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T-2 Toxin as an Emetic Factor in Moldy Corn

ROBERT A. ELLISON AND FRANK N. KOTSONIS
School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53711

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Extracts of *Fusarium poae* (NRRL 3287) grown either on sterile corn at 8 C or in Richards solution at room temperature were shown to have emetic activity in pigeons at nonlethal concentrations under conditions of oral and intravenous administration. The causative agent was found to be T-2 toxin (3-hydroxy-4,15-diacetoxy-8-[3-methylbutyryloxy]-12,13-epoxy-Δ^9-trichothecene). Oral and intravenous mean toxic dose values for this compound were found to be 0.72 and 0.15 mg/kg, respectively, as compared with an oral mean lethal dose of 2.75 mg/kg. The fact that T-2 toxin causes emesis at nonlethal concentrations may explain, at least in part, the observance of vomiting as a symptom resulting from ingestion of cereal grains infected with toxic *Fusarium* species containing T-2 or a similar toxin.

Among the various toxicoses associated with contamination of cereals by *Fusarium* species (8), there have been intermittent reports of vomiting as a distinct symptom occurring among both animals and humans. Outbreaks of emesis have been reported involving moldy rye (4), wheat (14), rice (11), barley (3), and corn (2). The chemical nature of the causative factor or factors remained unknown. Early attempts to characterize this emetic factor were carried out by Prentice and Dickson, who demonstrated that a water-soluble extract from field-grown corn infected with *F. graminearum* (synonymous with *F. roseum*) induced emesis in pigs, dogs, and pigeons. Subsequently they showed that several strains including those of *F. graminearum*, *F. culmorum* (synonymous with *F. roseum*), *F. moniliforme*, *F. nivale* and *F. poae* (synonymous with *F. tricinctum*, according to the system of Snyder and Hansen [10]) produced an emetic substance when grown on Richards solution for 12 to 40 days (9). In the case of *F. moniliforme* 111, final purification by preparative thin-layer chromatography (TLC) yielded two substances with emetic activity. The one of higher R_f was found to be active at doses of less than 100 μg when given intravenously to pigeons and was not lethal at this concentration. The material of lower R_f also produced emesis but killed the pigeon in less than 12 h. These compounds were not characterized except in a preliminary way.

In pursuing this problem further, we have reexamined the strains studied by Prentice and Dickson (9) and now present evidence that T-2 toxin (3-hydroxy-4,15-diacetoxy-8-[3-methylbutyryloxy]-12,13-epoxy-Δ^9-trichothecene), isolated from *F. poae* Northern Regional Research Laboratory (NRRL) strain 3287 (Fig. 1) is an emetic at sublethal concentrations and thus may be responsible for or contribute to emesis in the field.

MATERIALS AND METHODS

In this paper, TD refers to nonlethal toxic dose (i.e., emesis), as opposed to LD (lethal dose), which retains its conventional meaning.

Fungi. The following strains were obtained from the U.S. Department of Agriculture Northern Regional Research Laboratory, Peoria, Ill. and are the corresponding strains studied by Prentice and Dickson: *F. culmorum* (NRRL 3288), *F. moniliforme* (NRRL 3197), *F. nivale* (NRRL 3289), *F. poae* (NRRL 3287), and *F. roseum* (NRRL A-15,666).

The above strains were grown on moist sterile corn at 8 and 25 C and in Richards solution at 25 C in the Department of Plant Pathology, University of Wisconsin, by using previously described conditions (7, 9).

Pigeons. Healthy adult pigeons (300 to 400 g) were supplied and maintained by the animal care unit, University of Wisconsin. For dosing purposes the pigeons were weighed to the nearest gram.

T-2 toxin. Pure toxin for mean TD (TD_{50}) and mean LD (LD_{50}) studies was obtained in part by preparative isolation from cultures of *F. poae* (NRRL 3287) as described below and in part from a sample generously supplied by H. R. Burmeister of the NRRL, Peoria, Ill.

Oral toxicity. Samples were prepared by dissolving the appropriate amount of material along with commercial corn oil (0.5 ml) in chloroform followed by prolonged evaporation in vacuo at 50 C. Control samples of corn oil were similarly prepared. Crude oily

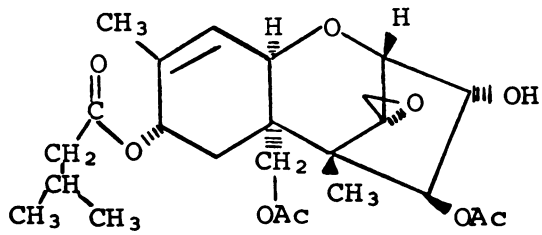

FIG. 1. *Structure of 3-hydroxy-4,15-diacetoxy-8-[3-methylbutyryloxy]-12, 13-epoxy-Δ⁹-trichothecene (T-2 toxin).*

ethylacetate extracts (0.5 ml) were fed directly. The pigeons could be accurately force-fed by delivering the sample from a plastic 1-ml tuberculin syringe (without needle). In order to achieve relatively uniform stomach content, food (but not water) was withheld for 20 h before testing. After receiving the samples, birds were observed for a period of 1.5 h for emesis and at least 1 week for death. A positive emetic response was defined as a distinct vomiting episode (retching and expulsion of stomach contents) occurring within 35 min, followed by several further episodes over a period of 1 h. For this study, a lethal dose was defined as that which caused death within 40 h.

Intravenous toxicity. Samples were prepared by dissolving appropriate amounts of T-2 toxin in 95% ethanol and adding 0.9% saline to give a 27% ethanol-saline solution (0.25 to 0.35 ml). These were conveniently injected via the wing vein of the pigeon by using 1-ml tuberculin syringes fitted with 0.5-in (approximately 1.25 cm) no. 27 needles. The birds were observed 1 h for emesis and at least 1 week for death as above.

Isolation of metabolites from F. poae (NRRL 3287). **A. Liquid culture.** The contents of 14 culture flasks each containing 500 ml of a liquid culture of *F. poae* (NRRL 3287) were filtered through Whatman no. 2 paper, and the filtrate was adjusted to pH 9.0 with saturated sodium carbonate followed by five extractions with chloroform. The chloroform layers were combined, dried over sodium sulfate, and evaporated in vacuo (40 C) to give an oily residue (861 mg) which was biologically active. Additional active material (86 mg) was obtained by extraction of the mycelial residue with water for 8 h followed by chloroform extraction of the filtrate as before.

Thin-layer chromatography (TLC) of the crude material on silica gel (F-254) with ethylacetate as solvent showed a number of spots which were visualized with ultraviolet light after spraying with 30% sulfuric acid in ethanol and charring at 120 C.

Column chromatography of the material recovered from the filtrate (662 mg) on silica gel (Merck E.M., 120 g, 2.7 by 46 cm) was effected with ethylacetate-Skelly B (85:15) as solvent with 1.0-ml (tubes 1 to 232) and 1.7-ml (tubes 233 to 300) fractions being collected. Metabolite fractionation was monitored by the above TLC system and eluate collected into six fractions which were subjected to bioassay. The fraction collected in tubes 200 to 287 was the only active fraction. Preparative TLC of this material (145 mg) on silica gel (Merck E.M. PF-254) with ethylacetate gave three bands. The upper and lower bands corresponded to inactive material contained in neighboring fractions from the column. The middle, biologically active band was removed and the material was recovered by extraction with 15% methanol in chloroform. The resulting oil (59 mg) proved identical on mixed TLC, proton magnetic resonance, and mass spectrum with an authentic sample of T-2 toxin.

B. Corn culture. The moldy corn, supplied as a powder (800 g), was extracted with ethylacetate (4 liters) for 46 h at 25 C with continuous stirring. The mixture was filtered, and the residue was washed with additional ethylacetate (1 liter). Evaporation of the solvent yielded a biologically active yellow oil which was partitioned between water (600 ml) and Skelly B (250 ml). After five extractions with Skelly B, the organic layers were combined and evaporated to give a yellow oil (31 g). The water layer was adjusted to pH 9.0 with saturated sodium carbonate and extracted several times with chloroform. The combined extracts were dried over sodium sulfate and evaporated in vacuo to give an oil (116 mg) which was biologically active. The oil from several work-ups was combined for further fractionation.

This oily residue (263 mg) was chromatographed on silica gel (5.5 g, 1.0 by 20.5 cm) with ethylacetate and collected in 0.4-ml fractions. Biological activity was found in a fraction consisting of tubes 27 to 98. This was re-chromatographed on silica gel (5.5 g, 1.0 by 2.05 cm) with Skelly B-ethylacetate (85:15) as eluant in 0.5-ml fractions. Tubes 21 to 23 contained a single compound (4 mg) which was biologically active and which was chromatographically identical with T-2 toxin.

Emetic activity was also found in the oil recovered from the Skelly B phase. A portion of this oil (200 mg) was chromatographed on silica gel (5.5 g, 1.0 by 20.5 cm) under linear gradient conditions by using Skelly B (100 g) and ethylacetate-Skelly B (3:2) (100 g) as eluant phases (1.1-ml fractions). Tubes 27 to 50 contained largely corn oil. Biological activity was detected in a combined fraction consisting of tubes 90 to 119. This material (12 mg), although not completely pure, consisted largely of a compound which was identical on mixed TLC with T-2 toxin.

RESULTS

Pigeon bioassay. The pigeon proved to be a convenient and reliable species for assaying emetic activity and has been used previously in this capacity (1, 5). In the case of active fractions, vomiting typically occurred within 35 min and in several cases as early as 10 min. The vomiting response as described earlier was distinct and easy to detect. It has been reported that pigeons injected with emetics typically develop a conditioned response such that subsequent injection of saline controls initiate vomiting (5). This was also observed in this study, and each pigeon was consequently subjected to only one injection. This phenomenon was much less pronounced with oral feeding, and whereas birds were used only one time for the toxicity

studies, in general they could be re-used after a waiting period of 5 days. In the case of oral feeding it was important to insure complete removal of solvent from oil samples, since in one case residual chloroform induced narcosis of the bird. The time periods for emesis and death were chosen to conform with general experience during the bioassay. Thus, in the case of emesis, 36 of 39 birds which vomited did so within 30 min regardless of route of administration. In the case of higher doses, 33 of 34 birds which died did so within 40 h.

Bioactivity of cultures. In initial experiments, ethylacetate extracts of each *Fusarium* strain grown on moist, sterile corn both at 8 and 25 C were fed orally to pigeons. No activity was evident in the cultures grown at 25 C, and of those grown at 8 C, only *F. poae* (NRRL 3287) proved active. In contrast to the results of Prentice and Dickson (9), *F. poae* was the only strain to exhibit emetic activity when grown in Richards solution at 25 C. Consequently, cultures of *F. poae* were grown in both media in large scale for further investigation.

Examination of F. poae metabolites. When fractions from chromatography of the chloroform extracts from either the corn or the liquid culture were subjected to bioassay, only one component consistently maintained activity. In each case this compound, when purified, was shown to be identical to an authentic sample of T-2 toxin upon comparison of the proton magnetic resonance spectrum, mass spectrum, and behavior on TLC. Further, after determining the emetic potency of T-2 toxin it was possible to attribute all the emetic activity of the crude extract to the T-2 toxin.

In the case of the 8 C corn culture, emetic activity was consistently found in the Skelly B fraction as well as in the chloroform extract and was again found to be due to T-2 toxin. The persistent distribution of the toxin in both aqueous and organic phases as well as retention by the mycelium makes quantitation difficult but it is estimated that in Richards solution the toxin is produced to the extent of 14% (by weight) of total metabolites. TLC comparison of the chloroform extracts from both media indicates a very similar pattern of metabolite production. Cursory examination of the remaining nonemetic fractions indicated the presence of at least eight other significant compounds, some of which appear to be trichothecenes. Due to their lack of activity, however, they were not investigated further.

Bioactivity of T-2 toxin. With the knowledge that T-2 toxin had emetic activity, it became important to determine whether this activity could be expressed without associated lethality. The results of force-feeding pigeons with increasing doses of pure toxin dissolved in commercial corn oil are shown in Fig. 2. The oral TD_{50} and LD_{50} were found to be 0.72 and 2.75 mg/kg, respectively. The results also show that an emetic dose range exists which is nonlethal. Whereas expulsion of unadsorbed toxin during vomiting might have been expected to reduce the precision of the results, in fact, reasonable, curves were obtained, permitting meaningful comparisons to be made. The corresponding intravenous TD_{50} was found to be 0.15 mg/kg.

Since certain compounds which are antiemetics in humans have been shown to be emetic in pigeons, it was necessary to determine the activity of T-2 toxin in higher species (1). Injection of an intravenous dose of approximately 2.5 mg of toxin into a 10-kg dog resulted in the initiation of vomiting after 35 min in a pattern similar to the pigeon, suggesting but not proving that the response is general.

DISCUSSION

The fact that four of the five original strains of *Fusarium* were found to be inactive as emetics after storage makes comparison with the earlier work difficult. Furthermore, only one of two reported emetic substances was detected. Hence, it is likely that the present strains are no longer identical with the original ones. However, although such comparisons are admittedly tenuous, when the same solvent and visualization systems of Prentice and Dickson were used (9), T-2 toxin had an R_f which coincided closely with their lethal emetic and suggests that they may in fact be the same compound. It is pertinent that none of the extracts of inactive corn cultures showed the presence of T-2 toxin on TLC and also that extracts of the 25 C corn

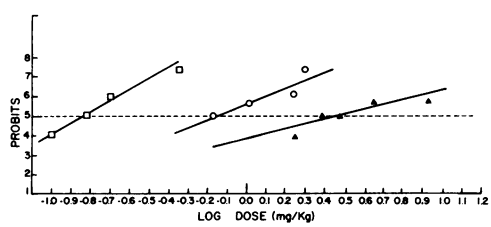

FIG. 2. *Dose-response curves for T-2 toxin in pigeons (6). Symbols: □, emesis resulting from intravenous administration. A total of 26 birds was used. TD_{50} = 0.15 mg/kg; ○, emesis resulting from oral administration. A total of 26 birds was used. TD_{50} = 0.72 mg/kg; △, death resulting from oral administration. A total of 47 birds was used. LD_{50} = 2.75 mg/kg.*

cultures of *F. poae* were inactive and contained no T-2 toxin on TLC. In the case of the liquid cultures, inactive extracts of 4-week cultures of *F. culmorum* and *F. roseum* showed some T-2 toxin on TLC, but total metabolite production was negligible. This corroborates the evidence that T-2 toxin is the only significant emetic compound produced by these strains under our conditions.

From the intravenous data, T-2 toxin appears to be as potent an emetic in the pigeon as digitoxin (TD_{99} = 0.2 mg/kg) but less potent than alkavervir, a mixture of *Veratrum* alkaloids (TD_{99} = 0.03 mg/kg) (1).

It can be seen that the TD_{90} for T-2 toxin corresponds approximately to the LD_{25} which clearly indicates that a dose range exists where emesis can be expected to be the predominant or exclusive biological activity. The observation of emesis in some cases as early as 10 to 15 min after oral administration, particularly at higher doses, suggests but does not prove that T-2 toxin and not a metabolite is responsible for the emetic activity. This also implies a quite rapid adsorption from the gastrointestinal tract which is consistent with the substantial lipid solubility of the toxin.

The recent report (12) that fusarenone-X (3,7,15-trihydroxy-4-acetoxy-8-oxo-12,13-epoxy-Δ^9-trichothecene), isolated from a strain of *F. nivale*, is both emetic (subcutaneous minimal effective dose = 0.4 to 0.5 mg/kg) and lethal (subcutaneous LD_{50} = 2 mg/kg) in 10-day-old Peking ducklings substantiates the fact that certain of the trichothecenes can act as emetics at nonlethal concentrations. Thus, whereas the apparently nonlethal and as yet unidentified emetic of Prentice and Dickson remains elusive, it appears likely that the intermittant but widespread reports of emesis associated with *Fusarium*-infected cereal grains may be due at least in part to certain trichothecenes, since these are known to widely occur in the genus *Fusarium* (13).

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LITERATURE CITED

1. Burkman, A. M., A. Tye, and J. W. Nelson. 1957. Antiemetics in the pigeon. J. Amer. Pharm. Ass., Sci. Ed. **46**:140.
2. Curtin, T. M., and J. Tuite. 1966. Emesis and refusal of feed in swine associated with *Gibberella zeae*-infected corn. Life Sci. **5**:1937.
3. Dickson, A. D., K. P. Link, B. H. Roche, and J. G. Dickson. 1930. Report on the emetic substances in *Gibberella*-infected barley. Phytopathology **20**:132.
4. Dounin, M. 1930. The fusariosis of cereal crops in European Russia in 1923. Phytopathology **16**:305.
5. Earl, A., R. L. Winters, and C. M. Schneider. 1955. Assay of reserpine based on emesis in pigeons. J. Pharmacol. Exp. Ther. **115**:55.
6. Goldstein, A., L. Aronow, and S. M. Kalman. 1968. Principles of drug action, p. 343. Harper and Row, New York.
7. Ikediobi, C. O., I. C. Hsu, J. R. Bamburg, and F. M. Strong. 1971. Gas-liquid chromatography of mycotoxins of the trichothecene group. Anal. Biochem. **43**:327.
8. Kadis, S., A. Ciegler, and S. J. Ajl. (ed.) 1971. Microbial toxins, vol. 7, section B, p. 107–316. Academic Press Inc., New York.
9. Prentice, N., and A. D. Dickson. 1968. Emetic material associated with *Fusarium* species in cereal grains and artificial media. Biotech. Bioeng. **10**:413.
10. Snyder, W. C., and H. N. Hansen. 1945. The species concept in *Fusarium* with reference to discolor and other sections. Amer. J. Bot. **32**:657.
11. Tsunoda, H., O. Tsuruta, S. Matsunami, and S. Ishii. 1957. Researches on the microorganisms on the stored cereals and grains. 14. Studies on rice parasitic moulds, *Gibberella* and *Fusarium*. Proc. Food Res. Inst. (Japan) **12**:27.
12. Ueno, Y., N. Sato, K. Ishii, K. Sakai, H. Tsunoda, and M. Enomoto. 1973. Biological and chemical detection of tricothecene mycotoxins of *Fusarium* species. Appl. Microbiol. **25**:699–704.
13. Ueno, Y., I. Ueno, Y. Iitoi, H. Tsunoda, M. Enomoto, and K. Ohtsubo. 1971. Toxicological approaches to the metabolites of *Fusaria*. 3. Acute toxicity of Fusarenon-X. Japan J. Exp. Med. **41**:521.
14. Urakura, U. 1933. On wheat damage. 8. Byochugai Zashi **20**:390.