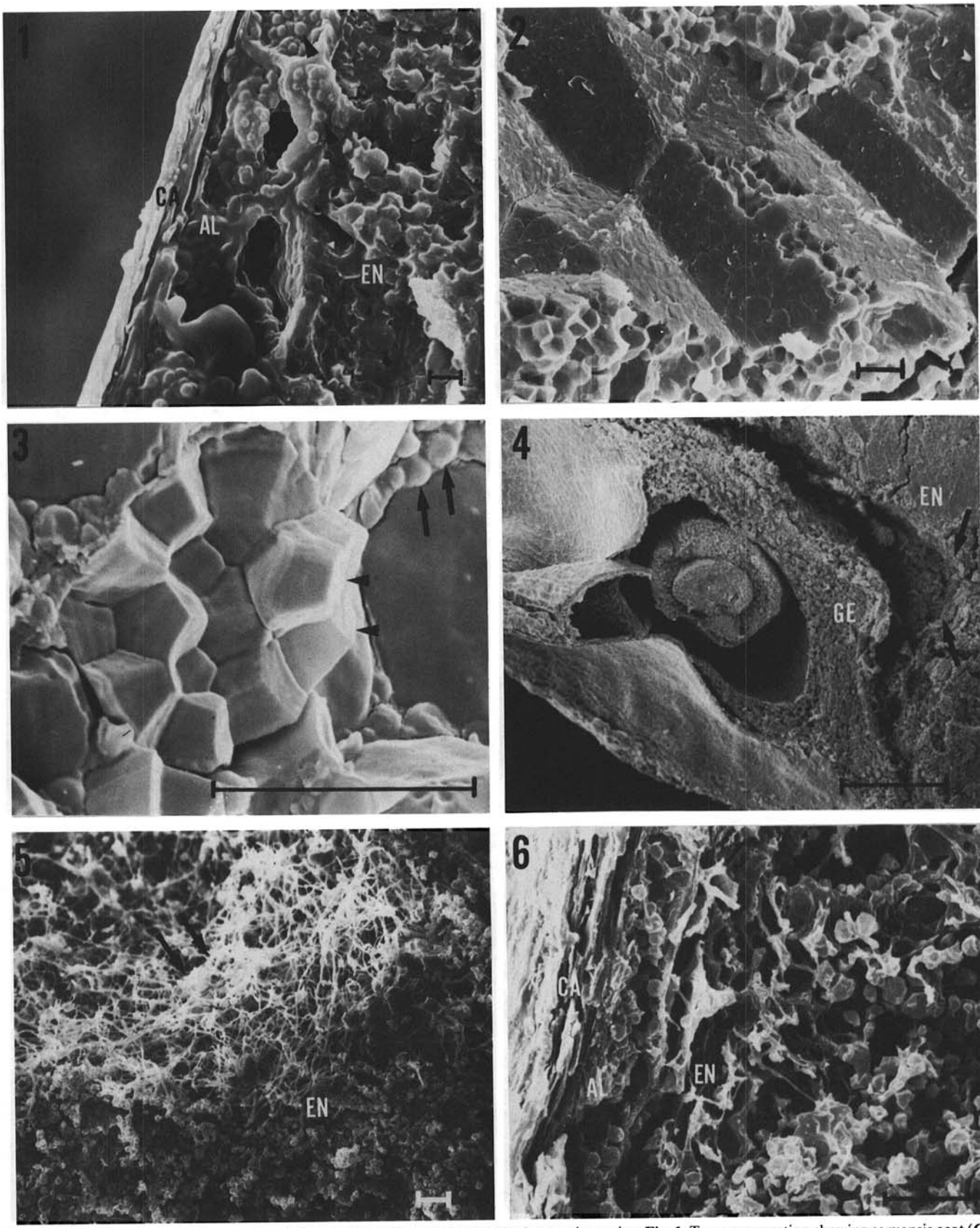
The brown rice was milled with a Kette Parrest test mill to give various milling yields (91.7–56.4%). The rice bran fractions so obtained were extracted with chloroform overnight and filtered with filter paper (Toyo-Roshi no. 2). The milled rice grains, after being crushed with a mortar and pestle, were also extracted and filtered. All the extract solutions were concentrated under reduced pressure and dissolved in 1 ml of acetone. Then, they were cleaned with Sephadex LH-20 column chromatography as previously described (Manabe et al 1973). The fraction containing sterigmatocystin was redissolved in 1 ml of acetone. An aliquot of the solution and 5–20 μl of the standard containing 0.1 mg of sterigmatocystin per milliliter were spotted on a Merck-Kiesel gel plate (type 60) and developed with benzene–methanol–acetic acid (90:5:5, v/v). After drying, the plate was sprayed with 20% ethanolic aluminum chloride and heated at 80°C for 15 min. Values of R_f and coloring of sample spots were examined using sterigmatocystin as standard, the R_f of which was 0.68. Sterigmatocystin was determined with a Shimazu Chromato-scanner CS-900 fluorodensitometer at excitation and emission wavelengths of 365 and 500 nm, respectively.

RESULTS

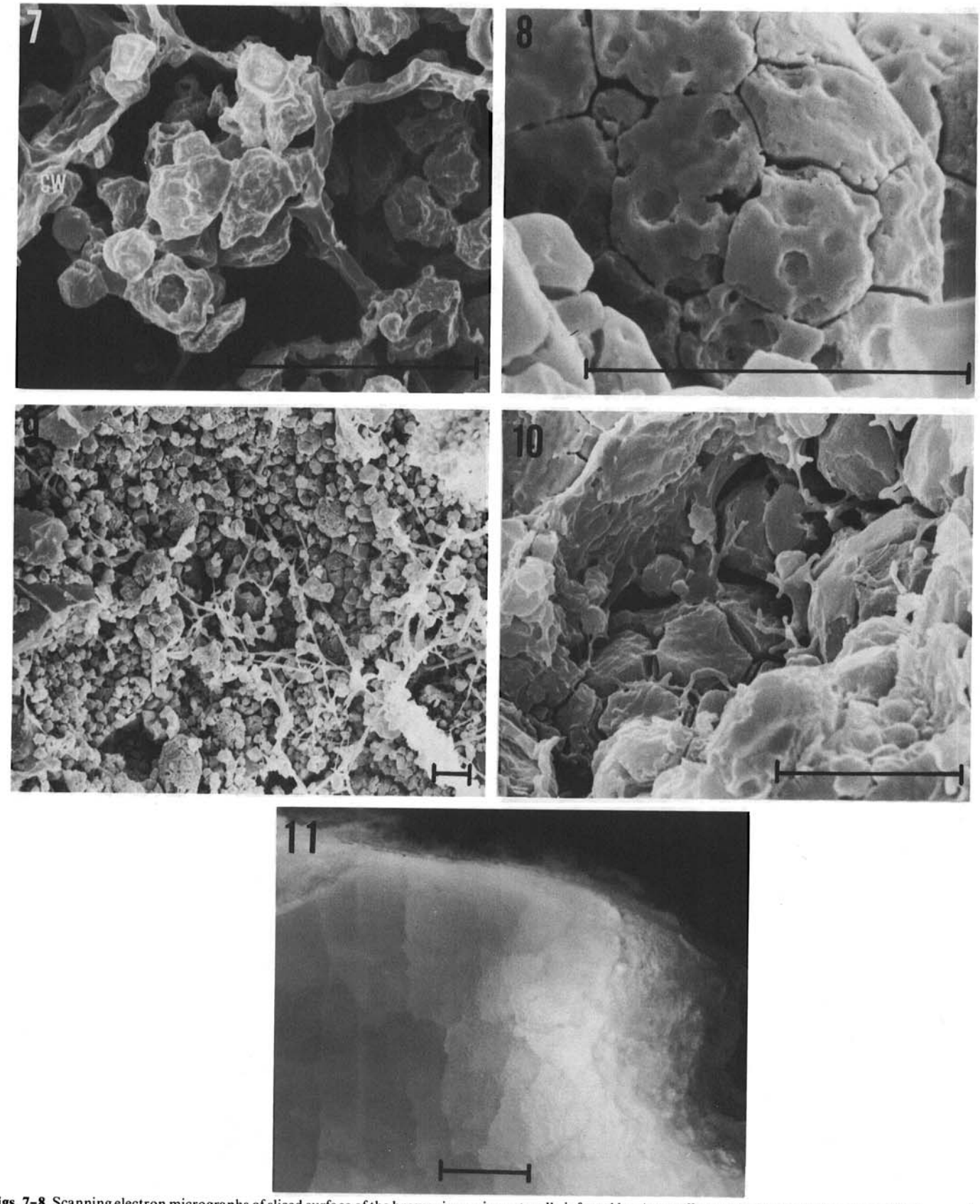
Scanning Electron Microscopy

A healthy mature brown rice kernel consists of three main parts, ie, caryopsis coat (pericarp and seed coat), germ, and endosperm, which is composed of the aleurone layer and starchy endosperm. The aleurone contains many aleurone grains (protein bodies) and lipid bodies (Fig. 1). Starchy endosperm, which is the major part of a rice kernel, is composed of polygonally shaped cells arranged tightly in a regular manner (Fig. 2). They were enclosed with ultrathin cell walls (Shibuya and Iwasaki 1978). Compound starch granules were embedded among the cementing materials and protein bodies (Fig. 3). The germ is usually tightly attached to the starchy endosperm, but these parts were easily separated during tissue processing (Fig. 4). These findings were consistent with those reported in the literature (Kiribuchi and Nakamura 1974).

The invading mycelium of *A. versicolor* was clearly observed in the adjacent germ, aleurone, and endosperm. In particular, a mycelial mass covered the surface of the endosperm adjacent to the germ (Fig. 5). The aleurone layer was also destroyed by fungal digestion (Fig. 6), in which aleurone grains and lipid bodies had



Figs. 1–4. Scanning electron micrographs of sliced surface of apparently healthy brown rice grains. **Fig. 1.** Transverse section showing caryopsis coat (**CA**), aleurone layer (**AL**), aleurone grains plus lipid bodies (arrowhead), and starchy endosperm (**EN**). This specimen was prepared without dehydration. **Fig. 2.** Polygonal pillar starchy cells arranged tightly in a regular manner in the endosperm. Numerous compound starch granules are closely packed in the cells. **Fig. 3.** Magnified image of a compound starch granule embedded in the cementing materials and protein bodies (arrows), consisting of many granula (arrowheads). **Fig. 4.** Longitudinal section around the germ showing the inner edge of the germ bordering the starchy endosperm. Germ (**GE**) is separate from the endosperm (**EN**), which results in the formation of space between them (arrows). **Figs. 5–6.** Scanning electron micrographs of sliced surface of the brown rice grains naturally infected by *Aspergillus versicolor*. **Fig. 5.** Longitudinal section showing the surface of the endosperm (**EN**) adjacent to the germ covered with an abundant mycelial mass (arrows). **Fig. 6.** Transverse section showing invading mycelium, and structures of the aleurone layer (**AL**) and the endosperm (**EN**) eroded extensively by fungal enzymes. CA = caryopsis coat. Scale bars indicate 10 μm, except in Fig. 4, in which the scale bars indicate 250 μm.



Figs. 7–8. Scanning electron micrographs of sliced surface of the brown rice grains naturally infected by *Aspergillus versicolor*. **Fig. 7.** The magnified image of the eroded endosperm. The starch granula and the wall of the starchy cells (**CW**) were partially digested by fungus. **Fig. 8.** The compound starch granule showing partial digestion by the fungal enzymes, which resulted in numerous pitlike and/or terraced depressions on the surface. **Figs. 9–10.** Scanning electron micrographs of surface of the milled rice grains naturally infected by *A. versicolor*. **Fig. 9.** Longitudinal section showing mycelia that were not removed by milling. The mycelia are wedged among individual starch granula. **Fig. 10.** Transverse section showing some spaces caused by the fungal digestion of the starch granules in the endosperm. **Fig. 11.** Fluorescence micrograph of the surface of longitudinally sectioned grains naturally infected by *A. versicolor*. The fluorescence due to the sterigmatocystin is brilliant in the surface layer of the brown rice grains, especially around the germ. Scale bars indicate 10 μm except in Fig. 11, in which the scale bar indicates 250 μm.

small depressions. Some lipid bodies also coalesced and formed large masses of amorphous material (not shown) similar to spherosomes in the moldy wheat grains (Anderson et al 1970). In the highly damaged grain, most of the bodies disappeared, and the layer was filled with mycelia (not shown). Mycelia occasionally invaded the starchy endosperm through the germ and/or aleurone layer. In some kernels, no mycelia could be observed in the endosperm even though mycelia were abundant in the aleurone. Invaded endosperm had cell walls, cementing materials, protein bodies, and starch granules that were partly or completely digested. A great number of individual starch granula were also seen (Fig. 6). Partially digested starch granula had pitlike and/or terraced depressions on their surfaces (Figs. 7 and 8). Generally, mycelia were observed on the surface of the brown rice grains, and rarely found in the starchy endosperm, except when the rice grains had been cracked (not shown).

Split surfaces of the milled rice (88% yield) obtained from the moldy brown rice showed that the fungal mycelium had penetrated into the endosperm through the germ (Figs. 9 and 10). Cell walls and storage proteins were lacking, and many partially digested starch granula were present.

Fluorescence Microscopy

Characteristic yellow fluorescence under ultraviolet light, probably due to sterigmatocystin, was observed in the germ, aleurone layer, and starchy endosperm adjacent to them, particularly around the germ (Fig. 11). The fluorescence generally became less intense as the milling yield decreased. The fluorescent yellow pigment and invading mycelium from the infected rice grains could not be completely removed even by the extensive milling. This yellow fluorescence was not observed in a noninfected brown rice and residual powder of infected brown rice after extraction with chloroform. In addition, no fluorescent yellow substance except the sterigmatocystin, was detected in the chloroform-extract solutions made from the moldy rice.

Distribution of Sterigmatocystin

Table I shows the contents of the sterigmatocystin in the milled rice and bran fractions at various milling levels. The concentration of the mycotoxin in the original brown rice was calculated from the total amount in the milled rice plus bran fraction. They were in the range of 3.8–4.3 ppm, and nearly consistent with that previously reported by Manabe and Tsuruta (1975). The concentration of the toxin in the milled rice grains decreased gradually with a decrease in milling yield. The ratios of distribution of the toxin in the milled rice ranged from 71.6 to 7.7%, depending upon the degree of milling (91.7–56.4%). Most of the mycotoxin in the brown rice was removed in the milling process.

The mycotoxin could not be removed completely from the contaminated rice by milling, nor was it possible to remove the invaded mycelium or the fluorescent glow of the mycotoxin. In addition, most of the milled rice grains were broken during milling at the minimum milling yield (56.4%) in our experiments. At the usual milling yields (93–91% for domestic use), about one third of the mycotoxin initially present in the brown rice was removed.

TABLE I
Distribution of Sterigmatocystin in the Milled Rice and Rice Bran Fraction at Various Milling Yield Levels

Milling Yield[a] (%)	Fraction	Weight[b] (g)	Concentration of Sterigmatocystin (ppm)	Distribution of Sterigmatocystin (%)
91.7	Milled rice	9.41	3.0	71.6
	Rice bran	0.85	13.2	28.4
86.9	Milled rice	9.92	2.0	41.3
	Rice bran	1.30	21.8	58.7
69.3	Milled rice	7.41	0.8	12.2
	Rice bran	3.29	12.2	87.8
56.4	Milled rice	5.88	0.6	7.7
	Rice bran	4.54	8.7	92.3

[a] Milling yield represents (weight of milled rice/weight of brown rice) × 100.
[b] Wet basis.

DISCUSSION

Fungi generally invade a cereal grain through the germ (Christensen and Kaufman 1974, Fennel et al 1973). In brown rice, invasion begins at the germ unless the grain is stored under high (100%) humidity or the caryopsis coat is injured (Naito 1955). Our observations on the moldy rice with the scanning electron microscope showed abundant mycelia at the starchy endosperm adjacent to the germ. Previous work suggested that the presence of nutrients and relatively higher water activity in the germ facilitated ready invasion by the mold (Naito 1954) because the surface of brown rice grains is completely enclosed by a hydrophobic cuticle (Bechtel and Pomeranz 1977), except near the germ (Takahashi et al 1982). The germ is probably a major path for an alternative transfer of moisture between a rice grain and the atmosphere.

Researchers recently observed that small spaces between pericarp and germ occurred in artificially moldy corn kernels (Tsuruta et al 1981). In moldy brown rice grains, as well, similar vacant small space might be formed between the germ and adjacent starchy endosperm because the germ would become smaller after drying after harvest. The germ is likely to separate from starchy endosperm by dehydration, as shown in Fig. 4, or by physical force such as milling. Bechtel and Pomeranz (1978) reported that the rice germ was easily removed from the caryopsis during milling, which probably depends on structural factors between the germ and starchy endosperm. Our micrographs show some larger spaces and vigorous growth of invading fungi in the spaces.

Previous work by Tsuruta et al (1974) on the mycoflora of the moldy rice revealed that *A. versicolor* was the major fungus because it could be detected in most of the grains tested. The majority of the sterigmatocystin produced (approximately 98%) was still present within the mycelia after 30 days in liquid culture (*unpublished data*). Sterigmatocystin is a typical endo-type toxin, which produces hepathocarcinogens (Purchase and van der Watt 1970). Therefore, the distribution of the mycotoxin would almost parallel that of the invaded fungal mycelium as well as the yellow fluorescence. Our results clearly showed that most of the sterigmatocystin was located in the adjacent germ, aleurone, and starchy endosperm of the moldy grains, particularly around the germ, and only a little toxin occurred in the starchy endosperm. In contrast, Lee et al (1967) determined and schematically diagrammed the distribution of aflatoxin, an exotoxin produced by *A. flavus*, within contaminated peanut. The aflatoxin (1,000 ppm) was distributed throughout the entire cotyledon.

As shown in Table I, at the usual milling yield range of 93–91% for domestic use, about one third of the sterigmatocystin in the brown rice was removed in the milling process. The data were concerned with brown rice having a high concentration of sterigmatocystin (3.4–4.3 ppm). Toxin levels in milled products were previously reported to vary with the content of the toxin in the cereal grains being milled (Schroeder et al 1968, Brekke et al 1976, Bennet et al 1976).

As a result, milling has played an important role in detoxifying rice physically. It is, however, also important to establish the safety of rice bran when the bran is used as a source of rice oil or livestock feed.

ACKNOWLEDGMENTS

We wish to thank C. W. Hesseltine, Northern Regional Research Laboratory, Peoria, IL; M. Ichinoe, National Institute of Hygenic Science, Tokyo, Japan; and N. Shibuya, Food Research Institute, Tsukuba-gun, Ibaraki, Japan, for their helpful suggestions. We are also grateful to H. Kurata for his encouragement throughout this work.

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[Received November 1, 1982. Accepted August 22, 1983]