Moniliformin, a Metabolite of *Fusarium moniliforme* NRRL 6322: Purification and Toxicity

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Fusarium moniliforme NRRL 6322 produced about 600 mg of recoverable moniliformin, a mycotoxic metabolite, per kg of corn grit medium. The moniliformin was extracted from the grits with methanol, purified by preparative thin-layer chromatography, and crystallized from ether. The 50% lethal dose for chicken embryos was 2.8 μ g per egg. For 1-day-old chicks dosed with moniliformin by crop intubation and for female and male mice injected intraperitoneally, the 50% lethal doses were 5.4, 20.9, and 29.1 mg per kg of body weight, respectively. The toxin did not cause a reaction on mouse skin.

Fusarium moniliforme, a field fungus common on many crops, including oats, pepper, flax, soybeans (20), millet (12), sorghum (2), barley, corn, and wheat, may be present in corn that appears to be perfectly sound (3). The fungus causes ear rot, kernel rot, and stalk rot of unharvested corn (8), and it may also be involved in advanced decay of stored high-moisture shelled corn (11). In laboratory experiments testing the development of fungi on stored sorghum, *F. moniliforme* was the dominant fungal competitor at moisture contents of from 15 to 29% (2).

Because *F. moniliforme* is present on unharvested and stored grain, knowledge concerning the mycotoxins it produces appears essential to maintaining animal health. In one study (6), extracts from 74 of 85 *F. moniliforme* strains induced toxic reactions on rabbit skin. Other reports (9, 17, 18) also indicate that a relatively high percentage of *F. moniliforme* strains produce substances toxic to mice. Chickens fed a ration infected with *F. moniliforme* developed a disease characterized by severe leg deformity (15). The fungus was also implicated in diseases of Equidae referred to as blind staggers, corn stalk disease, moldy corn disease, and leucoencephalomalacia, the latter a disease involving the central nervous systems of donkeys and horses (7, 19).

Although *F. moniliforme* has been for many years associated with animal toxicity, few mycotoxins have been identified from strains of this species. Fusariocin A, a compound toxic to HeLa cells (1), zearalenone, an estrogenic hormone (13), and moniliformin, a substance highly toxic to chickens (4), appear to be the only mycotoxins

characterized to date for this species. The structure of moniliformin (Fig. 1), 1-hydroxycyclobut-1-ene-3,4 dione (the potassium cation and one water of crystallization), was determined by X-ray structural analysis (16). Moniliformin also occurs as the sodium salt (4). Before characterization, the water-soluble molecule was found to be highly toxic to 1-day-old cockerals (50% lethal dose [LD₅₀], 4.0 mg/kg of body weight administered per os) and phytotoxic on corn and tobacco (4). Recently, moniliformin has been identified as a metabolite of *Fusarium fusarioides* (14). Several strains of *F. fusarioides* produce in laboratory culture more than 800 mg of moniliformin per kg of growth substrate.

Because of its toxicity to chickens, its phytotoxicity, and the prevalence of the producing fungus on grain, we report additional information about moniliformin detection, production, purification, and toxicity to test animals.

MATERIALS AND METHODS

Production of moniliformin. To 50 g of white corn grits in a 300-ml Erlenmeyer flask was added 15 ml of water before autoclaving and 10 ml of sterile water after autoclaving. Grits were inoculated with a conidial suspension of *F. moniliforme* NRRL 6322 and incubated in static culture at 28°C for 16 to 20 days. Fermented grits were extracted twice with 250 to 300 ml of methanol, and the solution was taken to dryness on a rotary evaporator. The soluble residue was dissolved in 50 to 75 ml of warm methanol and was added slowly to about 4 volumes of acetone to remove precipitable impurities. After filtration, the volume was reduced to 10 to 15 ml, and acetone insolubles were removed by a second precipitation. The solvents were evaporated, and the residual solids were taken up in 5 to 10 ml of methanol and streaked onto 2-mm-thick

FIG. 1. *Moniliformin*. $R = \text{Na or K}$.

thin-layer chromatography (TLC) plates (precoated PLC plates of silica gel 60 F-254 with fluorescent indicator; E. Merck AG, Darmstadt, Germany). Preparative plates were developed in a solvent system containing toluene, acetone, and methanol (5:3:2) and examined under short-wave, 254-nm UV light. A zone containing moniliformin was readily identified at an R_f of 0.25 to 0.3 by the intense quenching of the fluorescent indicator. Silica gel containing the moniliformin recovered from the plates was eluted with methanol. After charcoal filtration, the solution was reduced to 3 to 5 ml and added slowly to 25 ml of diethyl ether; usually tiny crystals formed along with an amorphous, brownish precipitate. The precipitate was rechromatographed on 2-mm-thick TLC plates, and the moniliformin was recovered in methanol and recrystallized from ether. The molar ratio of moniliformin was calculated from its sodium content obtained from a water solution by a Corning model 450 flame photometer and by UV absorption spectroscopy at 231 nm ($\epsilon = 20,300$; 10).

TLC limits for moniliformin detection. Quantities (1 μl) of methanol solutions (0.1, 0.2, 0.3, and 0.5 μg) were spotted on TLC plates with fluorescent indicator and developed in the solvent system described above. Developed plates were observed for quenching under short-wave UV light.

Toxicity tests with mice. Sterile aqueous solutions of moniliformin were injected intraperitoneally (0.2 ml) into five female and five male white mice, weighing about 25 g each, at concentrations equivalent to 0, 20, 25, 30, and 35 mg per kg of body weight. The mice were observed over a 4-day period, and LD_{50} values were determined by the computer program of Daum (5). The same program was used for LD_{50} determinations in chicks and chicken embryos.

Toxicity tests with chickens. A 0.5-ml amount of a moniliformin-water solution was intubated into the crop of 1-day-old chicks (seven chicks per dose level) at concentrations of 0, 1, 2, 4, 8, and 16 mg per kg of body weight. Chicks were fed and watered after treatment and observed during the 4-day test period.

Toxicity to chicken embryos. Chicken embryos (4 days old; 25 eggs per dose level) were injected in the air sac with 0.05 ml of toxin in aqueous solution per embryo. Eggs were incubated for a total of 20 days, and then the developed embryos were killed with chloroform and examined for teratogenic effects.

Mouse skin test. About 100 μg of moniliformin dissolved in dimethyl sulfoxide was applied (0.01 ml of a 10-mg-per-ml solution) to a 2-cm square on the shaved back of each of four white mice.

RESULTS AND DISCUSSION

Moniliformin was extracted from fermented grits with methanol. After the removal of acetone-precipitable impurities, the compound was purified by preparative TLC and crystallization from diethyl ether. Nearly 660 mg of yellowish crystals, which chromatographed as a single substance on TLC when examined under long-wave (366-nm) and short-wave (254-nm) UV light, were obtained from 1 kg of white corn grits fermented with *F. moniliforme* NRRL 6322. Upon further purification with preparative TLC and recrystallization from ether, 480 mg of tiny, cream-colored crystals was recovered. The crystals had the same R_f value and the same UV, infrared, and nuclear magnetic resonance spectra as the moniliformin standard and matched literature values (4, 10). An atomic absorption spectrometry analysis revealed that the crystals were 96% moniliformin and that *F. moniliforme* NRRL 6322 produced only the sodium salt of moniliformin. A purity of 93% moniliformin was estimated for these crystals by the UV absorption method of analysis. On TLC plates with fluorescent indicator, 0.1 μg of moniliformin was visually discernible at an R_f near 0.35. Estimation by TLC of diluted methanol extracts indicated that *F. moniliforme* NRRL 6322 produced between 7 and 10 g of moniliformin per kg of fermented substrate.

The LD_{50} value (Table 1) of 5.4 mg per kg of body weight for 1-day-old chickens was only slightly higher than the 4.0 mg per kg reported by Cole et al. (4). Chicks surviving the dosages of toxin showed no ill effects at any time during the 4-day observation period. Moniliformin was less toxic to mice, with an LD_{50} of 20.9 mg per kg for the females and 29.1 mg per kg of body weight for the males. As in the case of the chicks, mice surviving the toxin demonstrated no ill effects; the mice or chicks that died became recumbent in 4 to 6 h after treatment and died within 24 h. In 4-day-old chicken embryos, a sharp LD_{50} of 2.8 μg per embryo was obtained with no overt gross teratogenic effects in the survivors. We have not studied the chronic effects of moniliformin in animals, but reports (10, 14) of rats fed meal rations molded with moniliformin-producing strains of *Fusarium* provide data indicating reduced weight gains, histopathological lesions of internal organs, and death of animals consuming feeds that contain large amounts of toxin.

At present, it is not known whether moniliformin significantly affects animal health. However, the potency of the toxin, the quantities elaborated by the fungus, and the prevalence of moniliformin-producing *Fusarium* encourage its study.

TABLE 1. Toxicity of moniliformin in laboratory tests

Test system	Route of administration	LD ₅₀	99% Confidence interval
Chicken embryo	Air sac	2.8 µg/egg	2.3-3.5
Chickens, 1-day-old	Crop intubation	5.4 mg/kg	3.6-8.2
Mouse, male	Intraperitoneal	29.1 mg/kg	26.8-31.9
Mouse, female	Intraperitoneal	20.9 mg/kg	19.1-24.7

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