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Co-Identity of the Refusal and Emetic Principle from *Fusarium*-Infected Corn

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The structure of vomitoxin isolated from *Fusarium*-contaminated corn was proved to be 3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one. This same toxin is responsible for the "refusal phenomenon" exhibited by swine fed contaminated corn. In addition, two new substances believed to be trichothecenes were isolated from naturally infected corn. Vomitoxin was also isolated from rice inoculated with *F. graminearium* NRRL 5883.

Since 1919, a recurring problem for hog feeders in the United States has been *Fusarium*-infected grain, a problem exacerbated in 1972 by an unusually cool and wet year. *Fusarium*-contaminated grain when fed to swine or animals with simple stomachs results in either refusal or vomiting. The *Fusarium* spp. produce many 12,13-epoxytrichothec-9-enes that are associated with various diseases in animals (4). Many of these trichothecene-mycotoxins, when produced by Fusaria either in vitro or naturally, cause emesis in a variety of laboratory animals (3, 7, 8). In 1973 (9), we isolated a new trichothecene (vomitoxin) from *Fusarium*-contaminated field corn grown in northwestern Ohio. This corn was infected mainly with *Fusarium graminearium* although other fungi were also present. Intubation of 7 mg of vomitoxin into a pig caused vomiting within 25 min.

On the basis of spectroscopic data, we tentatively assigned a structure, 3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one, to this naturally occurring trichothecene (Fig. 1) (9). Morooka et al. (6) also isolated this same trichothecene from fusaria-infected barley and, initially, assigned it the trivial name, R_d toxin; later Yoshizawa and Morooka (10) described its structure and assigned a new trivial name, 4-deoxy-nivalenol. Incidences of refusal of *Fusarium*-infected grain were reported in 1928 (5) and again in 1966 (2). It has not been determined whether the factors causing emesis and refusal have the same structure or differ. Curtin and Tuite (2) suggested first that refusal and emetic factors differed. However, our results show a co-identity of emetic (vomitoxin) and refusal factor. Also, under laboratory conditions, we obtained this toxin on a solid substrate by the isolate *F. graminearium* NRRL 5883; spiking noncontaminated corn with either molded grain or vomitoxin from rice fermentation rendered it nonacceptable to swine.

This additional work confirms our tentatively proposed structure for vomitoxin (1). We converted vomitoxin to its triacetate and compared its nuclear magnetic resonance (NMR) to that of the diacetate of 3α-acetoxy-12,13-epoxy-7α,15-dihydroxytrichothec-9-en-8-one reported by Blight and Grove (1). We also report gas-liquid chromatographic (GLC) and mass spectral data of the trimethylsilyl ether derivative of vomitoxin.

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MATERIALS AND METHODS

Production on rice and isolation. *F. graminearium* NRRL 5883 isolated from naturally infected corn was maintained on hay agar slants (6% hay, 2% K_2HPO_4, 2% agar, and pH 6 to 6.5). A sterilized moist solid substrate, consisting of 350 g of rice and 100 ml of distilled water (50 ml added before autoclaving and 50 ml of sterile water added after the autoclaved rice was loosened with a stirring rod) in Fernbach flasks, was inoculated with 1 ml of a spore suspension prepared from a 7-day-old slant culture and about 1 ml of sterile distilled water. The flasks were incubated at 28 C for 13 days and shaken daily to prevent matting. The molded rice, which was dried 4 h at 80 C in a forced-air oven, was mixed with an equal weight of uncontaminated corn and offered to 40-pound (ca. 18,140 g) pigs; these pigs had been without feed for 20 h.

Vomitoxin was isolated from fermented rice. The rice was blender extracted twice for 5 min with butanol (1 liter/kg), and the butanol-extracted rice was washed with hexane and dried in a vented hood. The dry butanol-extracted rice was then blender extracted for 5 min with 40% aqueous methanol (1

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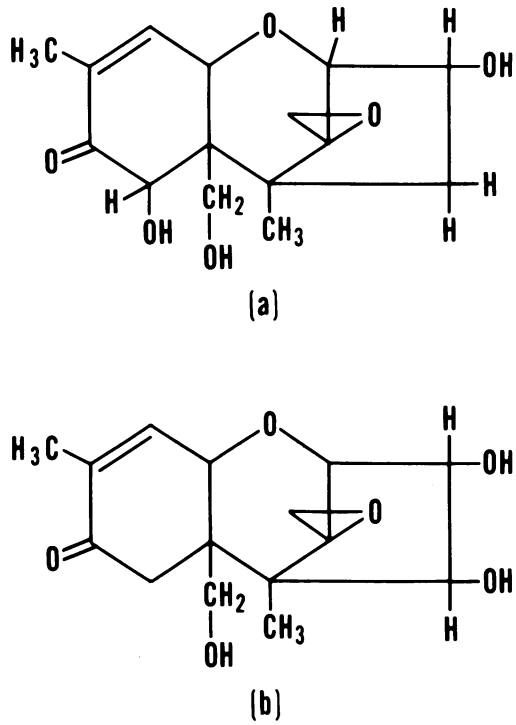

FIG. 1. *(a) Vomitoxin; (b) a trichothecene similar to vomitoxin.*

liter/kg, two times). These extracts were combined, concentrated to a small volume, and then freeze-dried to a brown solid. This residue was stirred with methanol at room temperature for 1 h, filtered, and taken to dryness with a rotor-evaporator. The solids were redissolved in a minimum amount of methanol and added to acetone with stirring to precipitate either sugars or other insoluble materials. The acetone-methanol solubles were repeatedly treated in this manner until no further precipitate occurred on acetone addition (usually three times). The methanol-acetone-soluble yellow oil was treated with 5% $NaHCO_3$ solution (pH 8 to 8.5) with stirring at room temperature for 0.5 h, taken to dryness under vacuum, and added to the top of a silica gel column presaturated with chloroform. The column was washed with a liter of chloroform followed by elution of the toxin with 300 to 400 ml of 3% methanol in chloroform. The crude vomitoxin was rechromatographed on silica gel with 2% methanol-chloroform. The flow rate of the column was 0.4 ml/min, and 7-ml fractions were collected. Pure vomitoxin was found in tubes 41 to 52 as evidenced by thin-layer chromatography (TLC), GLC, infrared (IR) spectral analysis, and specific rotation. This toxin was used to spike good corn before offering it to 40-pound pigs (ca. 18,140 g).

Isolation of vomitoxin from naturally infected corn. Vomitoxin was isolated from naturally *Fusarium*-contaminated corn as previously described (9) but with resin treatment of the ethanol-water solubles, Florsil chromatography, and preparative TLC omitted. The naturally infected corn was extracted with butanol and then methanol-water. The methanol-water extract was concentrated to small volume; absolute ethanol was added to precipitate a tan sticky residue. The ethanol-water solubles were concentrated to small volume and freeze-dried. This residue was treated with methanol, methanol-acetone in the same manner as the freeze-dried material from the rice fermentation. Crude vomitoxin from corn was further purified by silica gel column chromatography followed by $NaHCO_3$ treatment and again silica gel column chromatography, the procedure followed in isolating vomitoxin from rice fermentation. The vomitoxin was further purified by dissolving in acetone and applying the solution to a Sephadex LH-20 column (2.4 by 200 cm) presaturated with acetone. The column was eluted with acetone and 5-ml fractions were collected. Fractions 111 to 120 contained vomitoxin as evidenced by high resolution mass spectrometry (MS), optical rotation, and conversion of the toxin to a triacetate derivative. Toxin was spiked onto good corn and then fed to 40-pound (ca. 18,140 g) pigs.

Pig refusal. Each fraction obtained during the isolation procedure for vomitoxin from *Fusarium*-infected corn was assayed for refusal activity by spiking onto good corn an amount of extract equivalent to 500 g of moldy corn. The extract had been dissolved in 60 ml of ethanol and added in small amounts, with shaking, to the uncontaminated corn contained in plastic bags; the spiked corn was dried at 60 C in a forced-air oven. These samples of spiked corn were then fed in duplicate over a 24-h period to 40- to 60-pound (ca. 18,140 to 27,210 g) pigs that had been without feed for 17 to 20 h. Duplicate samples of spiked corn were randomly assigned to pigs so that no pig received the same sample during the 2-day feeding trial. Control samples of good corn and corn naturally infected with *Fusarium* were fed with each set of samples tested. Also, fermented rice was mixed with an equal weight of good corn and fed.

GLC. A Bendix gas chromatograph (model 1500) equipped with on-column flame ionization detectors was used for analysis of vomitoxin. The column (6 feet [ca. 182.88 cm] by 2 mm, glass) was packed with 3% OV-1 on Gas Chrom 100/200, the temperature was programmed from 150 to 250 C at 5 C/min, and the flow rate of nitrogen carrier gas was set at 40 ml/min. Vomitoxin (100 μg in 0.1 ml of pyridine) was converted to its trimethylsilyl (TMS) ether derivative by treatment with Regisil BSTFA plus 1% TMCS (40 μl) at room temperature for 15 min. Two microliters of this solution was injected into the column.

Combined gas chromatography-mass spectrometry (GC-MS). The TMS derivative of vomitoxin was analyzed on a Packard 7400 gas chromatograph connected through a membrane separator to a Nuclide 12-90-DF mass spectrometer. The mass spectra were recorded every 10 s of the GC peaks.

The IR spectra were recorded with a Beckman model IR-8 spectrophotometer from films deposited on KRS-5 plates (Wilks, Inc.). Melting point (uncorrected) was determined on a Fisher-John apparatus.

RESULTS

The refusal factor in *Fusarium*-infected corn was assayed by the response of swine to extracts of the moldy corn. Refusal activity was related to corn consumption and considered positive if the pigs' reluctance to consume each sample of corn containing the various extracts in a 24-h period was comparable with the pigs' acceptance of the naturally contaminated *Fusarium*-infected corn (Table 1). The swine neither refused nor were reluctant to consume good corn containing butanol extracts, ethanol precipitates, and acetone insolubles. Also, no unusual behavior by the pig, such as vomiting or diarrhea, was observed. The corn residue remaining after the methanol-water extraction was readily consumed by pigs and apparently had no adverse effects. Only fractions containing vomitoxin were refused by the swine to the same extent as the contaminated corn. These extracts prepared from 4 kg of naturally contaminated corn included the methanol-water, ethanol-water solubles, freeze-dried material, and methanol-acetone solubles.

The residue (6 g) from the methanol-acetone solubles was further fractionated by silica gel column chromatography; only the fraction that contained vomitoxin (300 mg, judged to be 55% pure as compared with known amounts of vomitoxin by TLC) caused a refusal response when put on good corn and fed to swine. Further purification of this fraction by silica gel chromatography gave 80 mg of vomitoxin (judged to be 90% pure by GLC). This purification was followed by Sephadex column chromatography that gave chromatographically pure vomitoxin, 20 mg of which was spiked into 500 g of good corn and fed to pigs. This corn was rejected to the same extent as naturally infected corn.

We were unable to crystallize vomitoxin by the usual techniques, such as solvent crystallization, high-vacuum distillation, and repeated Sephadex chromatography. The chromatographically pure vomitoxin gave an optical rotation in ethanol of $[\alpha]_D^{26}$ + 6.25 and a formula of composition $C_{15}H_{20}O_6$ (*m/e* calculated, 296.1259; found, 296.1260) as determined by high-resolution MS. These values, as well as the IR spectrum and NMR spectrum, are in agreement with those for deoxynivalenol as reported by Yoshizawa and Morooka (10). Vomitoxin was further analyzed as its TMS ether derivative by GLC and GC-MS. GLC revealed a major peak at 13.88 min and one minor peak at 14.22 min (Fig. 2). The GC-MS analyses showed the major peak to have a molecular ion at 512, which corresponds to addition of three TMS groups, and the minor peak, a molecular ion of 440 indicating two TMS groups; these data are consistent for the three hydroxyl groups contained in vomitoxin (Fig. 3).

On acetylation of vomitoxin (20 mg) with acetic-anhydride pyridine, we obtained a crystalline triacetate of vomitoxin with an mp 153 to 154 C (lit. 155 to 156 [1]; 155 to 157 C [10]). The NMR spectrum was identical to that of the diacetate of 3α-acetoxy-12,13-epoxy-7α,15-dihydroxytrichothec-9-en-8-one reported by Blight and Grove (1) (Table 2).

A fraction that preceded vomitoxin from the Sephadex column showed an *m/e* of 296 ($C_{15}H_{20}O_6$). The mass spectrum was similar in the lower regions to that of vomitoxin; since there was no major ion at *m/e* 248 corresponding to the loss of both water and formaldehyde

TABLE 1. *Refusal responses by pigs to naturally contaminated Fusarium corn versus good corn and good corn spiked with extracts from naturally contaminated corn and with vomitoxin*

Sample	Corn not consumed							
	Day 1 (g)				Day 2 (g)			
	0 h	2 h	6 h	24 h	0 h	2 h	6 h	24 h
Good corn	500	300	225	0	500	250	200	0
Fusarium-infected corn	500	490	400	325	500	400	400	385
Vomitoxin[a]	500	470	470	322	500	475	450	432
Fusarium-infected rice	450	440	440	418	500	475	450	410
Butanol extract	500	250	50	0	500	200	100	0
Methanol-water extract	500	350	325	300	500	350	300	200
Ethanol insoluble	500	400	100	0	500	200	0	0
Methanol insoluble	500	400	300	0	500	100	0	0
Acetone insoluble	500	450	300	0	500	200	25	0
Chloroform eluate	500	425	110	0	500	400	300	0
Methanol-$CHCl_3$ eluate	500	450	400	260	500	400	300	200
Corn residue	500	325	0	0	500	200	50	0

[a] First-day corn contained 20 mg of vomitoxin isolated from naturally infected corn; day 2 corn contained 20 mg of vomitoxin isolated from rice fermentation.

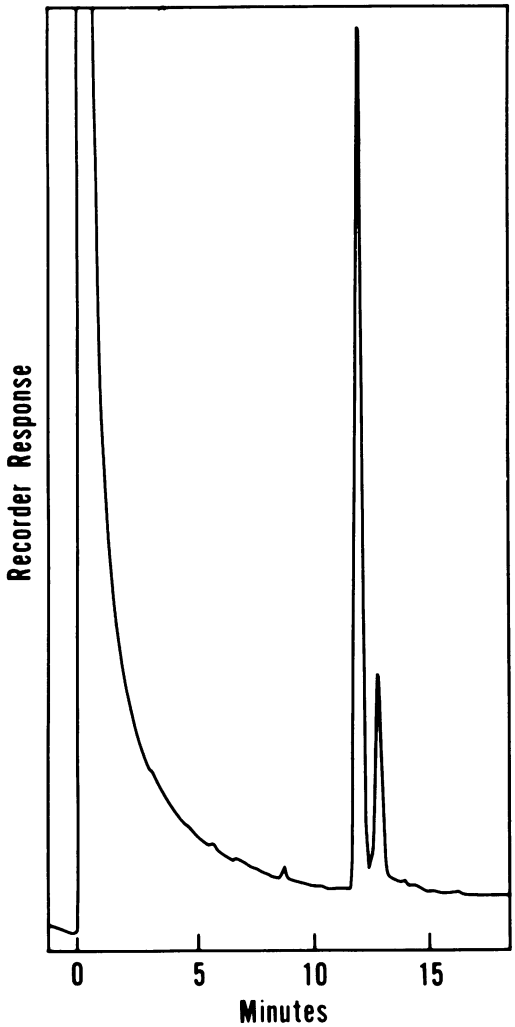

FIG. 2. *Analysis by GLC of TMS derivative of vomitoxin.*

for the hydroxyl group adjacent to the C- keto group, probably this compound is another trichothecene, similar to vomitoxin, but with the hydroxyl group on the C4 rather than the C6 position (Fig. 1b). Also IR bands at 1,680 and 3,400 cm^{-1} are consistent for the tentatively proposed structure of this substance. The mass spectrum of the second trichothecene appears as Fig. 4. Additionally, another fraction from the Sephadex column preceding the vomitoxin-like compound gave IR bands at 3,400, 1,730, and 1,670 cm^{-1}, suggestive of a partially acetylated trichothecene. These two components were isolated in amounts of approximately 3 mg/4 kg of *Fusarium*-infected corn.

The isolate, *F. graminearium* NRRL 5883, was grown on corn and rice to confirm that it was the fungus responsible for producing refusal factors. This isolate produced the refusal factor both on corn and rice. We chose to work further with the rice substrate because of greater ease of purification. A 13-day-old rice fermentation of the isolate NRRL 5883 was mixed with an equal amount of good corn and then pelletized. These pellets were rejected by the swine. From each kilogram of fermented rice, 60 mg of chromatographically pure vomitoxin was isolated. Its physiochemical properties were the same as those found for vomitoxin isolated from naturally contaminated corn. Vomitoxin from the rice substrate had an optical rotation of $[\alpha]_D^{26}$ + 6.65 (ethanol) and the same GLC pattern and GC-MS analysis. It was spiked onto good corn in an amount of 20 mg/500 g; pigs rejected this spiked corn.

DISCUSSION

Fusarium spp. have been widely reported for their ability to produce toxins, which have been implicated in many disorders of farm animals.

TABLE 2. *NMR of vomitoxin and the comparison of vomitoxin triacetate with the diacetate of 3α-acetoxy-12,13-epoxy-7α,15-dihydroxytrichothec-9-en-8-one*

C atom	Vomitoxin	Diacetate[a] (δ)	Vomitoxin triacetate (δ)
2	3.60 d (5)	3.99 d (5)	3.89 d (5)
3	4.50 m	5.3 m	5.2 m
4	2.16 m	2.25 m	2.28 m
7	4.80 m	6.14 s	6.04 s
10	6.60 m	6.64 d (5.5)	6.05 d (5.5)
11	4.80 m	4.82 d (5.5)	4.76 d (5.5)
13	3.10 ABq (4)	3.00 ABq (4)	2.95 ABq (4)
14	1.44 s	1.00 s	0.96 s
15	3.82 s	4.41 s	4.30 s
16	1.88 s	1.92 s	1.86 s
OAc		1.96 s	1.90 s
		2.20 s	2.12 s
		2.26 s	2.20 s

[a] Blight and Grove (1). d, Doublet; s, singlet; m, multiplet (J/Hz given in parentheses).

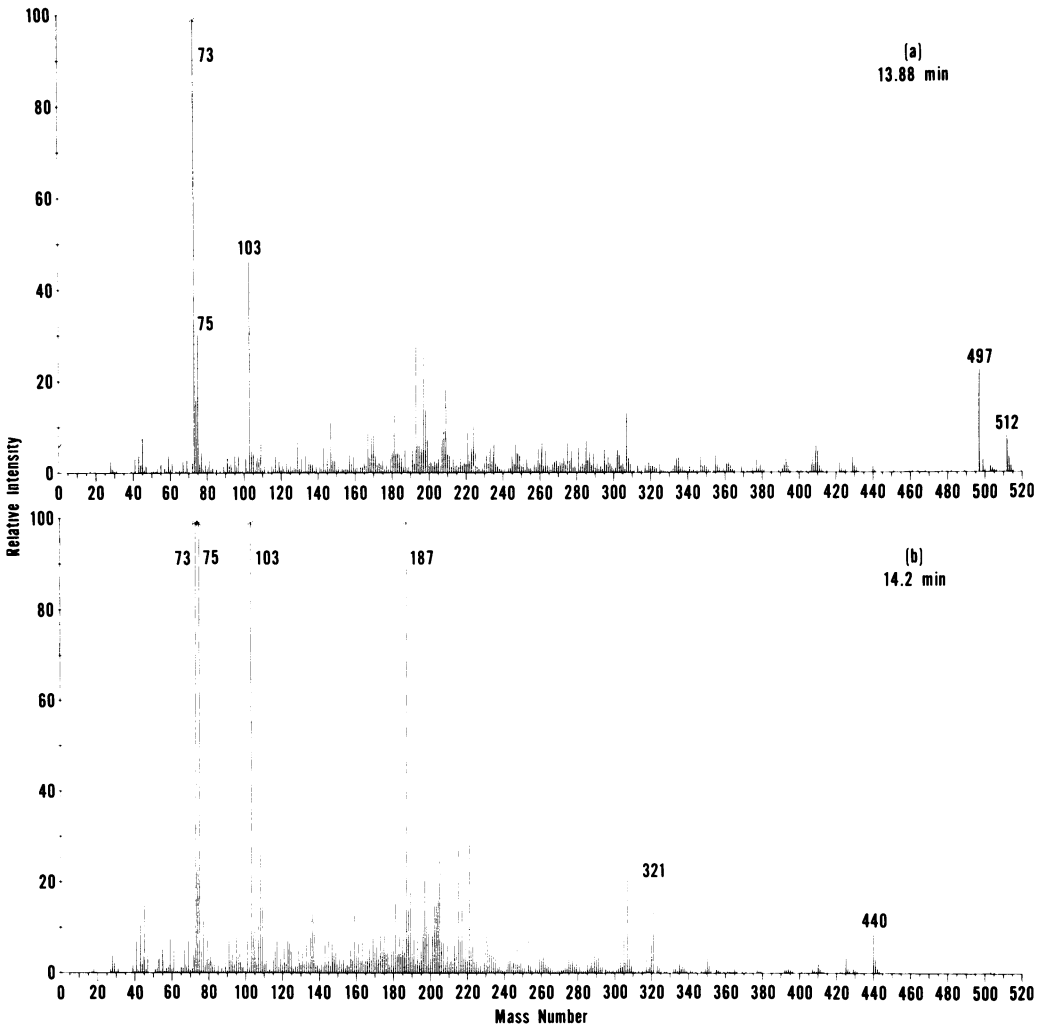

FIG. 3. *Plots of mass spectra of TMS ether of vomitoxin of GLC peaks at 13.88 min and 14.2 min.*

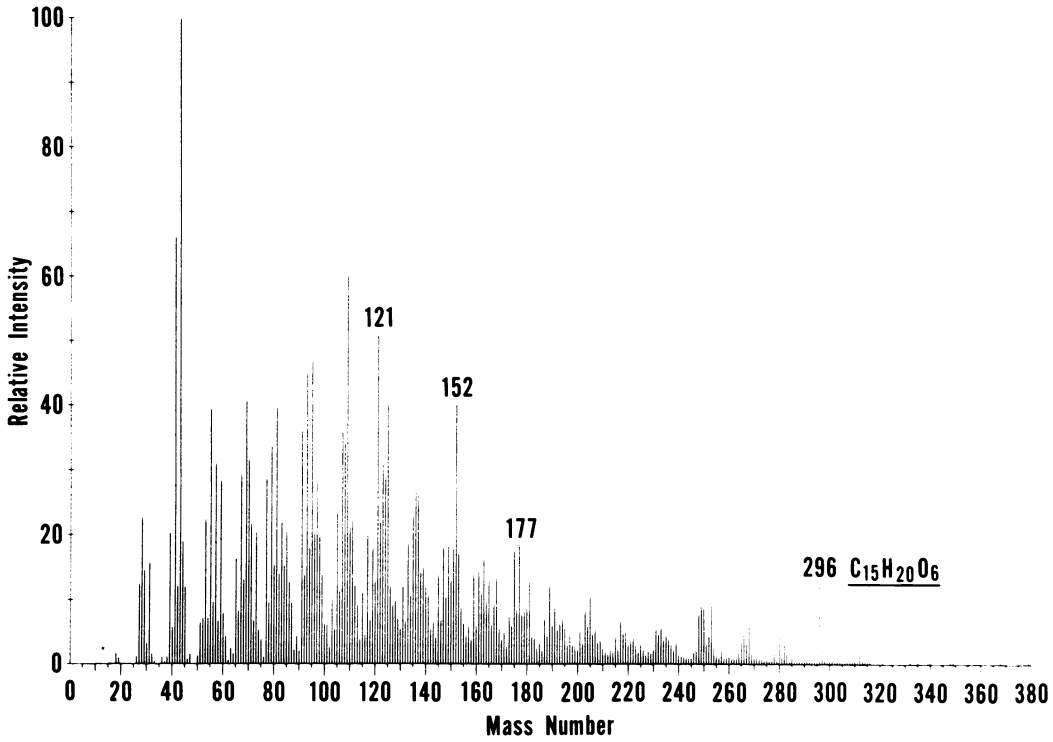

FIG. 4. *Mass spectrum of a compound closely related to vomitoxin.*

Here we show that *F. graminearum* isolated from naturally contaminated corn produces a toxin, vomitoxin, that is responsible for the rejection phenomenon in pigs. Previously, we had demonstrated that the same toxin at lower doses caused vomiting in swine which consumed corn that had been spiked. Hence, the levels of toxin present may be the factor which determines whether or not pigs will consume sufficient corn to elicit the vomiting response. We also present evidence for the concurrent presence of a new trichothecene and a partially acetylated trichothecene. Although these compounds are in low concentrations, they may contribute to both rejection and vomition. However, vomitoxin appears to dominate in both phenomena. Additional research will be required to determine the structures of the new trichothecenes and under what circumstances, if any, would they accumulate in higher concentrations.

LITERATURE CITED

1. **Blight, M. M., and J. F. Grove.** 1974. New metabolic products of *Fusarium culmorum*: toxic trichothec-9-en-8-ones and 2-acetylquinazolin-4(3H)-one. J. Chem. Soc., p. 1691-1693.
2. **Curtin, T. M., and J. Tuite.** 1966. Emesis and refusal of feed in swine associated with *Giberella zeae*-infected corn. Life Sci. 5:1937-1944.
3. **Ellision, R. A., and F. N. Kotsonis.** 1973. T-2 toxin as an emetic factor in moldy corn. Appl. Microbiol. 26:540-543.
4. **Kadis, S., A. Ciegler, and S. J. Ajl (ed.).** 1971. Microbial toxins, vol. 7, section B, p. 107-316. Academic Press Inc., New York.
5. **Mains, E. B., C. M. Vestal, and P. B. Curtis.** 1930. Scab of small grains and feeding trouble in Indiana in 1928. Ind. Sci. Proc. 39:101-110.
6. **Morooka, N., N. Uratsuji, T. Yoshizaiwa, and H. Yamamoto.** 1972. Studies on the toxic substances in barley infected with *Fusarium* spp. Jpn. J. Food Hyg. 13:368-375.
7. **Ueno, Y., K. Ishii, N. Sato, and K. Ohtsubo.** 1974. Toxicological approaches to the metabolites of Fusaria. VI. Vomiting factor from moldy corn infected with *Fusarium* spp. Jpn. J. Exp. Med. 44:123-127.
8. **Uneo, Y., I. Ueno, Y. Iitoi, H. Tsunoda, M. Enomoto, and K. Ohtsuba.** 1971. Toxicological approaches to the metabolites of Fusaria. III. Acute toxicity of Fusarenon-X. Jpn. J. Exp. Med. 41:521-539.
9. **Vesonder, R. F., A. Ciegler, and A. H. Jensen.** 1973. Isolation of the emetic principle from *Fusarium*-infected corn. Appl. Microbiol. 25:1008-1010.
10. **Yoshizawa, T., and N. Morooka.** 1973. Deoxynivalenol and its monoacetate: new mycotoxins from *Fusarium roseum* and moldy barley. Agric. Biol. Chem. 37:2933-2934.