Assay of Tall Fescue Seed Extracts, Fractions, and Alkaloids Using the Large Milkweed Bug

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Tall fescue infected with the endophyte *Acremonium coenophialum* is a source of diverse types of alkaloids: ergopeptines, ergoamides, saturated pyrrolizidines, diazaphenanthrenes, β-carbolines, and assorted amines. The large milkweed bug, *Oncopeltus faciatus*, was used to determine the toxicity of tall fescue extracts, subfractions of extracts, single alkaloids, and binary mixtures of alkaloids. Oral ED_{50} values for six alkaloids were measured (in units of micrograms/bioassay): *N*-formylloline, 2; ergonovine, 21; ergocryptine, 21; halostachine, 280; perloline, 380; norharmane, 590. Statistical analysis of the data for synergism between alkaloids indicated that one pair, perloline–ergocryptine, produced higher mortalities than that expected for each alkaloid acting independently.

Tall fescue (*Festuca arundinacea* Schreb.) is a cool-season pasture grass used extensively in the southeastern United States and in other countries throughout the world. Reports from Australia, New Zealand, and the United States related that tall fescue can be toxic to livestock and other animals (Yates, 1962). Much research to determine the cause of this problem has been done in the ensuing years (Bush et al., 1979). Progress in determining the etiology of tall fescue's toxicity was limited, however, until Bacon et al. (1977) reported the presence of an endophytic fungus (*Epichloe typhina*), now referred to as *Acremonium coenophialum* (Morgan-Jones and Gams), in tall fescue. Porter et al. (1979, 1981) reported that this endophyte produced clavine and ergopeptine alkaloids in liquid culture.

The ergopeptine alkaloids (ergovaline, ergosine, and trace amounts of other ergopeptines) have been suspected contributors to fescue foot and summer syndrome in cattle grazing tall fescue (Yates et al., 1985; Porter et al., 1981); the pyrrolizidine alkaloids (*N*-formylloline, *N*-acetylloline, and lesser amounts of other pyrrolizidines) were related to the problem by Robbins et al. (1972). Perloline, a diazaphenanthrene alkaloid, was shown to contribute to the malady by Boling et al. (1975). Davis et al. (1983) related toxicity of tall fescue to the synergistic action between the phenethylamine halostachine and the β-carboline alkaloids harmane and norharmane. Other compounds, which appear to be ergonovine and similar amides of lysergic acid, are also present in alcohol extracts of endophyte-infected tall fescue (EITF) seed (Yates and Powell, 1988), and these alkaloids, like ergonovine, are expected to be physiologically active. Reported concentrations of some representative alkaloids present in EITF are shown in Table I.

Johnson et al. (1985) reported that methanol extracts of EITF seed were deleterious to the large milkweed bug, *Oncopeltus fasciatus* (Dallas), and that these extracts contained loline-type alkaloids. However, chromatographic analysis revealed the presence of other alkaloid types as well; therefore, it was not clear whether the insect toxicity was due only to loline amides, and the present study was undertaken to determine the relative toxicities of the EITF alkaloids to the milkweed bug. Preliminary fractionation of methanol extracts, guided by the milkweed bug assay, was initiated for the purpose of isolating other physiologically active subfractions. However, ambiguous results were frequently encountered, perhaps because of epimerization of ergopeptine alkaloids, volatilization of lolines, or the fact that a mixture of alkaloids was required for complete activity. Therefore, representatives of six classes of alkaloids known to be present in EITF were evaluated for toxicity to the large milkweed bug. Since the question of synergism was raised by Davis and co-workers (1983), alkaloids were tested in pairs as well as singly.

Table I. Reported Concentrations of Some Representative Alkaloids Present in Endophyte-Infected Tall Fescue

alkaloid	reference	concn, μg/g dry wt: seed	forage
ergovaline	Yates et al., 1988	3	
	Yates et al., 1985		0.3
ergonovine	Yates et al., 1988	detected	
loline alkaloids	Robbins et al., 1972	2000	1800–5000
halostachine	Davis et al., 1983		1
perloline	Hovin and Buckner, 1983		2000–3000

MATERIALS AND METHODS

Milkweed Bug Assays. Nymphs (0–1 day old) were deprived of food and water for 24 h before being used in an assay. Compounds and mixtures to be tested were appropriately diluted and added to dental cotton disks. Crude extracts, dissolved in their respective solvents, were tested at the milligram level, while pure standards dissolved in either methanol, water, or dilute formic acid were tested in the microgram range. After application of extracts or standards, cotton test pads were placed in a hood to dry. Control pads were treated with the solvent used to extract samples or to dissolve standards. Each test set consisted of five 1-oz creamer cups (five nymphs per cup). Three hundred microliters of water was injected into each treatment or control pad, and one milkweed seed was placed on each treatment or control pad to attract bugs to the pad. Because the milkweed bugs imbibe water from the pads, effects of the toxins are probably due to injestion, but some contact toxicity is also possible. Each cup was covered with a gas-permeable cardboard cover.

Endophyte-infected seed extracts were compared to extracts of endophyte-free seed and to controls, prepared by evaporating an appropriate volume of solvent. Subfractions of endophyte-infected seed extracts were compared to the corresponding subfractions from endophyte-free seed extracts and to controls.

Each alkaloid was evaluated at two dosage levels; these levels were based on preliminary experiments, so that low and high doses were chosen to kill approximately 30% and 70% of the test insects, respectively. Each test (25 nymphs) was repeated three times and compared the toxicity of alkaloid pairs (low–low, high–high, low–high,

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high–low), the toxicity of each alkaloid dosed singly (low level, high level), and controls (50 or 75 nymphs) that were prepared with the appropriate solvents. Mortality was recorded after 6 days. An additional 300 μL of water was placed on each pad every two days.

Seed Extracts and Pure Standards. Endophyte-free and endophyte-infected tall fescue seed samples were obtained through the courtesy of the University of Kentucky or purchased from Lambert Seed Co., Camden, AL. The latter seed was certified as 100% endophyte-infected. All seed material was ground to pass a 3.0-mm screen in a Brinkmann Model 192 centrifugal grinding mill. One hundred grams of seed meal was defatted with hexane and then extracted with methanol or ethanol, followed by 50% aqueous alcohol and then water. This extraction scheme was chosen to provide extracts containing compounds of increasing polarity. Each extract was reduced in volume in a rotory evaporator and was diluted to exactly 100 mL for bioassays.

Ergonovine, ergocryptine, and norharmane were purchased from Sigma Chemical Co., St. Louis, MO., and halostachine was purchased from Aldrich Chemical Co., Milwaukee, WI. Perloline was isolated by S.G.Y. (Yates et al., 1975). *N*-Formylloline was isolated by S.G.Y. (unpublished); its identity was confirmed by GC–MS. Mass spectral data of the latter sample were as follows, m/z (%): 41 (22), 42 (45), 55 (16), 69 (16), 80 (21), 82 (100), 83 (17), 95 (25), 96 (9), 110 (12), 111 (11), 123 (10), 139 (1), 154 (15), 182 (1). Gas chromatographic analysis of this sample indicated 0.26 g/mL total loline alkaloid (80% *N*-formylloline, 20% related loline alkaloids), calculated as *N*-formylloline dihydrochloride. Several impurities in the loline alkaloid sample were characterized: UV analysis indicated 0.001 g/mL of perloline hydrochloride. HPLC analysis indicated 72 μg/mL of ergovaline, plus the presence of like amounts of other uncharacterized fluorescing compounds. Titration with a standard base indicated the presence of 0.16 g/mL of formic acid.

Chromatography. High-performance liquid chromatography was a modification of the methods of Scott and Lawrence (1980) and Yates and Powell (1988). Thin-layer chromatography utilized 0.25-mm silica gel plates, developed with chloroform–methanol (90:10, 80:20, 50:50). Alkaloids were visualized with a modified Ehrlich's reagent (0.5 g of 4-(dimethylamino)benzaldehyde in 100 mL of cyclohexane; after spraying, plates were placed in a chamber saturated with HCl) or with potassium iodoplatinate (1 g of platinic chloride in 10 mL of water mixed with 250 mL of 4% potassium iodide and diluted to 500 mL).

Data Analysis. Effectiveness of individual standard alkaloids in the milkweed bug assay, compared to controls, was evaluated by analysis of variance. For this analysis, data were pooled over all experiments in which a particular single alkaloid occurred. The response variable was the percent mortality in each group of 25 insects. The days on which experiments were set up were used as blocks. No data transformation was used because very few data points were near 0% or 100%.

In order to estimate ED_{50}'s for the alkaloids, it was necessary first to separate effects of the alkaloids from effects due to experimental conditions (control mortality). This was done by applying Abbott's formula: $P = (X_A - X_{control})/(100 - X_{control})$, where X_A and $X_{control}$ are the mean percent mortalities for the alkaloid and control, respectively. P represents the percent mortality caused by the alkaloid, considering only the insects that survive the control conditions (Finney, 1971a). ED_{50}'s were then estimated graphically by plotting the probits of these values versus the logarithms of the doses (Finney, 1971b).

Table II. Milkweed Bug Assay of Sequential Extracts[a]

seed description	extract[b]	seed eq,[c] mg	mortality,[d] %
endophyte-free	hexane	107	20
	ethanol	45	40*
	ethanol–water	26	48**
	water	89	8
endophyte-infected	hexane	128	4
	ethanol	64	96***
	ethanol–water	18	92***
	water	64	76***
control	ethanol		16

[a] Data from day 3. [b] Extract weight 1 mg/cup containing five insects. [c] Milligrams of seed required to provide 1 mg of appropriate extract after solvent removal. [d] Key: *, **, and *** indicate mortality significantly different from control, at 0.05, 0.01, and 0.001 levels, respectively (χ^2 tests; 25 insects used for each treatment, 50 for control).

The two-alkaloid combinations were also examined by analysis of variance for evidence of synergism. Again, percent mortality was the response variable. For each pair of alkaloids, the interaction contrast, IC, was calculated: $IC = X_{(A+B)} - (X_A + X_B - X_{control})$, where X indicates mean percent mortality and A, B, and A+B represent two individual alkaloids and their combination, respectively. In this formula, $X_{(A+B)}$ is the observed mean mortality in the presence of the alkaloid combination, and $X_A + X_B - X_{control}$ represents the "expected" mortality in the absence of synergism. The observed and expected mortalities will not differ (IC will not be significantly different from zero, relative to appropriate standard error) if the two alkaloids act independently. On the other hand, if the contrast is significantly greater than zero, there is evidence for the activity of the combined alkaloids being greater than the sum of the two separate effects. The "expected" mortality, as defined above, is based on Abbott's formula (Finney, 1971a) and the model of simple independent action (Finney, 1971c). The interaction contrast provides a reasonable test for synergism when both single alkaloids cause mortality only slightly above control levels. This analysis was conducted only for the lower alkaloid doses. Mortalities at the upper doses of single alkaloids were too high for meaningful discussion of synergism.

RESULTS AND DISCUSSION

The milkweed bug assay demonstrated that hexane extracts of endophyte-infected tall fescue (EITF) seed were nontoxic and that ethanol, ethanol–water, and water extracts were toxic (Table II). Equivalent extracts of endophyte-free tall fescue (EFTF) seed were much less toxic although not completely innocuous. This mild toxicity is attributed to normal plant constituents; no alkaloids were detected in EFTF seed extracts. Fractionation of ethanol extracts of EITF seed by silica gel chromatography yielded some subfractions toxic to the milkweed bug when tested at a level equivalent to 4× the level of the original extract (Table III). Testing of the subfractions was set at the 4× level to overcome dilution of toxicity due to separation of toxins into more than one fraction, separation of possible synergists, and/or physical loss of sample. Equivalent subfractions from EFTF seed were nontoxic.

Chromatographic analysis of extracts and subfractions showing biological activity in the milkweed bug assay indicated the presence of loline- and ergopeptine-type alkaloids in two of the chloroform–methanol fractions (90:10 + 80:20) (Table IV). Perloline and perlolidine do not occur in the seed (Yates, 1983) and, therefore, would not

Table III. Milkweed Bug Assay of Subfractions of Ethanol Extracts of Endophyte-Infected and Endophyte-Free Tall Fescue[a]

	endophyte-free		endophyte-infected	
sample[b]	seed eq,[c] mg	mortality,[d] %	seed eq,[c] mg	mortality,[d] %
ethanol extract	45	8	1	48***

	endophyte-free		endophyte-infected	
subfraction	% extract	mortality,[b] %	% extract	mortality,[d] %
chloroform insolubles	56	4	27	36***
chloroform 100%	10	8	4	0
chloroform 100%	4	4	4	4
chloroform–methanol (95:5)	0	5	0	4
chloroform–methanol (90:10)	8	12	12	56***
chloroform–methanol (80:20)	6	4	12	80***
chloroform–methanol (50:50)	8	12	13	12
methanol 100%	2	8	2	8
recombined subfractions	94	12	74	96***
control		10		6

[a] Data from day 3. [b] Extract weight = 1 mg/cup containing five insects; subfraction weight = 4 mg/cup containing five insects. [c] Milligrams of seed required to provide 1 mg of appropriate extract. [d] Key: *, **, ***, indicate mortality significantly different from control at 0.25, 0.01, and 0.001 levels, respectively (χ^2 tests; 25 insects used for each treatment, 50 for controls).

Table IV. Indication of Loline-Type and Ergopeptide-Type Alkaloids in Subfractions from an Alcohol Extract of Endophyte-Infected Tall Fescue Seed

	HPLC[a]		TLC[b]	
fraction	fluorescence	UV[c]	Ehrlich's[c]	PtK_2I_6[d]
chloroform insolubles		neg	neg	pos
chloroform 100%		neg	neg	neg
chloroform–methanol (95:5)		neg	neg	neg
chloroform–methanol (90:10)	pos	pos	pos	pos
chloroform–methanol (80:20)	pos	pos	pos	pos
chloroform–methanol (50:50)		neg	neg	pos
methanol 100%		neg	neg	pos

[a] High-performance liquid chromatography (Yates and Powell, 1988). [b] Thin-layer chromatography (silica gel 60; chloroform–methanol, 90:10). [c] Indicates ergopeptine-type alkaloids. [d] Indicates loline-type alkaloids.

be detected. The presence of other alkaloids found in EITF—the β-carboline alkaloids harmane and norharmane and the phenethylamine halostachine—was not investigated for lack of convenient analytical procedures.

Eventually, a fraction was obtained in which ergovaline and related ergopeptine alkaloids were separated from what appeared to be simpler amides of lysergic acid, such as ergonovine. This latter fraction also contained *N*-formylloline and related alkaloids and was always highly toxic. The ergopeptine alkaloid-enriched fraction proved relatively nontoxic although commercial ergopeptines, ergocryptine or ergotamine, were toxic. Total separation of loline-type alkaloids from simple lysergic amides was not realized; therefore, these alkaloids could not be tested separately. Because our procedure had not led to pure compounds as was originally intended, further fractionation and assay were temporarily discontinued. Instead, representatives of the various alkaloid types found in EITF were evaluated. Testing of pairs of alkaloids was an effort

Table V. Bioassay Results for Individual Alkaloids

	dose, μg		mean mortality,[a] %				calcd P[b]		
alkaloid	high	low	high	low	control	n	high	low	ED_{50}
ergocryptine	20	5	60***	27	22	10	49	7	21
ergonovine	20	5	63***	39*	29	10	49	15	21
halostachine	500	250	85***	56***	21	15	81	45	280
N-formylloline	2.5	0.5	72***	34	29	8	61	7	2
norharmane	500	250	55***	36***	21	15	44	20	590
perloline	375	75	62***	33	25	10	50	10	380

[a] Differences from the control significant at the 0.05 and 0.001 levels indicated by * and ***, respectively. [b] $P = (X_{alkaloid} - X_{control})/(100 - X_{control})$, where X is the mean percent mortality. P represents the percent of the insects killed by the alkaloid, considering only those surviving the control conditions.

Table VI. Activities of Combinations of Alkaloids (Both at the "Low" Levels)[a]

	alkaloid b				
alkaloid a	ergocryptine	ergonovine	halostachine	*N*-formylloline	norharmane
ergocryptine					
ergonovine	56 (35)				
halostachine	59 (48)	96 (89)			
N-formylloline	20 (32)	44 (48)	43 (67)		
norharmane	53 (47)	68 (56)	64 (79)	25 (39)	
perloline	80*** (36)	60 (53)	64 (69)	67 (47)	35 (30)

[a] Values are observed and (expected) percent mortalities at the indicated alkaloid combinations. Expected mortalities calculated as explained in Materials and Methods. (***) indicates interaction contrast significant at the 0.001 level; that is, there is evidence for synergism. No other combinations were significant at even the 0.05 level.

to evaluate the possibility of synergism among the various types of alkaloids present [cf. Davis et al. (1983)].

Ergovaline, the principal alkaloid of EITF, was not available, and commercially available ergocryptine was used as a substitute. Results with this alkaloid, rather than with ergovaline, are expected to differ somewhat at the same dose level, but not in specific physiological activity. Perloline was included because it has been recorded to be present in tall fescue forage in concentrations as high as 3000 $\mu g/g$ (Hovin and Buckner, 1983) and has been shown to contribute, though only slightly, to tall fescue toxicity (Boling et al., 1975).

The high dose of each alkaloid caused mortality that was significantly greater than that of the controls (Table V). The low doses of halostachine, norharmane, and ergonovine were also significantly toxic. Although the estimated ED_{50}'s are rather crude (being derived from only two dose levels), it is clear that, on a weight basis, *N*-formylloline is far more potent than halostachine, norharmane, and perloline; ergonovine and ergocryptine have intermediate activity (Table V).

Of the 15 pairs of alkaloids tested for synergistic activity, only the combination of perloline and ergocryptine caused significantly higher than expected mortality in the milkweed bug (Table VI). In general, interactions among these classes of alkaloids appeared to be subtle and are probably of little practical consequence to the milkweed bug. The relationship between our observations concerning milkweed bug toxicity and the effects of these same compounds in mammals remains to be determined.

Registry No. Ergocryptine, 511-09-1; ergonovine, 60-79-7; halostachine, 495-42-1; *N*-formylloline, 38964-33-9; norharmane, 244-63-3; perloline, 7344-94-7.

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