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Mouse Bioassay for *Fusarium* Metabolites: Rejection or Acceptance when Dissolved in Drinking Water

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Ten metabolites of *Fusarium* species (butenolide, diacetoxyscirpenol, equisetin, fusaric acid, gibberellic acid, moniliformin, NRRL 6227 peptide, T-2 toxin, vomitoxin, and zearalenone) were added to the drinking water of mice to determine whether they were consumed or refused. Of the 10, only the trichothecenes—diacetoxyscirpenol, T-2 toxin, and vomitoxin—were refused. Refusal of 2 mg of the trichothecenes per liter was not enhanced by adding 100 mg of zearalenone per liter.

Fusarium species are often found on corn, barley, oat, and wheat kernels and, as agents of major storage rots, may produce toxins that contaminate human and animal food. In laboratory culture, selected strains of most species of *Fusarium* are capable of producing secondary metabolites toxic to animals (15). Not only are those metabolites toxic to animals, but some of them may also reduce the palatability of feedstuffs for certain farm animals. Feed refusal by farm animals, particularly swine, has been a recurring problem in the north central United States (5, 10, 17, 22): in several years, when the preharvest season was cool and moist, a relatively large portion of the corn crop was affected. Ear rot of dent corn caused by *Gibberella zeae* (Schw.) Petch, conidial-stage *Fusarium graminearum* Schwabe, was evident in one-third of the Indiana counties in 1972 (17). Swine refused to eat freshly harvested corn contaminated with this mold. Several counties in northwest Ohio reported similar feeding problems in the years 1970, 1972, 1975, and 1977 (22). Investigations to determine the fungal product that was specifically responsible for the feed rejection led Vesonder et al. (20) to vomitoxin (also known as deoxynivalenol), a compound which they purified from field-contaminated corn and identified as a member of the trichothecene family. Other studies indicated that many trichothecenes caused feed rejection and emesis in laboratory animals (18). In nine cases in which feeding corn or mixed feeds caused either food refusal, vomiting, or physiological damage to animals, Mirocha et al. (13) found low levels of either diacetoxyscirpenol (DAS), T-2 toxin, or vomitoxin and zearalenone. Both vomitoxin and zearalenone are often produced by the same strain of *F. graminearum* and, consequently, are found together in the same grain sample (10, 11, 13). Analysis of feed samples refused by swine may yield levels of vomitoxin too low to account for refusal (10, 11). This observation has led some investigators to surmise that vomitoxin, zearalenone, and possibly other factors are interacting to cause feed refusal (10, 11).

The trichothecenes have no unique properties that provide an easy identification by physicochemical analysis, although gas-liquid chromatography and gas chromatography-mass spectrographic procedures have been applied to the analysis of feed samples suspected of containing these toxins (14). Before the instrumental analysis can be made, however, column and thin-layer chromatographies and other separation techniques are required to separate the relatively small amounts of toxin from interfering substances (9, 12). Biological tests for trichothecenes are not definitive, but they provide a way to reduce the need for physicochemical procedures by indicating samples most likely to contain active compounds. Feeding swine (5, 7, 19, 21) or rodents (6, 10, 19) *F. graminearum*-contaminated rations or rations spiked with a rejection factor is one way to determine the level of metabolite required to cause feed rejection. Cereal products, however, are not easy to mix uniformly and are not readily recovered for accurate weighings. To overcome these obstacles, we developed a mouse bioassay based on the consumption of the mycotoxin in a solution of ethanol and water to determine the level of acceptance of three trichothecenes and seven other metabolites produced by *Fusarium*. The assay is relatively quick and simple and defines the relation between the metabolite concentration and the amount consumed.

MATERIALS AND METHODS

***Fusarium* metabolites.** DAS was obtained from

Makor Chemicals Ltd., Jerusalem, Israel, and gibberellic acid was obtained from Calbiochem, La Jolla, Calif. Zearalenone was a gift from Commercial Solvents Corp., Terre Haute, Ind. The remaining compounds were produced at the Northern Regional Research Center by previously described procedures: butenolide (8), equisetin (2), fusaric acid (23), moniliformin (3), NRRL 6227 peptide (4), T-2 toxin (1), and vomitoxin (20, 21).

Test of metabolites as feed refusal factors. Each metabolite was dissolved in 95% ethanol to the desired concentration (see Tables 1 and 2) and added to distilled water so that solutions contained 5% of the ethanol-metabolite solution. Zearalenone was not soluble at this concentration of ethanol-water, but a stable milky-appearing suspension was formed. Ten milliliters of the metabolite solution was pipetted into test tubes (18 by 150 mm), which were then closed with a rubber stopper fitted with a glass tubing. A 5% solution of the ethanol in distilled water served as a control. Before the testing of the metabolites, mice were given the 5% ethanol control fluid for 1 week; mice consuming less than 4 ml/day were not used in the study. The solutions were presented to adult Swiss Webster female white mice that were caged individually, fed a commercial feed, and housed in a 25°C, constant-temperature room. After 24 h, the tubes were removed from the cages, and the volume imbibed by each mouse was recorded. Mice were given a control solution for at least 2 days before being presented another test solution.

Each compound, except DAS and vomitoxin, was presented at a concentration of 100 mg/liter to each of seven mice. Solutions of metabolites consumed at about the same rate as the control were not tested further. The metabolites known to have rejection properties, the trichothecenes, were tested further with seven mice per treatment at levels of 0, 2, 5, 10, 20, and 40 mg/liter. Zearalenone (100 mg/liter) and each of the trichothecenes, DAS, T-2 toxin, and vomitoxin (2 mg/liter), were tested together (seven animals per test solution) to determine whether there was an interaction of zearalenone and the trichothecenes.

Data consisting of the milliliters of water consumed per animal were examined by use of analysis of variance. When a series of concentrations was tested, lines relating water consumption to logarithm concentration were calculated for each compound. The significance of differences between compounds was tested by standard statistical methods (16). The standard deviation per mouse was 0.97 ml, and the standard error at a mean based on seven mice per test was 0.37.

RESULTS AND DISCUSSION

Solutions of the drinking fluid containing 100 mg of the nontrichothecene metabolites—butenolide, equisetin, fusaric acid, gibberellic acid, moniliformin, NRRL 6227 peptide, and zearalenone—per liter were consumed (about 6.0 ml per mouse) at a rate about equal to the control, whereas mice presented the solutions containing T-2 toxin drank only 13.5% (0.8 ml) of that consumed by the animals on the control solution (Table 1). The data clearly show that mice refuse drinking water containing either T-2 toxin (Tables 1 and 2), vomitoxin, or DAS (Table 2).

Butenolide, like T-2 toxin and DAS, is a skin vesicant (24) and a likely refusal factor, if the cause of refusal is due to irritation brought about by dermatic effects in the alimentary tract. Butenolide, however, was not rejected. Moniliformin was consumed at a rate (18 mg/kg of body weight, assuming that a mouse weighs 33 g) near its intraperitoneal 50% lethal dose value (20.9 mg/kg) for female mice (3) with no adverse effects. In fact, all of the mice in the study remained active and appeared healthy several months after cessation of the testing.

Each of the three trichothecenes caused a substantial reduction in the consumption of fluid at 40 mg/liter (Table 2). The intake was reduced 66, 80, and 70%, respectively, for DAS, T-2 toxin, and vomitoxin. As with rats and pigs offered solid feeds amended with mycotoxins, the consumption of drinking water by mice was reduced as the concentration of trichothecene was increased. The 24-h dose consumption response for each of the toxins indicated that the bioassay relation was semilogarithmic for each of the

TABLE 1. *Mean water consumption for seven mice with various Fusarium metabolites at 100 mg/liter*[a]

Treatment	Mean (ml/day)
Control	6.9
Moniliformin	6.6
Equisetin	6.5
Butenolide	6.7
Control	6.1
NRRL 6227 peptide	6.0
Zearalenone	5.6
Gibberellic acid	6.4
Control	6.0
T-2	0.8
Fusaric acid	6.5

[a] Least significant difference at 5% level, 1.03; 95% limits, 0.73.

TABLE 2. *Mean water consumed by seven mice for three trichothecenes at various concentrations*[a]

Trichothecene	Water consumed (ml/day) at following concn (mg/liter):						
	0[b]	2	5	10	20	40	100
T-2 toxin	5.6	4.0	3.4	2.3	2.1	1.1	0.8
Diacetoxyscirpenol	5.6	4.7	2.4	2.5	2.1	1.9	
Vomitoxin	5.6	5.2	3.1	2.8	2.4	1.7	

[a] Least significant difference at 5% level, 0.89.

[b] Control mean, which represents 35 mice in five separate tests. The range of variation between test controls was 5.0 to 7.2 ml/day.

toxins and parallel at the precision level of the assay (Fig. 1). A 2.5-fold increase in refusal factor concentration resulted in a reduction of approximately 1 ml of drinking solution per mouse.

Table 3 shows the results of a number of feeding trials conducted in different laboratories. In comparing rejection rates of trichothecenes in the mouse bioassay, one must keep in mind that experimental procedures and the aliment amended varied considerably. Despite the lack of uniformity of test conditions, the rejection rate of each trichothecene by rodents and swine was reasonably consistent. For example, the range of rejection for DAS was 66 to 75% at the 40-μg/g level, even though the three tests were carried out with different animal species and the toxin was presented to each species on a different aliment. Rejection rates at the 40-μg/g level for T-2 toxin was 78% for swine presented amended whole corn, 75% for rats fed corn kurls, and 80% for mice imbibing toxin in drinking water. A considerably larger range of rejection rates occurred in the vomitoxin tests at 40 μg/g than with either DAS or T-2 toxin. The rejection values varied from 54% for rats fed corn kurls to 90% for swine given naturally contaminated corn. In the mouse bioassay, the refusal rate for vomitoxin was 70%, a value 10% less than the volume of T-2 toxin-contaminated water imbibed and 4% more than the refusal of DAS. Although literature data for feeds amended with less than 40 μg/g of a refusal factor are fewer, comparisons of feed refusal of each test at the lower test levels indicate that the sensitivity of mice consuming a refusal factor dissolved in water is comparable to that of swine or rats presented trichothecene-amended feeds. In fact, the results of a swine feeding test by Vesonder et al. (19) at levels of 10, 20, and 40 μg of T-2 toxin per g vary no more than 3% from that of the mouse bioassay. At trichothecene levels of

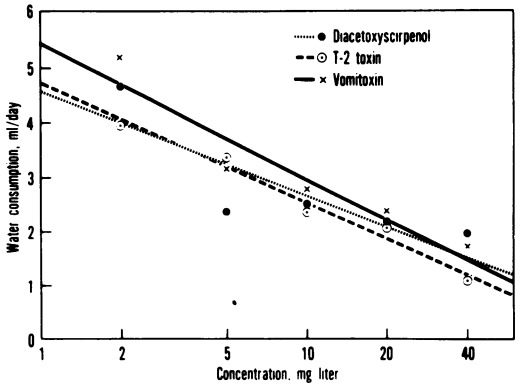

FIG. 1. *Comparison of consumption by mice of solutions of three trichothecenes at increasing concentrations.*

TABLE 3. *Comparison of various tests for animal refusal of three trichothecenes*

Trichothecene	Concn (μg/g)	Feed	Animal	% of control rejected	Reference
Vomitoxin	3.6	Corn	Pig (4)[a]	20	7
	7.2	Corn	Pig (4)	43	7
	7.2	DS[b]	Mouse (7)	32	—[c]
	40.0	Corn	Pig (4)	90	7
	40.0	Corn	Pig (4)	75	19
	40.0	Corn kurls	Rat (18)	54	19
	40.0	DS	Mouse (7)	70	Table 2
T-2 toxin	2.0	Corn	Pig (4)	8	19
	2.0	DS	Mouse (7)	29	Table 2
	5.0	Ground corn	Rat (5)	54	10
	5.0	DS	Mouse (7)	39	Table 2
	10	Corn	Pig (4)	58	19
	10	DS	Mouse (7)	59	Table 2
	20	Corn	Pig (4)	65	19
	20	DS	Mouse (7)	62	Table 2
	40.0	Corn	Pig (4)	78	19
	40.0	DS	Mouse (7)	80	Table 2
	40.0	Corn kurls	Rat (14)	75	19
	50.0	Ground corn	Rat (5)	74	10
DAS	40.0	Corn	Pig (4)	75	19
	40.0	Corn kurls	Rat (14)	74	19
	40.0	DS	Mouse (7)	66	Table 2

[a] Number of animals in test.
[b] DS, Trichothecene dissolved in drinking solution. Data from this manuscript.
[c] Concentration extrapolated from Fig. 1.

below 10 μg/g, results of the different assays varied considerably more than they did at higher levels, suggesting that more standard tests need to be made to determine the validity of comparing refusal rates of swine and rodents at trichothecene levels of below 10 μg/g.

Concentrations of 100 mg of zearalenone per liter and 2 mg each of DAS, T-2 toxin, and vomitoxin per liter were chosen to test whether the refusal rate of the mouse was influenced by an interaction of zearalenone and the trichothecenes. This test was suggested for the following reasons: (i) zearalenone and vomitoxin are usually found together in preharvested corn, and zearalenone, along with T-2 toxin, has been found in a mixed feed sample (13); (ii) *Fusarium*-contaminated feeds containing zearalenone and vomitoxin are reportedly rejected at a rate much greater than can be accounted for by the measured vomitoxin concentration (10, 11); and (iii) a concept is developing, based on circumstantial evidence, that feed refusal rates are influenced by the presence of zearalenone (10, 11). In our study, mice did not refuse solutions containing 100 mg of zearalenone per liter (Table 1), and there was no apparent interaction of zearalenone with any of the trichothecenes to influence the consumption rate (Table 4). Kotsonis et al. (10) likewise found no interaction to enhance feed refusal between pure zearalenone (50 mg/kg) and T-2 toxins (5 mg/kg) fed to rats, but a positive correlation of refusal was noted when a larger amount of T-2 toxin was added to the feed.

The mouse bioassay data suggested that zearalenone was not a refusal factor, and it did not alter the intake of water containing 2 mg of either of the three trichothecenes per liter. Whether zearalenone would interact with a trichothecene to enhance refusal at higher trichothecene concentrations is not known. We believe, however, that the noted enhancement of refusal of *F. graminearum*-contaminated feeds by swine (10, 11) is more likely caused by either incomplete measurement of the actual amount of vomitoxin in the feed or unidentified refusal factors, rather than by an interaction of zearalenone and vomitoxin.

TABLE 4. *Mean water consumed for tests of 2 mg of each trichothecene per liter with and without 100 mg of zearalenone per liter*[a]

Compound	Water consumed (ml/day)	
	Trichothecene only	Trichothecene plus zearalenone
Control	5.2	6.4
Diacetoxyscirpenol	4.7	4.0
T-2 toxin	4.0	4.7
Vomitoxin	5.2	4.8

[a] Least significant difference at 5% level, 0.98.

Measurement of the acceptance or rejection of solutions of *Fusarium* toxins by mice appears to be an efficient, economical way to differentiate between metabolites involved in rejection of feed by swine and metabolites not involved in feed refusal. The small amount of metabolite required for each mouse permits the use of more animals, which increases the precision of comparisons among compounds. The mouse test should be advantageous in screening for trichothecenes in feed refusal cases and also in examining *Fusarium* cultures for refusal potential. With mice as indicators of refusal factors, strains of *Fusarium* that are good producers of known trichothecenes or new refusal factors may be easily selected for further study.

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