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Identification of the Toxic Principle in a Sample of Poaefusarin

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A sample of poaefusarin (a mycotoxin suspected of being one of the toxins involved in alimentary toxic aleukia in the U.S.S.R.) was received from a Soviet scientist for evaluation and comparison with other mycotoxins. Although poaefusarin is presumed to be a steroid, analyses by thin-layer chromatography, gas-liquid chromatography, and infrared, ultraviolet, and mass spectrometry could not confirm the presence of a steroid structure. However, 2.5% of the sample was made up of the trichothecene T-2 toxin, an amount sufficient to explain the toxicity found in the rat and rabbit skin toxicity tests. In addition, neosolaniol (0.14%), T-2 tetraol (0.6%), and zearalenone (F-2) (0.43%) were present in the sample. Since the toxicity was found to be associated only with T-2 toxin, no attempt was made to determine the nature of the other nontoxic components of the sample.

According to Joffe (7, 8), alimentary toxic aleukia (ATA), or septic angina, is a disease recorded in the U.S.S.R. since the 19th century and was perhaps most serious among the population of the Orenburg district of the U.S.S.R. during World War II. Its signs in humans include fever; a hemorrhagic rash; bleeding from the nose, throat, and gums; necrotic angina; extreme leukopenia; agranulocytosis; sepsis; and exhaustion of the bone marrow. The disease is associated with consumption of grain (wheat, rye, oats, buckwheat, millet) overwintered in the field and colonized by many fungi, most important of which are *Fusarium poae* and *F. sporotrichiodes*.

This disease was particularly rampant during and after World War II when manpower shortages caused unusually large amounts of grain to be left to overwinter in the fields. Joffe (8) reports that more than 10% of the population in the Orenburg district was affected during this time.

The toxins identified by the Russian scientists as most likely responsible for the disease were called poaefusarin from *F. poae* and sporofusarin from *F. sporotrichiodes*, the structure of which can be found in Fig. 1 (8). The steroid glycoside called poaefusarin and isolated by Olifson (10, 11) contained xylose as the sugar. The aglycone moiety was designated poaefusariogenin ($C_{24}H_{28}O_5$). Sporofusarin, on the other hand, contains a ribose and two glucose sugars and, upon hydrolysis, yields sporofusariogenin, aglycone, with an empirical formula of $C_{24}H_{30}O_4$. Both toxins are C_{24} steroids, having as a side chain a doubly unsaturated six-member lactone ring at C_{17} and a 14-β-hydroxyl group. These compounds are almost identical to that reported by Fialkov and Serebriany (5) and are called stachybotryotoxin, which has been reported to be a fungal metabolite toxic to horses. Furthermore, poaefusarin is structurally similar to bufadienolides, poisons isolated from the Chinese toad (*Bufo asiaticus*) and from the Chinese drug called Ch'an Su in China and Senso in Japan.

In the United States, *F. sporotrichiodes* is usually known by the synonym *F. tricinctum*. Smalley et al. (12) found that *F. tricinctum* was one of the fungi most frequently isolated from corn associated with toxicity in domestic animals in Wisconsin. In contradistinction to the results of the Soviet scientists, Bamburg et al. (2, 3) found the toxin of *F. tricinctum* to be diacetoxyscirpenol and T-2 toxin (Fig. 1). These toxins are sesquiterpenoids and derivatives of the trichothecene group of toxins and are different from those described by Olifson (10, 11). Bamburg and Strong (3) reviewed the literature on the trichothecene natural products from fungi and suggest that, more likely, ATA may be largely due to trichothecene toxins rather than to the steroidal toxins reported by Olifson. More recently, Hsu et al. (6) found T-2 toxin in

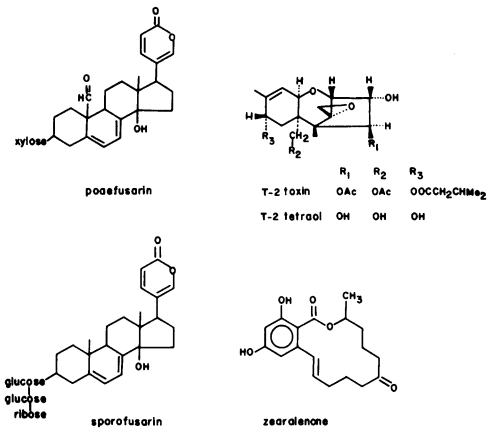

FIG. 1. *Structures of various mycotoxins reported to be produced by species of Fusarium.*

moldy corn associated with a lethal toxicosis in dairy cattle. This is the first report of the T-2 toxin being found in a field case of suspected mycotoxicosis.

We established collaboration with Soviet scientists in an effort to gain more information on the chemistry of the toxic principles involved. Through the kindness of V. I. Bilai and I. P. Misiurenko of the Institute of Microbiology and Virology, Kiev, Ukraine, U.S.S.R., we have received a small sample of poaefusarin described as unpurified but which, upon chromatographic analysis, was to yield a toxin identical to poaefusarin as described by L. E. Olifson (11). The toxin was produced by *F. sporotrichiella* var. *poae* and described as being "dermatoxic." The toxin, as prepared by the Russian group, was produced by growing *F. sporotrichiella* var. *poae* in submerged culture in a synthetic medium as described by Olifson (10) and then extracting a residue, left over after extraction of the fungus mycelium with diethyl ether, with ethyl alcohol. The growing controversy regarding the existence of poaefusarin prompted us to examine the sample with the intention of identifying poaefusarin, a steroid glycoside, and other active principles of the mixture. Only the major components of the mixture were analyzed for toxicity due to limitation of sample availability.

MATERIALS AND METHODS

GLC. A Varian 1500 series custom model chromatograph equipped with a hydrogen flame detector was used for gas-liquid chromatography (GLC) analyses of the poaefusarin sample. The flow rate of nitrogen, the carrier gas, was 20 ml/min through a stainless-steel column (3 feet by ⅛ inch; 91.44 by 0.318 cm) packed with 3% OV-1 on 100/120 mesh gas chrom Q. The temperature of injector port and the detector cell was 275 C.

A 320-μg portion of the poaefusarin sample was reacted with 20 μliters of Tri-Sil-BT (Pierce Chemical Co., Rockford, Ill.) in a Pierce Reactivial. The mixture was kept at 70 C for 1 h. One microliter of this solution was injected into the column programmed from 150 to 275 C at 6/min.

The standards used were T-2 toxin [4-β,15-diacetoxy-8-α-(3 - methylbutyryloxy) - 12,13-epoxy-Δ^9-trichothecen-3-α-1], zearalenone [6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid lactone], xylose, *d*-glucose, and arabinose, and they were chromatographed under the same conditions as poaefusarin.

Separation of various components present in the poaefusarin sample was carried out by thick-layer chromatography. Poaefusarin (50 mg) was dissolved in 0.1 ml of methanol. The solution was applied to a preparative silica gel P254 plate (20 by 20 cm) and developed in chloroform-methanol (90:10 vol/vol). One section of the thick-layer chromatography (TLC) plate was sprayed with concentrated sulfuric acid and charred over a hot plate to locate various components. Five major bands were located and eluted with methanol. The methanol solution of each band was concentrated under a nitrogen stream to approximately 100 μliters.

GC-MS. Low resolution mass spectra were recorded on a LKB-9000 combination gas chromatograph-mass spectrometer. A sample of 1 μliter of poaefusarin Tri-Sil-BT reaction mixture was injected into the gas chromatography-mass spectrometry (GC-MS) system for analysis, and mass spectra were recorded in 4 to 6 s on the apex of the GC peak. Background spectra were obtained before the appearance of the first peak and after the last (seventh) peak.

A 10-μliter sample of each band, separated by TLC and subsequently eluted, was transferred into separate vials. Methanol was evaporated by a stream of nitrogen and the residue was reacted with 10 μliters of Tri-Sil-BT. One microliter of this reaction mixture of each band was analyzed by GC-MS as described above.

Low resolution mass spectra of the five bands (underivatized) were run on a LKB-9000 by using the direct inlet system. A 1-μliter sample of a methanol solution of each band was placed into a small glass ampoule and the methanol was removed under vacuum. The ampoule was then inserted into the probe and the spectrum was recorded at 70 eV of ionization potential. The inlet temperature was high enough to give an observable spectrum.

An infrared spectrum was recorded on a Perkin-Elmer model 257 grating infrared spectrophotometer. A 0.7-mg portion of poaefusarin sample was ground with 50 mg of spectro-grade KBr and pressed into a disk, and the spectrum was recorded. In addition, a methanol solution of band 4 was dried thoroughly and a few drops of alcohol-free chloroform were added. An infrared spectrum was taken by using a 0.025-mm sodium chloride cell.

An ultraviolet spectrum of poaefusarin was recorded in both water and methanol solution at

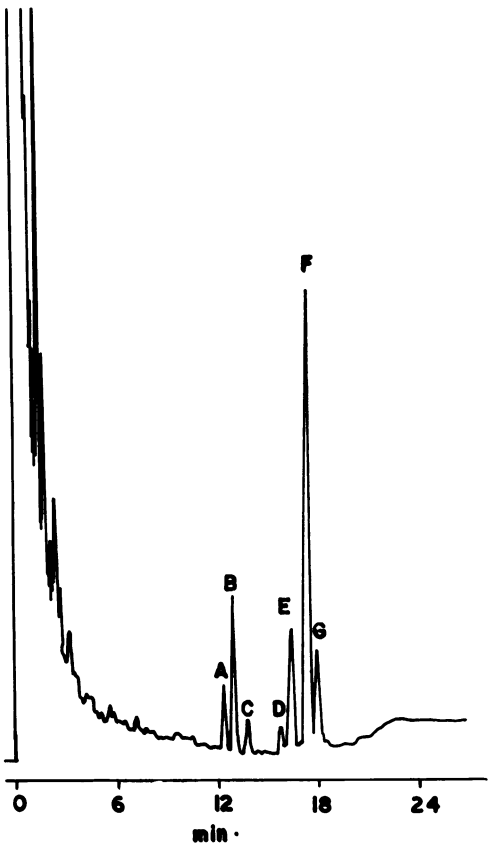

FIG. 2. *Analysis by GLC of TMS ether derivatives of the constituents of the poaefusarin sample.*

concentrations of 10 μg/ml, 50 μg/ml, and 350 μg/ml.

In studies involving hydrolysis of poaefusarin, 5 mg was placed in a vial containing 0.5 ml of 2 N HCl solution. The vial was kept at 70 C for 1 week. A 100-μliter sample of the hydrolysate was transferred to a vial and dried under a nitrogen stream. It was then reacted with 50 μliters of Tri-Sil-BT in 50 μliters of pyridine. The reaction mixture was analyzed by GC-MS. The remainder of the hydrolysate was diluted with 1 ml of water and neutralized with excess of sodium bicarbonate and then extracted twice with 3 ml of *n*-butanol. The butanol extract was concentrated and analyzed by TLC and GC-MS.

For determination of toxicity, a 2- by 3-cm area on the back of a rat (Holtzman-white, female, 21-day-old weanling) was shaved, and 1 mg of poaefusarin in 0.2 ml of 95% ethanol was applied daily for 10 days. The rat was examined every day. The same skin test was carried out on New Zealand white rabbits at the same level as the rat but for 5 days.

RESULTS

Analysis by GLC of the trimethylsilyl (TMS) ether derivatives of the poaefusarin sample revealed at least seven major components (Fig. 2). Peak enhancement as well as mass spectrometry were used to identify the components of the mixture. The components separated by GLC (Fig. 2) are compared with the component separated by TLC (Fig. 3) and are shown in Table 1. Component G was identified as zearalenone (F-2) by GC-MS. Component F, the major component detected by GLC of the silylated poaefusarin sample, was T-2 toxin. Mass spectra of authentic T-2 toxin and band 4 were compared by direct probe as well as their TMS derivatives by GC-MS (Fig. 4). The fragmentation pattern of band 4 (Fig. 3) and component F (Fig. 2) were identical to those of their respective standards, T-2 toxin and TMS ether T-2.

An infrared spectrum of band 4 in chloroform was compared with that of authentic T-2 toxin and found to be identical. The major bands of

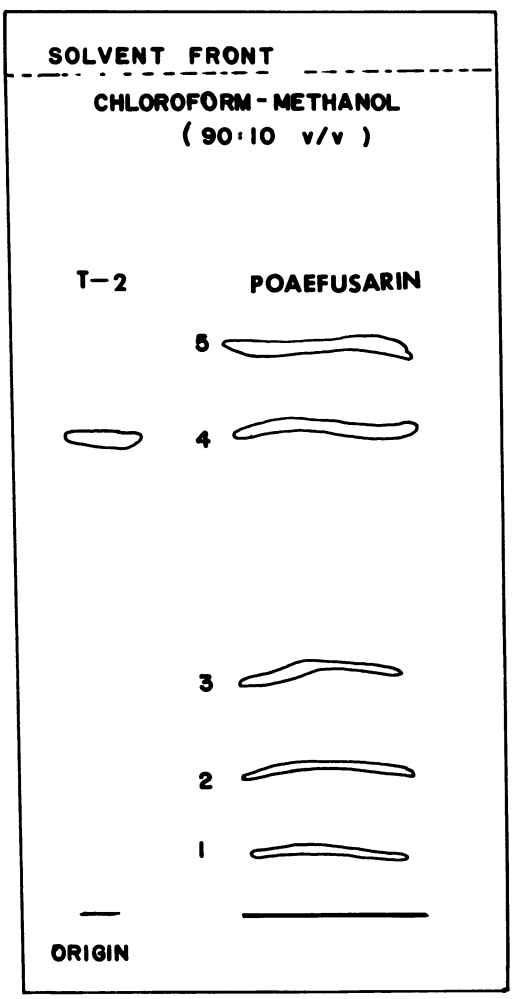

FIG. 3. *Separation by TLC of the constituents of the poaefusarin sample.*

TABLE 1. *Comparison and identification of the major components resolved by GLC (Fig. 2) and TLC (Fig. 3)*

GLC peak	TLC band	Major mass spectrum fragments[a]		
		Direct probe	TMS derivative	Identification
B	Band 3	298 (0.6), 203 (28), 121 (100), 109 (28)	586 (8.4), 496 (67), 483 (30), 204 (100)	T-2 tetraol
F	Band 4	364 (19), 278 (19), 121 (100)	436 (12.5), 350 (25), 122 (100)	T-2 toxin
G			462 (41), 447 (61.8), 429 (23), 333 (100)	F-2 or zearalenone
D		466 ( )	466 (12), 193 (100), 117 (64)	Neosolaniol

[a] The fragment ions are listed first followed by percent relative abundance in parentheses.

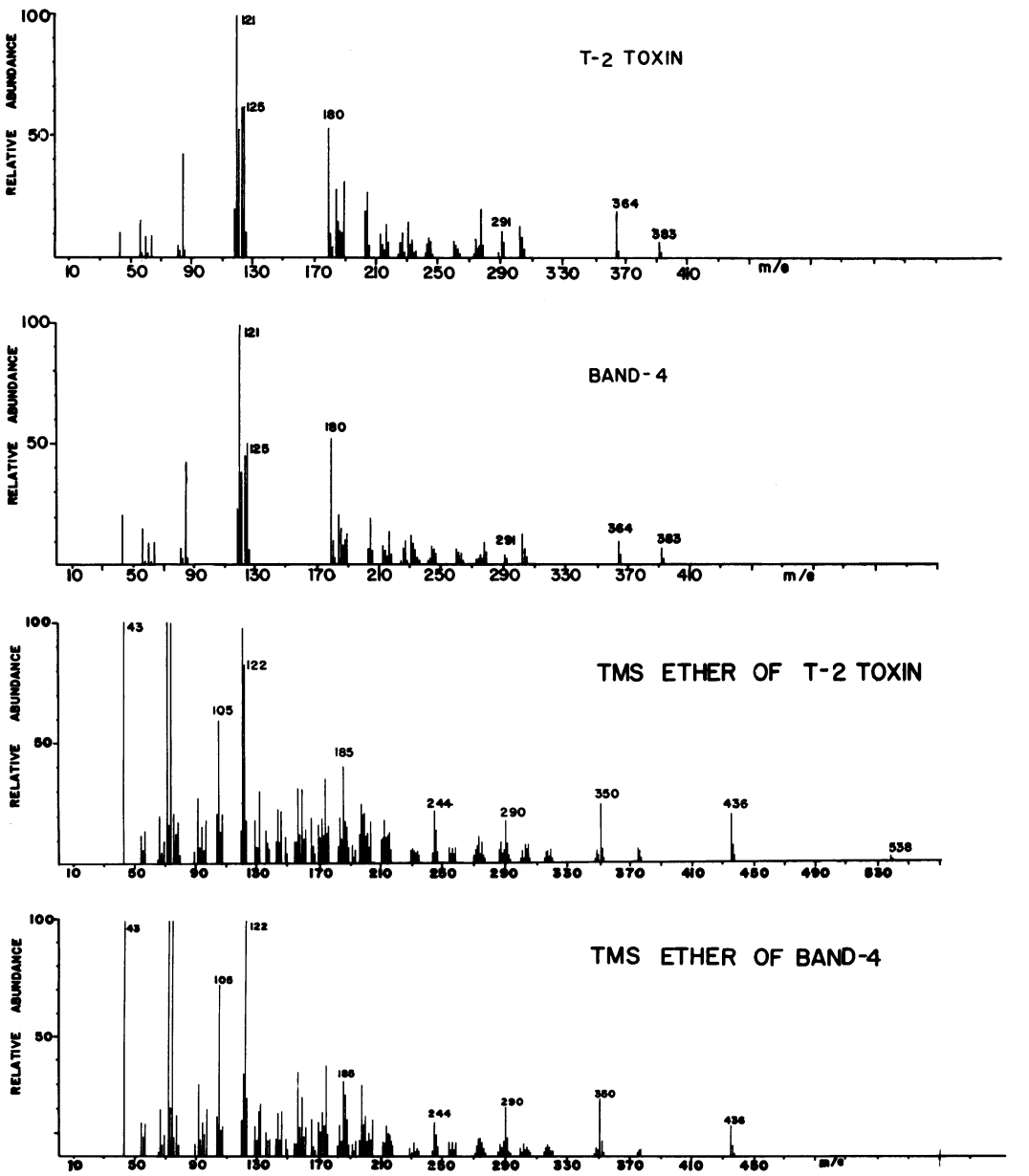

FIG. 4. *Plots of the mass spectra of T-2 toxin and the major constituent (band 4), as determined by GLC, of the poaefusarin sample.*

absorption were: 3,540 cm^{-1} (OH); 3,010 cm^{-1} (H—C=C); and 1,720 cm^{-1} (C=O).

Quantitative estimation of the amount of T-2 toxin present in the sample labeled poaefusarin was carried out by GLC by using peak height as a criterion. T-2 toxin in Tri-Sil-BT was used as the primary standard. Three separate determinations were made and are shown in Table 2. About 2.5% of the poaefusarin sample was T-2 toxin.

Band 3 of Fig. 3 and component B of Fig. 2 were identified by mass spectrometry as the T-2 tetraol (3-α,4-β,8-α,15-tetrahydroxy-12,13-epoxy-Δ^9-trichothecene). Similarly, component D (Fig. 2) was identified as neosolaniol (3α,8α-dihydroxy, 15-diacetoxy-12,13-epoxy-Δ^9 trichothecene. By comparison with the amount of T-2 present, the T-2 tetraol was estimated to be about 25% the concentration of T-2 or about 0.6% of the total, and neosolaniol was about 0.14% of the total.

The biological activity of the poaefusarin sample was determined by the skin test using both white rats and New Zealand white rabbits. In the rat skin test, no reaction was observed until the 4th day when edema was noticed which became progressively more severe, developing into a heavy scab and hemorrhaging by the 7th day. The rabbit was more sensitive than the rat, because edema was noticed on the 2nd day which turned into a scab and then hemorrhaged on the 4th day.

DISCUSSION

Olifson (10, 11) isolated and purified poaefusarin and according to Bilai (4) reported its absorption maximum of 300 nm. It is further characterized as having an aldehyde at C_{10} (Fig. 1). Its empirical formula as the glycoside is $C_{29}H_{36}O_9$ which, upon alkaline hydrolysis, yields the aglycone ($C_{24}H_{28}O_5$) and xylose. There is no reason to believe that this compound does not exist, because we have isolated a metabolite similar to poaefusarin in cultures of *Stachybotrys atra* grown on oats (unpublished data). Furthermore, Olifson et al. (11) and Misiurenko (9) recently published a method of isolation and production of this toxin further affirming its biological activity. However, none of the U.S. investigators who have worked with this group of *Fusarium* spp. has been able to find this toxin in their cultures although, in all cases, they have found one or more trichothecenes which are highly toxic.

Recently, Misiurenko (9) reported the purification of a toxic component from *F. sporotrichiella* var. *poae* by extracting the free and bound lipids with hot ethyl alcohol and subsequent purification by preparative TLC developed in petroleum ether-diethyl ether-glacial acetic acid (70:30:2 vol/vol/vol). He reported that the most toxic principle is very polar, remains near the origin, and has an R_f value of 0.06. Olifson et al. (11) report that toxic millet, rye, and wheat have only two fluorescent substances when viewed under ultraviolet light, and both are toxic. From these toxins, the fluorescent toxic lipid substance, fraction 2a, with an R_f value of 0.06, is the toxic steroid identical with sporofusarin. The second toxic steroid has an R_f value of 0.22 and is also a steroid but its identity is not known. As a developing system, petroleum ether-diethyl ether-glacial acetic acid (70:30:2 vol/vol/vol) was used. The same developing solvent was used by Misiurenko (9). We found that the T-2 toxin on a TLC plate of Silica Gel G migrates to approximately R_f 0.06 in petroleum ether-diethylether-glacial acetic acid (70:30:2), thus indicating that T-2 toxin might be the active principle involved. Furthermore, cultures of *F. sporotrichiodes*, originally obtained from the U.S.S.R., were grown on rice and extracted by the procedure of Olifson et al. (11). TLC on Silica Gel G, PF 254 of the extract, and authentic samples of T-2 toxin, F-2, diacetoxyscirpenol, neosolaniol, and butenolide using Olifson's solvent system revealed the presence of two fluorescent spots at R_f 0.06 and 0.32 from the extract. The spot at R_f 0.32 was identified as F-2. Concentrated sulfuric acid spray and heat treatment of the plate yielded two charred spots at R_f 0.06 and 0.00. The spot at R_f 0.06 was identified as T-2 toxin. Neosolaniol and butenolide did not move from the origin. The above solvent system was unable to resolve T-2 toxin from the fluorescent substance on Silica Gel G.

TABLE 2. *Quantitative determinations of the concentration of T-2 toxin present in the poaefusarin sample as determined by GLC*

Test	Poaefusarin sample (μg/μliter)	T-2 toxin found (μg/μliter)	% T-2
1	320/20	0.37	2.3
2	160/10	0.42	2.6
3	70/10	0.19	2.7

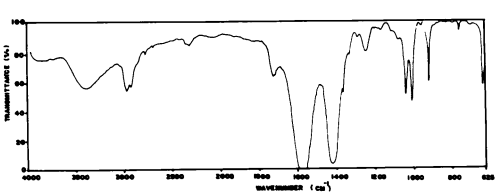

FIG. 5. *Infrared spectrum of the crude sample of poaefusarin.*

The infrared spectrum of the poaefusarin sample in Fig. 5 shows a very strong band at 1,600 cm^{-1} and indicates a carboxylate ion. A moderate absorption at 1,720 cm^{-1} is due to the acetyl carbonyl of T-2 toxin. The overall spectrum is indicative of a low-molecular-weight carboxylate salt. Measurements of pH indicated absence of potassium or sodium salt. We did not carry out elemental analyses due to the small amount of sample available. The poaefusarin sample shows only absorption at 219 nm. Lastly, no sugar or amino acid was detected in the hydrolysis studies.

Dermal toxicity determined by topical administration in the skin test showed that 5 and 10 mg of the poaefusarin sample is needed to cause necrosis and hemorrhaging in the treated skin of the rabbit and rat, respectively. Bamburg and Strong (3) report that about 100 μg of pure T-2 toxin is sufficient to incite necrosis and hemorrhaging in the rat and rabbit skin test. It appears then, from the foregoing, that the observed toxicity in our test was due to the T-2 toxin present in the sample (2.5%).

The presence of the T-2 tetraol was unexpected. We believe that the sample as obtained had not been treated with base. Mild alkaline hydrolysis of T-2 toxin does yield T-2 tetraol (3). No evidence is available to show the conversion of T-2 toxin into T-2 tetraol in vivo, yet the possibility of such transformation by microbes cannot be denied.

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