Mycotoxins as a Possible Cause of Fescue Toxicity

S. G. Yates, H. L. Tookey, J. J. Ellis, W. H. Tallent, and I. A. Wolff

Sporadic outbreaks of fescue foot occur in cattle grazing toxic pastures of tall fescue. The clinical signs of fescue foot can be produced by giving cattle extracts of the toxic hay. An alkaloidal fraction containing the major alkaloid festucine, prepared from such an extract, was not toxic to cattle. The sporadic and seasonal occurrence of fescue toxicity suggests a fungal involvement. One mold isolated from toxic fescue, *Fusarium tricinctum*, produces three toxins: 4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone, 8α-(3-methylbutyryloxy)-4β,15-diacetoxyscirp-9-en-3α-ol, and an unknown. Of 200 fungal isolates from toxic fescue or from nearby pastures (controls), almost all the toxin-producing molds belong to the genus *Fusarium*. The specific relationship, if any, of *Fusarium* toxins to fescue foot remains to be determined by tests in cattle.

Tall fescue, *Festuca arundinacea* Schreb., has long been associated with sporadic outbreaks of lameness in cattle (see Yates, 1962, for review). Goodman (1952) reported that such lameness, called fescue foot, had existed in Colorado for many years, but was confused with ergotism, foot rot, or similar diseases. In spite of its occasional toxicity, tall fescue is widely used in the United States as pasture for cattle. Tall fescue is a bunch grass, which may grow to a height of 3 to 4½ feet. Its heavy fibrous root system penetrates deep into the soil (Phillips Petroleum Co., 1960). These roots are tough and coarse, and produce a good sod (Cowan, 1956). Tall fescue is economically important because it is a good winter pasture, grows well on marginal land, and produces a high yield of dry matter per acre (Cowan, 1956). On the other hand, when outbreaks of fescue toxicity occur, the financial consequences are often severe to whole agricultural areas, as well as to individual farmers. The outbreaks usually come during cold weather and seem to recur every few years. They are especially prevalent in an area that extends from eastern Kansas through Missouri, southern Illinois, and southern Indiana, to Kentucky.

DESCRIPTION OF FESCUE FOOT

Clinical signs of fescue foot may appear as early as 3 days after animals are placed on fescue pasture (Pulsford, 1950). One of the earliest indications is a reluctance of cattle to get up in the morning. Later signs may include lameness, loss of weight, arched back (Figure 1), swelling in the hind legs in the region of the fetlock, cracks showing separation of the hooves from the feet (Figure 2), dry gangrene of the extremities, especially the rear legs, sloughing of the hooves (hence the name fescue foot, Figure 3), altered hoof and horn growth, and elevated body temperatures and respiration rates (Jacobson *et al.*, 1963).

Other clinical signs associated with fescue toxicity may accompany the lameness and subsequent sloughing of the hooves. Rossi (1959) reported tumefactions in the region of the ribs. Whereas some authors mention a loss of appetite (Merriman *et al.*, 1955), others do not (Price and Miller, 1959; Rossi, 1959). Although occasionally reported in cattle on fescue, abortion does not appear to be typical of the fescue foot syndrome (Pulsford, 1950). Cattle affected so severely they can no longer walk often give birth to normal, full-term calves. Fescue foot is sometimes fatal, but usually cattle recover if taken off the toxic pasture before dry gangrene causes permanent damage (Pulsford, 1950).

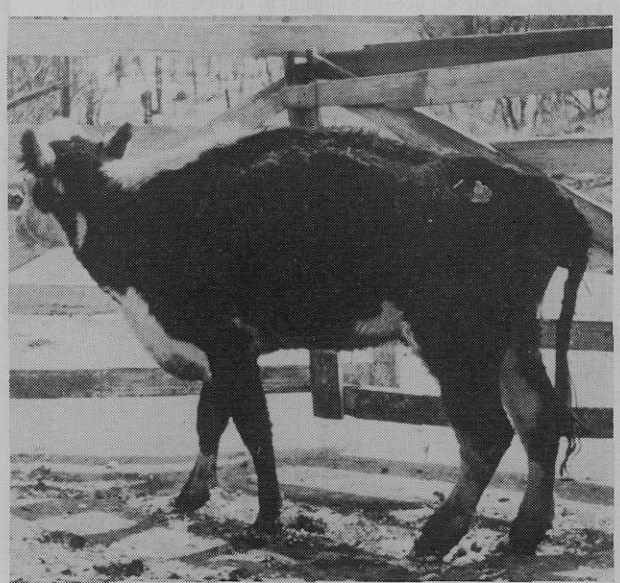


Figure 1. Cow with advanced symptoms of fescue foot

Certain facts establish that fescue foot is distinct from other diseases such as ergot poisoning, foot rot, frozen feet, or selenium poisoning. Results of several studies indicate that ergot is not involved (Cunningham, 1949; Jacobson and Miller, 1961; Jacobson *et al.*, 1963; Trethewie *et al.*, 1954). Of the many reportedly toxic fescue hay samples present examined, none contained ergot sclerotia. Foot rot, unlike fescue foot, is an

Northern Regional Research Laboratory, U. S. Department of Agriculture, Peoria, Ill. 61604

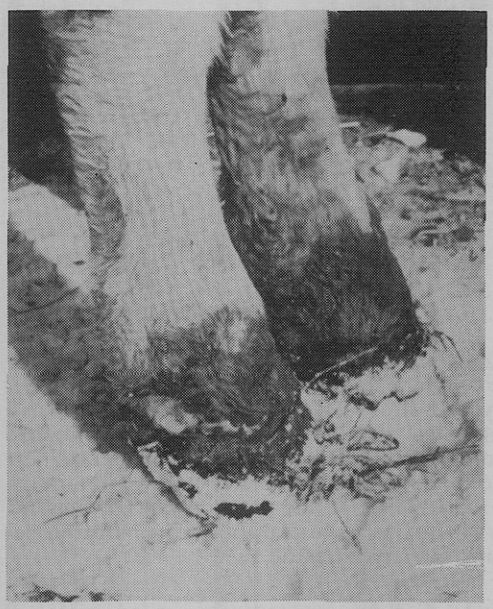


Figure 2. Rear legs of the cow shown in Figure 1. Hooves are beginning to crack away from the foot

Figure 3. Sloughed hoof of cow grazing a toxic tall fescue pasture

(courtesy of Dixon Springs Exp. Station, Robbs, Ill.)

infectious disease of cattle that occurs less often on the range than in the feed lot (Blood and Henderson, 1963). Frozen feet would not ordinarily be restricted to cattle on fescue pastures. Selenium poisoning, like frozen feet, affects many different species of animals, whereas fescue foot has so far appeared only in cattle. Alkali disease, a chronic form of selenium poisoning resembling fescue foot, may be diagnosed in borderline cases by the selenium content of the hair (Kingsbury, 1964). Veterinarians familiar with fescue poisoning in cattle can distinguish fescue foot from these and similar diseases.

Although fescue foot symptoms are sometimes seen in warm weather (Merriman *et al.*, 1955), they are most frequently observed and are more severe in cold weather (Goodman, 1952; Price and Miller, 1959). Whittow (1962) studied the significance of the extremities of the ox in thermoregulation. The legs, tail, ears, and dewlap account for almost one-third of the total surface area. Whittow observed the variation of the skin temperatures of the extremities over a range of environmental temperatures from 45° to −5° C. These variations in skin temperature were caused by varying blood flow to these areas: to conserve heat in cold weather, the ox apparently decreases blood flow to the extremities. A possible rationale to explain fescue foot is suggested by this effect. In cold weather, blood flow to the extremities may stop completely if vasoconstrictors are present in toxic fescue being consumed by the animal.

FRACTIONATION OF TOXIC HAY

The first approach to the problem of fescue foot was to prepare an extract from toxic fescue hay, assay this extract in cattle, and then fractionate the toxic material. This procedure was repeated on succeeding subfractions to concentrate the toxic material. An 80% ethanol extract was prepared from toxic hay supplied by the Kentucky Agricultural Experiment Station. Intraruminal administration of this extract (concentrate I, Figure 4) produced the typical symptoms of fescue foot in a cow (Jacobson *et al.*, 1963). This was the first time any extract from fescue proved toxic to cattle. A thick lipid-like layer (lipid III, Figure 4) of a similar extract was skimmed off, and the remaining aqueous phase (aqueous II, Figure 4) produced gangrene in the end of the tail. This aqueous phase was further subfractionated to give a crude alkaloid phase (alkaloid V), a chloroform phase (chloroform IV), and an aqueous residue (aqueous VI). Only aqueous VI was toxic. Skin temperature near the end of the tail was monitored before and after daily administration of samples. The mean difference between the environment and the tail skin temperature was about 21° C. for animals given alkaloid V, chloroform IV, or for the negative control, but only 8.6° C. for the animal given aqueous VI. In addition, aqueous VI caused purple discoloration at the end of the tail.

All these assays were performed in the winter, in an open or unheated barn, and required the extract from 300 to 600 pounds of hay for each test (Jacobsen *et al.*, 1963). To test hay extracts throughout the year, a constant-temperature room was constructed in which several cattle could be housed at 16° C. To reduce the amount of hay extract required, the tail skin temperature assay was tried. Unfortunately, at 16° C., the results with this assay were erratic. Simultaneously, the Pharmacology Laboratory of the Western Utilization

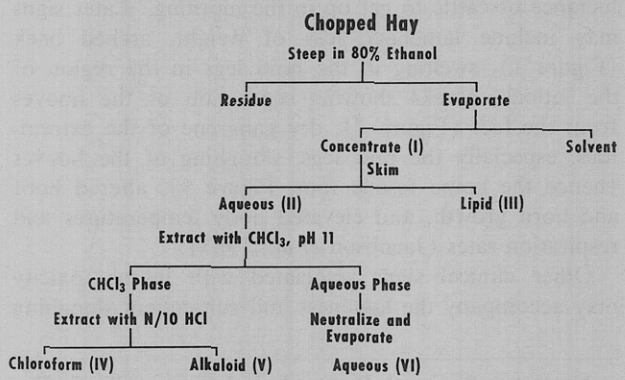


Figure 4. Fractionation of tall fescue hay

Research and Development Division (ARS, USDA) attempted unsuccessfully to correlate several small animal assays with toxicity of fescue samples. Tests for stimulators of smooth muscle were ambiguous; false positives were obtained from grass samples showing no toxicity to cattle.

ALKALOIDS OF TALL FESCUE

Concurrently with the fractionation studies, the alkaloids of tall fescue were investigated. An alkaloid extract of fescue hay was made by a method similar to the scheme in Figure 4 and from this, nine alkaloids were separated by paper chromatography (Yates, 1963). The major alkaloids of toxic and nontoxic fescue and of rye grass produce a similar array of spots on a paper chromatogram sprayed with potassium iodoplatinate (Figure 5).

The major alkaloid festucine was separated by preparative paper chromatography and shown to have the structure given in Figure 6 (McMillan and Dickerson, 1964; Yates and Tookey, 1965). The structure of festucine was first reported to be isomeric with that of loline, an alkaloid from *Lolium cuneatum* Nevski seed (Yunusov and Akramov, 1955, 1960). A revised loline structure (Akramov and Yunusov, 1965) is identical to that of festucine. Earlier, Yunusov and Akramov (1960) suggested the identity of norloline (des-*N*-methylloline) and despropionyldecorticasine, an alkaloid from the legume *Adenocarpus decorticans* Boiss (Alonso de Lama *et al.*, 1959). By direct comparison of certain derivatives or the alkaloids themselves, Ribas (1967; see also Ribas and Pazo, 1967) showed that festucine, loline, and despropionyl-*N*-methyldecorticasine were indeed identical. Interestingly, temuline, an alkaloid of unspecified structure from *Lolium temulentum*, has the same empirical formula as des-*N*-methylfestucine (Merck Index, 1960).

When alkaloids of normal fescue were compared with those from a sample of fescue diseased with the fungus *Stemphylium*, festucine and one or more of the other fescue alkaloids were absent (Yates, 1963). This comparison shows that fungi may affect the production of alkaloids or cause their transformation to other compounds. If an invading fungus so alters alkaloids, possibly it will also affect other metabolites. Such an effect suggests an interesting, but experimentally untested, possibility concerning the cause of fescue foot. In response to pathogens, plants often elaborate substances that inhibit growth or reproduction of the invading organisms (U. S. Dept. Agr., 1968). Possibly, in fescue, synthesis of compounds toxic to cattle may be triggered by the presence of certain fungi.

TOXIC FUNGI FROM FESCUE

Fescue foot is neither contagious nor infectious. It is sporadic, occurs seasonally, and appears to be regional. These factors support the concept that a mold is involved in fescue foot. Cold weather, which seems necessary for an outbreak of the disease, might stimulate a mold present on the grass to produce toxins. Joffe (1960) showed that alimentary septic angina in man and livestock was caused by toxic fungi on overwintered cereals. Many isolates of fungi, especially *Fusarium poae, Fusarium sporotrichioides*, and *Cladosporium epiphyllum*, from

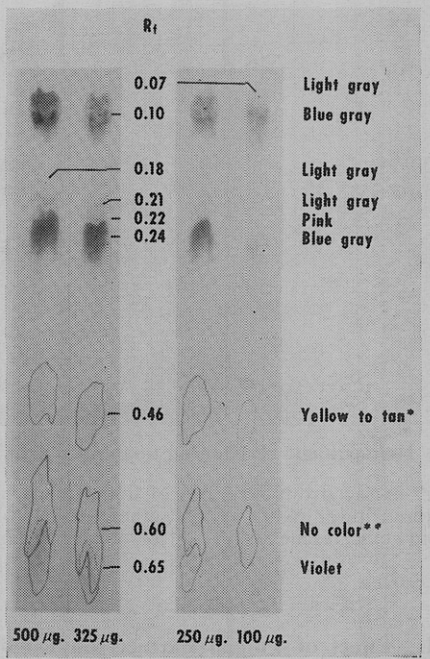


Figure 5. Paper chromatogram of tall fescue alkaloids sprayed with potassium iodoplatinate. Festucine, $R_f = 0.10$.

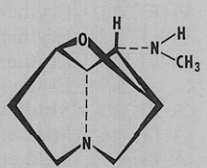


Figure 6. Festucine, an alkaloid of tall fescue

overwintered cereals were toxic, whereas fungal isolates from summer-harvested cereals were not.

Beginning in March, 1962, the authors received samples of toxic hay from a farm near Sturgeon, Mo., at approximately 2-week intervals. These samples were examined for fungi that appeared to be infrequent or unusual. There was no evidence that an unusual fungus was present on toxic fescue hay and absent from nontoxic fescue hay; hence, attention turned to the more common fungi.

Keyl *et al.* (1967) extracted 11 accessions of toxic fescue hay with ether. Five of the 11 extracts produced a hemorrhagic reaction on the skin of a rabbit (Figure 7). Keyl found that one sample of hay would give a positive test, whereas another sample of the same accession, but perhaps from another bale, would be negative (Keyl, 1964). This discrepancy indicated that only portions of a field were toxic—i.e., toxicity is localized within any one field. From one of the toxic hay samples, 24 fungi were isolated. These were cultured at 3° C. and extracts of the cultures were tested on a rabbit's back. Of the 24 isolates, nine (Table I) produced materials that caused a skin reaction (Figure 7). Three of the nine organisms were grown in Sabouraud's maltose broth at 3° C., and filtrates or their 10-fold concentrates killed mice on intraperitoneal (i.p.) injection. These were *Fusarium tricinctum* NRRL 3249, *Epicoccum nigrum* (A-13,312), and *Cladosporium cladosporioides* (A-13,310) (Keyl *et al.*, 1967).

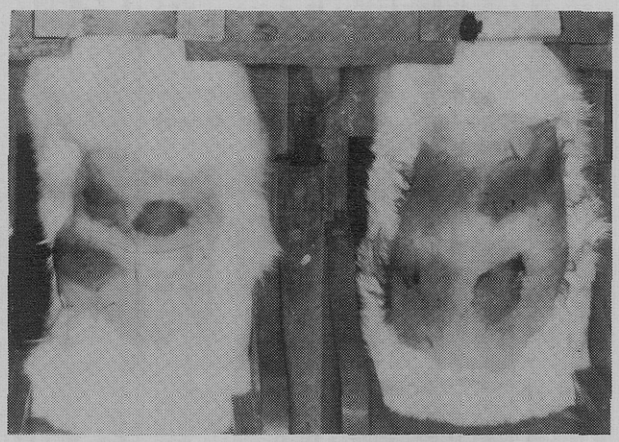


Figure 7. Hemorrhagic reaction on unabraded skin of rabbit

Left, ether extract from 50 grams of toxic fescue hay (control quadrant, lower right). Right, ether extracts of various fungi from toxic fescue hay (control quadrant, upper left)

Table I. Effect of Fungal Extracts on Rabbit Skin

Species	NRRL No.	Toxic Signs
Cladosporium cladosporioides	A-13,309	Erythema, edema
Cladosporium sp.	A-13,310	Erythema, edema
Epicoccum nigrum	A-13,311	Erythema, edema
Epicoccum nigrum	A-13,312	Erythema, edema
Fusarium sp.	A-13,313	Erythema, edema, necrosis
Fusarium tricinctum[a]	3249	Erythema, edema, necrosis
Sphaeropsidales sp.	A-13,315	Mild erythema, edema
Mucor fragilis	A-13,316	Mild erythema, edema
Mucor fragilis	A-13,317	Mild erythema, edema

[a] First identified as *F. nivale* NRRL A-13,318 (Keyl *et al.*, 1967; Yates *et al.*, 1967, 1968).

Nontoxic, sterile fescue hay was inoculated with aqueous spore suspensions of *F. tricinctum* NRRL 3249. The cultures were kept at 7° C. for 2 to 3 weeks, with occasional warming to room temperature to simulate field conditions. Glucose, peptone, and salts were added to the hay to produce good growth. The moldy hay was extracted with 80% ethanol, and the extract was tested in cattle. Extract equivalent to 1¾ pounds of moldy hay given orally killed one out of two animals within 24 hours. The second animal died after receiving another dose equivalent to 1 pound of moldy hay. However, in a later 15-day test at a lower daily dose (equivalent to 0.4 pound of hay), the extract did not produce toxic symptoms (Jacobson, 1965). Hay infected with *Cladosporium* was similarly tested, but was nontoxic.

In January, 1967, a herd of 100 cattle was placed on an almost pure fescue pasture near Fayette, Mo. Approximately a month later, 11 of the animals were severely affected with fescue foot (Garner, 1967). Samples of grass from the areas where the animals had apparently grazed, and samples from other fields nearby were examined for fungal flora. Fungi isolated were identified according to genera (Table II).

All isolates were incubated at a cool temperature to preserve any ability to produce mycotoxins. Duplicate Sabouraud's agar plates were made from each isolate. One plate was extracted with dichloromethane and the other plate with 95% ethanol. Solvent was removed and the extractives from 1/10 of an agar plate were tested by i.p. injection into mice.

The toxic fungi were almost exclusively in the genus *Fusarium* (Table II). Thirteen per cent of all fungi tested and 46% of the *Fusarium* isolates were toxic. Organisms classified as questionably toxic are scattered among the major genera.

Toxic fungi are not found exclusively on fescue grass (Table II). The possibly toxic fescue and the nontoxic control (orchard grass) actually contained a higher proportion of toxin-producing fungal isolates than did the known toxic fescue grass. However, these data do not show the relative abundance of toxic organisms in a toxic pasture; that is, how much of a field may be infected or how heavy is the infection. Lack of such evidence precludes any definite statements about the involvement of mycotoxins in fescue foot. Nevertheless, there are *Fusarium* fungi on toxic tall fescue, and about half of these organisms produce toxins on synthetic media at low temperatures. In short, *Fusarium* organisms are a potential hazard and may be involved in the toxicity of tall fescue to cattle.

MYCOTOXINS FROM *FUSARIUM TRICINCTUM*

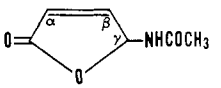
Yates and Burkhardt independently isolated a butenolide from *F. tricinctum* NRRL 3249 and characterized it as 4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone (Figure 8) (Yates *et al.*, 1967, 1968). This structure has been verified by synthesis (Burkhardt *et al.*, 1968). White (1967) has also published the isolation, characterization, and synthesis of this butenolide. He obtained his metabolite from two strains of *F. equiseti* grown on a glucose–ammonium nitrate medium at 25° C. Our isolation of the butenolide was guided by a rabbit skin assay. The butenolide can be extracted with dichloromethane, ether, or ethyl acetate. Its presence can easily be detected and its relative abundance estimated in dichloromethane extracts of the fungus. Three carbonyl bands in its infrared spectrum between 1700 and 1800 $cm.^{-1}$ are characteristic of this mycotoxin (Yates *et al.*, 1967). Two bands at 1760 and 1790 $cm.^{-1}$ are typical of $\Delta\alpha,\beta$-butenolides. These bands possibly result from Fermi resonance and are said to be quite sensitive to changes in polarity of the solvent (Jones *et al.*, 1959). The third band at 1705 $cm.^{-1}$ is due to amide C=O absorption. Bands for free and bonded N—H absorption at 3440 and 3340 $cm.^{-1}$ indicate that the mycotoxin is a monosubstituted amide. Gas-liquid chromatography showed the presence of acetamide in the alkaline hydrolysate of the butenolide (Yates *et al.*, 1968). The position of the acetamido group was confirmed by the hydrogenated mycotoxin giving a silver mirror test, showing the presence of a potential aldehyde on the γ-carbon. Nuclear magnetic resonance (NMR) data on the mycotoxin and its hydrogenation product (Yates *et al.*, 1967, 1968) support the structure shown in Figure 8. Despite the presence of a chiral center at the γ-carbon, the isolated butenolide had no optical rotation.

The butenolide is produced by *F. tricinctum* NRRL 3249 not only on Sabouraud's agar held at 15° C. or cycled between 7° and 20° C., but also in Sabouraud's maltose broth at 3° C. (Yates *et al.*, 1968). Little of

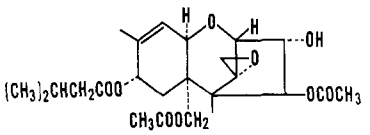
Table II. Results of Mouse Assay of Extracts of Fungi from Collected Grass Samples

	Source of Isolate								
	Known Toxic Fescue			Possibly Toxic Fescue[a]			Orchard Grass[b]		
Genus	Total	Toxic[c]	Questionable[c]	Total	Toxic	Questionable	Total	Toxic	Questionable
Alternaria	18	0	5	3	1	1	3	0	0
Cladosporium	28	0	7	2	0	0	5	0	0
Epicoccum	28	1	2	4	0	1	3	0	0
Fusarium	29	13	5	16	7	4	5	3	0
Mucor	11	0	0	6	0	0	0	0	0
Other	32	0	3	5	0	0	2	0	1

[a] This grass was from a farm near Sturgeon, Mo., which apparently is toxic every year. Since there were no cattle on the field, we do not know if the field was toxic when these samples were taken.
[b] This grass was gathered for a control from a field near the known toxic fescue.
[c] "Toxic" means that one or both extracts killed two of two mice within 4 days. "Questionable" means that one or both extracts killed one of two mice or made two of two mice noticeably sick within 4 days.



4-Acetamido-4-hydroxy-2-butenoic acid-γ-lactone



8α-(3-methylbutylryloxy)-4β,15-diacetoxyscirp-9-en-3α-ol

Figure 8. Mycotoxins from *Fusarium tricinctum* NRRL 3249

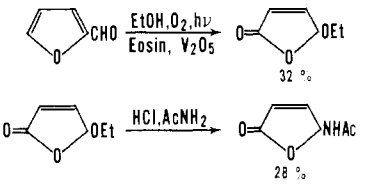


Figure 9. Synthesis of 4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone

the compound is produced at room temperature. Isolates from the parent strain may lose the ability to produce the butenolide through repeated transfers on agar slants incubated at room temperature. This loss may occur after only one transfer on agar slants and is usually accompanied by a change in pigmentation. The usual yellow and red pigments are replaced by dark reddish-brown pigments, and the colony appears tan.

The butenolide has an LD_{50} in mice of 43.6 mg. per kg. body weight by i.p. injection and an oral toxicity of 275 mg. per kg. (Keyl, 1966). It has weak antibiotic properties (Yates *et al.*, 1968). At a concentration of 10 mg. per ml., 11 of 14 bacteria and two of three molds were markedly inhibited. Only slight activity was detected at 1 mg. per ml. and none at 0.1 mg. per ml. Useful antibiotics are active at concentrations of 0.1 mg. per ml. or less. However, microbial inhibition might serve as an assay method at concentrations of 10 mg. per ml. or more (Yates *et al.*, 1968).

To make material available for tests in cattle, investigators at the Research Triangle Institute (Wall, 1968) synthesized 4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone by the method shown in Figure 9. They found that furfural was a better starting compound than furan (Schenck, 1953) for photochemical conversion to 4-ethoxy-4-hydroxy-2-butenoic acid-γ-lactone. This product was purified by distillation, and in the next step the ethoxy group was replaced by the acetamido group. The butenolide was isolated by chromatography, precipitation, and sublimation in an over-all yield of 9%.

In addition to the butenolide, Burkhardt isolated 8α-(3-methylbutyryloxy)-4β,15-diacetoxyscirp-9-en-3α-ol (Figure 8) from *F. tricinctum* NRRL 3249. The organism was grown either on Sabouraud's maltose broth at 3° C. for 20 to 30 weeks or on winter wheat at 3° C. for 10 to 12 weeks. Burkhardt was in the process of characterizing this mycotoxin when he learned of a similar scirpenol described at the American Chemical Society's 152nd National Meeting (Bamburg *et al.*, 1966). Samples from the two laboratories proved to be identical by direct comparison. There was no detectable depression of a mixed melting point, their infrared spectra were identical, and the samples had similar optical rotations (Yates *et al.*, 1968). This mycotoxin was originally named as a derivative of scirpenol; however, Godtfredsen *et al.* (1967) proposed an alternative nomenclature which refers to such sesquiterpenes as derivatives of trichothecane.

NMR was used to identify 8α-(3-methylbutyryloxy)-4β,15-diacetoxyscirp-9-en-3α-ol in crude extracts of *F. tricinctum* NRRL 3299 (obtained from E. B. Smalley, University of Wisconsin). An ethyl acetate extract of an agar culture was evaporated to dryness, and the residue triturated with CCl_4. The CCl_4 extract from a single petri plate was sufficient for an NMR spectrum to show key peaks reported (Bamburg *et al.*, 1968) for the pure scirpenol derivative.

Burkhardt also isolated a third toxin of unknown structure from Sabouraud's maltose broth (Yates *et al.*, 1968). This isolate had about the same order of toxicity as the butenolide. In Sabouraud's maltose broth, the *F. tricinctum* NRRL 3249 organism produced the three toxins in the ratio of 87:8:3 for butenolide, unidentified toxin, and 8α-(3-methylbutyryloxy)-4β,15-diacetoxyscirp-9-en-3α-ol (Yates *et al.*, 1968).

CONCLUSIONS

Alkaloids from *Festuca arundinacea* apparently do not cause fescue foot, at least by themselves. Toxic substances are produced by a strain of *Fusarium tricinctum*

isolated from a pasture where cattle developed fescue foot after grazing. Larger quantities of these toxins were produced when the cultures were subjected to cool temperatures. Of all the molds from samples of tall fescue taken from a pasture after a later outbreak of fescue foot occurred, approximately 20% were *Fusarium*. About one-half of these *Fusarium* isolates produced toxins on synthetic media at 15° C. However, about the same proportion of toxic *Fusarium* isolates were obtained from orchard grass. If *Fusarium* toxins are crucial in the etiology of fescue foot, other factors may be involved, such as intensity of infection of the pasture and synergism with other compounds in fescue. Alternatively, the combination of cold environment and ingestion of the responsible mycotoxin may be sufficient to cause the disease; accordingly, it may have become associated with tall fescue simply because this grass is so commonly used as a winter pasture. In any event, *Fusarium* organisms cannot be conclusively blamed until substances produced by them have been shown to produce typical fescue foot symptoms in cattle.

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Received for review November 4, 1968. Accepted January 21, 1969. Presented at the symposium on Natural Food Toxicants—(B) Mycotoxins. Division of Agricultural and Food Chemistry, 156th Meeting, ACS, Atlantic City, N. J., September 1968. The Northern Regional Research Laboratory is headquarters for the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.