Activity of Tall Fescue Alkaloids and Analogues in in Vitro Rumen Fermentation

Lowell P. Bush,* Harold Burton, and James A. Boling

The alkaloid perloline inhibited rumen fermentation in vitro at 0.04 to 1.0 mM as measured by cellulose digestion. The perlolidine moiety of perloline also inhibited cellulose digestion, but the inhibitory effect of perlolidine was equal to that of perloline only at the higher concentrations. Perloline methyl ether was less inhibitory than perlolidine. The veratrole moiety of perloline and perlolyrine did not inhibit cellulose digestion below 10 mM. Phenanthridine inhibited cellulose digestion at 0.1 to 1.0 mM; however, phenanthridone, 4-(2′-amino)phenyl-2-pyridone, and 4-(2′-formylamino)phenyl-2-pyridone did not inhibit in vitro rumen fermentation. Associated with inhibition of cellulose digestion was a decreased production of volatile fatty acids and bacterial protein production. Biological activity of these substances was greatly influenced by the substitution at the C-5 position of perloline.

The alkaloids of tall fescue (*Festuca arundinacea* Schreb.) and ryegrass (*Lolium perenne* L.) have been implicated in the etiology of animal diseases (Aasen et al., 1969; Bush et al., 1972; Yates, 1963). Of the many alkaloids that have been isolated from tall fescue, perloline and perlolidine are the most predominant. Tall fescue and ryegrass alkaloids have been separated into nine or ten components by paper chromatography (Jeffreys, 1964; Yates, 1963). Most of these components have not been identified.

Perloline, the principal alkaloid found in tall fescue forage, inhibits in vitro growth of rumen cellulolytic bacteria and in vitro and in vivo ruminal cellulose digestion. Volatile fatty acid (VFA) production was inhibited, and the composition of the VFA was altered by perloline (Bush et al., 1972; Boling et al., 1975). Bush et al. (Bush et al., 1970; Bush and Buckner, 1973) suggested that perloline may be involved in the observed poor performance of cattle grazing tall fescue in summer. They hypothesized that the in vivo effect of perloline in tall fescue is the inhibition of microflora activity in the rumen, particularly cellulolytic activity, and subsequent decrease in the energy and nutrient availability to the animal. The influence that other, identified or nonidentified, components of the alkaloid fraction have in our in vitro system or the influence they would have in an in vivo system is not known. The objectives of this investigation were: (1) to determine the effects perlolidine and perlolyrine have on in vitro rumen fermentation as compared with perloline, and (2) to modify perloline to determine the biological active portion of the alkaloid in in vitro rumen fermentation. Answers to the second objective would be useful for investigation on identification of important unknown components of the alkaloid fraction.

MATERIALS AND METHODS

Perloline (Ia) was isolated from tall fescue as described by Bush et al. (1970). Perlolidine (II), 4-(2′-amino)-phenyl-2-pyridone (VIa), and 4-(2′-formylamino)phenyl-2-pyridone (VIb) were synthesized using the procedures described by Akhtar et al. (1967). Phenanthridine (IV) was obtained by cyclodehydration of 2-formylaminobiphenyl. Veratrole and phenanthridone V were obtained from K&K Laboratories. Perlolyrine III was supplied by Dr. J. A. D. Jeffreys, University of Strathclyde, Glasgow, Scotland. Structures of these compounds are shown in Figure 1.

Department of Agronomy (L.P.B., H.B.) and Department of Animal Sciences (J.A.B.), University of Kentucky, Lexington, Kentucky 40506.

Cellulose digestion was measured by a modification of the in vitro rumen fermentation technique of Baumgardt et al. (1962). Ruminal fluid was obtained from a ruminally fistulated steer that was fed a daily ration of 2.7 kg of ground shelled corn (*Zea mays* L.) and grass–legume hay ad libitum. The rumen fluid was strained and mixed with the buffer–mineral medium 1:2 (v/v). Glucose and urea were added to provide a final concentration of 0.05% each. Isolated wood cellulose (Solka Floc, Brown Co., Berlin, N.H.) was used as substrate. Each fermentation tube contained 100.0 mg of cellulose and 30 ml of rumen fluid–mineral solution.

The alkaloids were solubilized in water or 0.1 N acetic acid and added to the individual fermentation tubes just prior to the beginning of the 24-h fermentation period. One milliliter of alkaloid solution and/or water was added to each tube. The perloline concentrations used in this study (0.04 to 1 mM, 14 to 350 mg/l.) could occur in the rumen of animals grazing tall fescue in the summer (Bush and Buckner, 1973). Percent cellulose digestion and percent inhibition of cellulose digestion were calculated according to Bush et al. (1972). Volatile fatty acids (VFA) in each fermentation tube were determined by the procedure of Erwin et al. (1961). Bacterial protein production was determined on the whole fermentation mixture by the trichloroacetic acid precipitable nitrogen procedure of Winter et al. (1964).

RESULTS

Inhibition of in vitro ruminal cellulose digestion occurred with the addition of perloline, perloline methyl ether, and perlolidine to the media (Figure 2). Perloline was most effective in inhibiting cellulose digestion and the perlolidine moiety was most important in causing the inhibitory effect as perlolidine and perloline methyl ether were almost as inhibitory as perloline. The veratrole moiety of perloline was inhibitory only at high concentrations. The indole alkaloid perlolyrine was not so inhibitory as the other alkaloids but followed a pattern similar to that of veratrole.

The perlolidine moiety was structurally modified to determine the portion of the ring system and, also, which substitution on the ring was necessary for inhibitory activity in the in vitro ruminal bioassay system. Opening the B ring as in 4-(2′-amino)phenyl-2-pyridone and 4-(2′-formylamino)phenyl-2-pyridone decreased inhibitory activity (Table I). Phenanthridine has the ring system intact but the nitrogen at position 3 has been replaced with a carbon and the keto function is absent at the C-4 position and the inhibitory activity is equal to that observed for perlolidine. However, phenanthridone, with substitution at the 5 position of perloline, did not have inhibitory activity equal to phenanthridine. Perloline methyl ether,

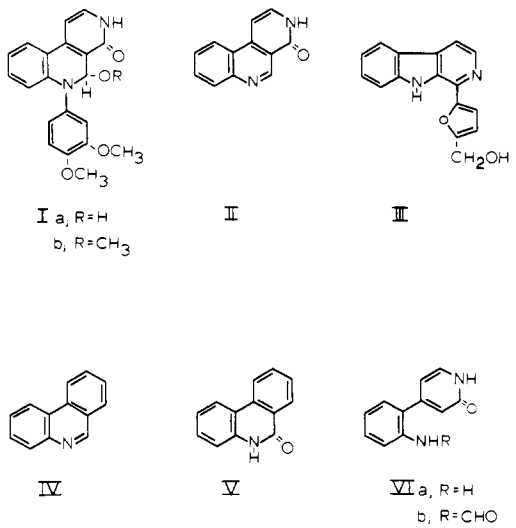

Figure 1. Ia = perloline, Ib = perloline methyl ether, II = perlolidine, III = perlolyrine, IV = phenanthridine, V = phenanthridone, VIa = 4-(2′-amino)phenyl-2-pyridone, VIb = 4-(2′-formylamino)phenyl-2-pyridone.

with the methyl ether at the 5 position, the same position as the substitution in phenanthridone, inhibited cellulose digestion less than perloline or perlolidine (Figure 2).

Inhibition of in vitro cellulose digestion by perloline has been well documented, but the mode of action is not understood. Phenanthridine, perloline, and the perloline moieties, perlolidine and veratrole, were used in a study to determine the influence of bacterial protein production on cellulose digestion. At 0.08 mM perloline stimulated bacterial protein production by 6% and stimulated cellulose digestion by 20% (Figure 3A).

Cellulose digestion was completely inhibited at 0.4 mM perloline with a 16% reduction of bacterial protein production. The perlolidine moiety of perloline completely inhibited cellulose digestion at 0.8 mM, with a 16% reduction in bacterial protein production (Figure 3B). Veratrole, the other perloline moiety, inhibited bacterial protein production 22% and cellulose digestion 100% at 10 mM (Figure 3C). Bacterial protein production was inhibited 16% with 0.1 mM veratrole but cellulose digestion was inhibited by only 2%. Phenanthridine inhibited bacterial protein production 36% when 100% inhibition of cellulose digestion occurred (Figure 3D).

Table I. Inhibition of Cellulose in in Vitro Ruminal Fermentation

Material	% inhibition at concn (mM) 0.01	0.1	1	10
Perloline		12	100	
Perlolidine		5	100	
Veratrole		4	1	30
Perlolyrine	2	3	10	34
4-(2′-Amino)phenyl-2-pyridone	6	−2	10	8
Phenanthridone	0	4	9	
Phenanthridine	3	3	100	98
4-(2′-Formylamino)phenyl-2-pyridone	0	5	18	
LSD, $P = 0.05$		12.3	8.1	21.3

VFA content of in vitro fermentation samples followed inhibition of cellulose digestion and bacterial protein production closely (Table II). The VFA values presented in this table are a summation of acetic, propionic, butyric, isovaleric, and valeric acids. Perloline, at 0.08 mM, increased VFA content 9% coincident with a 6% stimulation of protein production and a 20% increase in cellulose digestion. At 0.8 mM perloline, VFA content was reduced 12% when bacterial protein production and cellulose digestion were inhibited 16 and 100%, respectively. Perloline methyl ether, phenanthridine, and perlolyrine also caused increased VFA content at alkaloid concentrations that did not completely inhibit cellulose digestion. For all alkaloids VFA content was significantly decreased when cellulose digestion was inhibited 100%.

Acetic and propionic acids accounted for most of the VFA. No significant changes in VFA composition occurred during the 24-h fermentation period with phenanthridine, perloline methyl ether, perloline, and 4-(2′-formylamino)phenyl-2-pyridone. With perlolyrine, phenan-

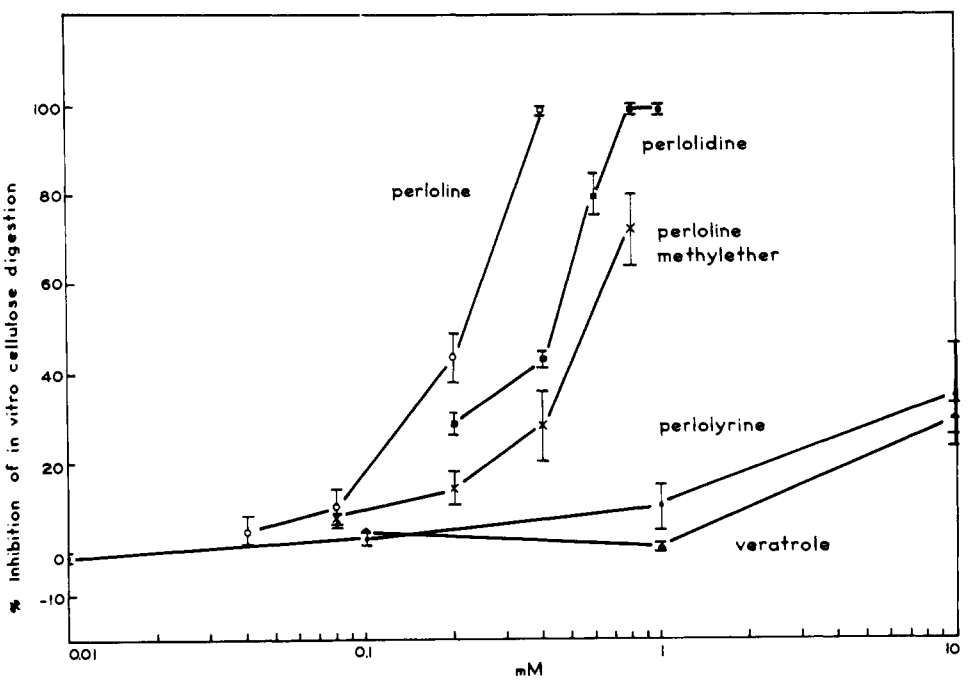

Figure 2. Inhibition of in vitro ruminal cellulose digestion; $I = S_{\bar{x}}$.

Table II. Influence of Perloline and Derivatives on VFA Content after 24 h of in Vitro Rumen Fermentation

	mmol of VFA/tube					
mM	Perloline	Perlolidine	Veratrole	Perloline methyl ether	Perlolyrine	Phenanthridine
0	1.84	1.00	0.72	1.80	0.75	1.90
0.04	2.01					
0.08	2.02			2.04		
0.1			0.78		0.95	2.28
0.2	1.83	0.83		1.98		
0.4	1.61	0.94		1.78		
0.6		0.95				
0.8	1.62	0.54		1.66		
1.0		1.30	0.84		0.62	1.60
10.0			0.42		0.66	1.80
LSD, $P = 0.05$	0.11	0.21	0.12	0.18	0.04	0.16

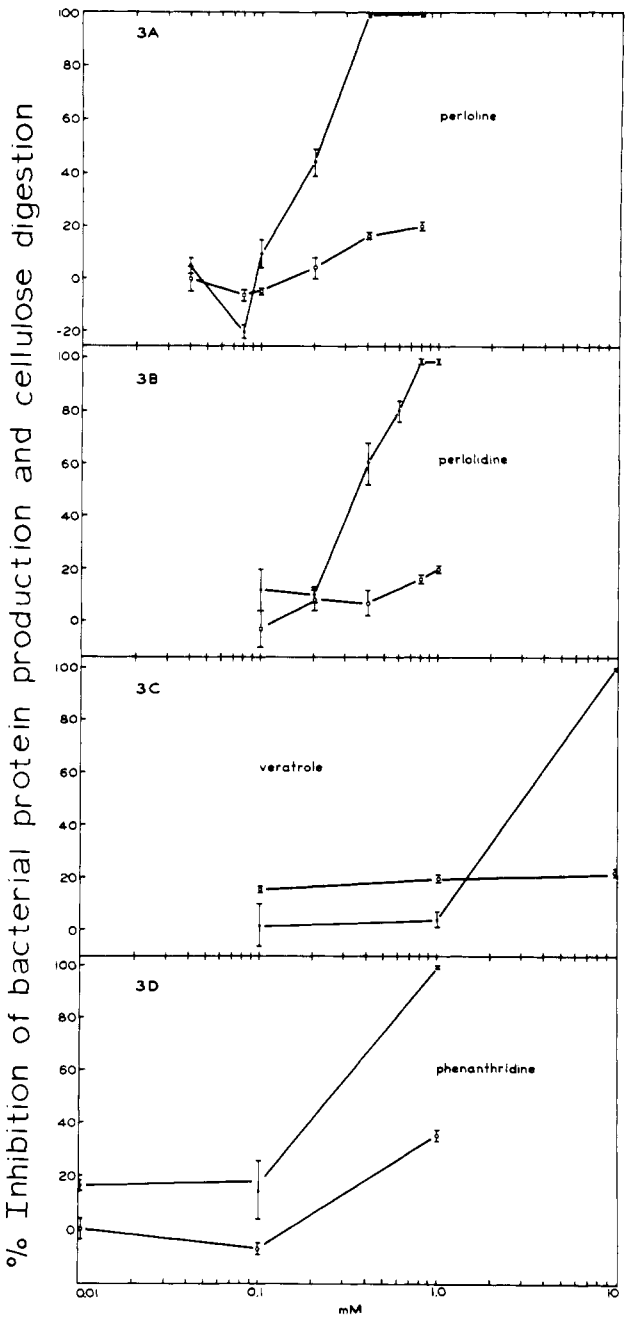

Figure 3. Inhibition of bacterial protein production (○) and inhibition of cellulose digestion (●); $I = S_{\bar{x}}$.

thridone, veratrole, and perlolidine the percentage of propionic acid decreased during the fermentation period and acetic acid increased or was not significant (Table III).

Table III. Influence of Perlolidine and 4-(2'-Amino)phenyl-2-pyridone on VFA Composition

	Molar % of perlolidine		Molar % of 4-(2'-amino)-phenyl-2-pyridone	
mM	Acetic acid	Propionic acid	Acetic acid	Propionic acid
0	56	32	55	30
0.01			56	28
0.1			49	34
0.2	68	24		
0.4	64	25		
0.6	66	22		
0.8	71	20		
1.0	71	18	55	28
10.0			52	32
LSD, $P = 0.05$	4.8	2.6	3.2	3.8

4-(2'-Amino)phenyl-2-pyridone decreased acetic acid and increased propionic acid content during the fermentation period (Table III).

DISCUSSION

The perlolidine moiety of the alkaloid perloline was primarily responsible for the inhibition of rumen fermentation in vitro (Figures 2 and 3A–C). Perlolidine at 0.2, 0.4, and 0.6 mM was 64, 43, and 80% as inhibitory as an equivalent concentration of perloline on in vitro cellulose digestion. The veratrole moiety was only 23, 1, and 30% as inhibitory to in vitro cellulose digestion as perloline at 0.1, 1.0, and 10 mM. Perloline methyl ether inhibited cellulose digestion less than perlolidine and perloline. Perloline methyl ether at 0.08, 0.2, 0.4, and 0.8 mM was 80, 32, 28, and 72% as inhibitory as perloline on in vitro rumen cellulose digestion. If the nitrogen at the 3 position in perlolidine is replaced with carbon and the carbonyl function removed from the C-4 position (phenanthridine) in vitro cellulose digestion is inhibited equally as well as with perlolidine. This indicates that the nitrogen in the A ring and the keto function at the C-4 position are not important biologically active sites. However, with a keto substitution at the 5 position of phenanthridine (phenanthridone), cellulose digestion was inhibited only 9% at 1.0 mM. Opening the B ring and having a formyl group at the 5 position carbon or removing the 5 position entirely greatly decreased inhibitory activity. From these observations we conclude that the inhibitory activity of perloline is due to the perlolidine moiety and that an intact B ring with no substitution on the 5 position is essential for maximum biological activity in our system.

Bacterial protein production was significantly inhibited by all of the treatment compounds when cellulose digestion was inhibited greater than 50%. A 16 to 36% reduction

in bacterial protein production resulted in 100% inhibition of cellulose digestion. Bacterial protein production is a measure of growth and the amount of reduction in growth required to completely inhibit cellulose digestion from rumen inoculum is much less than the relationship between perloline concentrations and growth of cellulolytic bacteria observed in pure cultures (Bush et al., 1972). Perloline concentrations that inhibited growth in pure cultures of cellulolytic bacteria from 9 to 24% inhibited cellulose digestion 30%. Perloline levels that inhibited cellulose digestion 100% inhibited growth of the cellulolytic bacteria from 44 to 80%. The association between in vitro rumen cellulose digestion and inhibition of bacterial protein production supports our earlier observations with perloline and growth of pure cultures of cellulolytic bacteria. The differences in response to perloline treatments cannot be explained with present data, but in vitro rumen cellulose digestion occurs as a result of the interaction of many organisms and not just cellulolytic bacteria. The production of VFA was probably a consequence of bacterial protein production and inhibition of cellulose digestion. The increased VFA content with 0.04 and 0.08 mM perloline corresponded with an enhanced bacterial protein production and cellulose digestion (Table II and Figure 3A). Bacterial protein production and VFA content were increased at 0.1 mM phenanthridine, but cellulose digestion was not increased. Composition of the VFA in response to perloline and related substances appears to be variable. In this study no change occurred with the addition of many of the materials, whereas in others the percentage of propionic acid decreased. Acetic acid percentage decreased and propionic acid percentage increased with addition of 4-(2′-amino)phenyl-2-pyridone to the fermentation tubes. Previous in vitro experiments reported no change in acetic acid percentage and a decrease in propionic acid with increased perloline levels (Bush et al., 1972). However, in vivo experiments with lambs showed that propionic acid increased with addition of perloline to the diet and that acetic acid decreased or did not change with perloline added to the diet (Boling et al., 1975).

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LITERATURE CITED

Aasen, A. J., Cluvenor, C. C. J., Finnie, E. P., Kellock, A. W., Smith, L. W., *Aust. J. Agric. Res.* **20**, 71 (1969).
Akhtar, M. A., Brouwer, W. G., Jeffreys, J. A. D., Gemenden, C. W., Taylor, W. I., Seelye, R. N., Stanton, D. W., *J. Chem. Soc.*, 859 (1967).
Baumgardt, B. R., Taylor, M. W., Cason, J. L., *J. Dairy Sci.* **45**, 62 (1962).
Boling, J. A., Bush, L. P., Buckner, R. C., Pendlum, L. C., Burrus, P. B., Yates, S. G., Rogovin, S. P., Tookey, H. L. *J. Anim. Sci.* **40**, 972 (1975).
Bush, L. P., Boling, J. A., Allen, G., Buckner, R. C., *Crop Sci.* **12**, 277 (1972).
Bush, L. P., Buckner, R. C., in "Anti-quality Components of Forages", Crop Science Society of America, Special Publication, No. 4, 1973.
Bush, L. P., Streeter, C., Buckner, R. C., *Crop Sci.* **10**, 108 (1970).
Erwin, E. S., Marco, G. J., Emery, E. M., *J. Dairy Sci.* **44**, 1768 (1961).
Jeffreys, J. A. D., *J. Chem. Soc.*, 4504 (1964).
Winter, K. A., Johnson, R. R., Dehonity, B. A., *J. Dairy Sci.* **47**, 793 (1964).
Yates, S. G., *J. Chromatogr.* **12**, 423 (1963).

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Analysis of T-2 Toxin (and HT-2 Toxin) by Mass Fragmentography

Stephen R. Pareles,[1] George J. Collins, and Joseph D. Rosen*

A method for the rapid screening of T-2 toxin in milk is presented. At the 1.25-ppm level, recovery is about 70%. The lower limit of detectability is estimated at 300 ppb. Although more research involving the use of deuterated T-2 toxin as an internal standard is needed before the method can be used as a routine quantitative procedure, an acceptable alternative to the rabbit-skin assay confirmation procedure is provided for use with both milk and corn. Furthermore, the proposed method can be used for the simultaneous semiquantitation and confirmation of both T-2 and HT-2 toxins.

Several outbreaks of toxicoses associated with moldy corn and other grains have been observed in recent years (Smalley et al., 1970; Hsu et al., 1972). One of the mycotoxins implicated in these episodes is the highly toxic 3-hydroxy-4,15-diacetoxy-8-(3-methylbutyrloxy)-12,13-epoxy-Δ^9-trichothecene (T-2 toxin). These outbreaks generally occur after cold, late harvest seasons and after the corn is stored during the following winter. It has been shown that 8 °C is the ideal temperature for T-2 toxin formation (Bamburg et al., 1968). Because of the possibility that dairy animals ingesting this corn (or other infected grains) may produce milk containing T-2 toxin, and there are no methods presently available for the analysis of this mycotoxin in milk, an attempt was made to devise a method for the analysis of T-2 toxin in milk.

Department of Food Science, Cook College, Rutgers University, New Brunswick, New Jersey 08903.
[1]Present address: McCormick & Co., Cockeysville, Md.

EXPERIMENTAL SECTION

Instrumentation. A DuPont 21-490 mass spectrometer equipped with a digital mass marker and interfaced to a Varian Model 2740 gas chromatograph (equipped with flame ionization detector) via a glass, single-stage jet separator was used. A mass fragmentography accessory of our own design (Pareles and Rosen, 1974) was set to detect only those gas chromatograph effluents whose mass spectra exhibited ions at m/e 436 or 350 for determination