crease glycoalkaloid content.

LITERATURE CITED

Allen, R. J.; Marlar, R. J.; Chesney, G. F.; Helgeson, J. P.; Kelman, A.; Weckel, K. G.; Traisman, E.; White, J. W., Jr. *Teratology* **1977**, *15*, 17.
Alvarado, R. M.S. Thesis, University of Massachusetts, Amherst, MA, 1977.
Bushway, R. J.; Barden, E. S.; Bushway, A. W.; Bushway, A. A. *J. Chromatogr.* **1979**, *178*, 533.
Bushway, R. J.; Barden, E. S.; Bushway, A. W.; Bushway, A. A. *Am. Potato J.* **1980**, *57*, 175.
Emanuel, I.; Sever, L. E. *Teratology* **1973**, *8*, 325.
Hanson, A. A. *Science (Washington, D.C.)* **1925**, *61*, 340.
Keeler, R. F.; Brown, D.; Douglas, D. R.; Stallknecht, G. F.; Young, S. *Bull. Environ. Contam. Toxicol.* **1976**, *15*, 522.
Maga, J. A. *J. Food Process. Preserv.* **1980**, *4*, 50.
Mun, A. M.; Barden, E. S.; Wilson, J. M.; Hogan, J. M. *Teratology* **1975**, *11*, 73.
Renwick, J. H. *Br. J. Prev. Soc. Med.* **1972**, *26*, 67.
Sinden, S. L.; Webb, R. E. *U.S., Dep. Agric., Tech. Bull.* **1974**, *No. 1492*.
Sizer, C. E.; Maga, J. A.; Craven, C. J. *J. Agric. Food Chem.* **1980**, *28*, 578.
Thornton, R. E.; Sieczka, J. B., Eds. "Commercial Potato Production in North America"; Potato Association of America: Orono, ME, 1980.
Willimott, S. G. *Analyst (London)* **1933**, *58*, 431.
Wilson, G. S. *Mon. Bull. Minist. Health Public Health Lab. Serv. (G.B.)* **1959**, *18*, 207.

Received for review January 2, 1981. Accepted April 30, 1981.

Effect of Phenolic Compound Removal on in Vitro Forage Digestibility

Hans-Joachim G. Jung and George C. Fahey, Jr.*

The effect of phenolic compounds on in vitro digestion of forages was studied by using polyvinylpolypyrrolidone (PVP) to remove forage phenolics prior to digestion. Phenolic removal appeared to increase cellulose and protein digestibilities of alfalfa (*Medicago sativa*) but not crown vetch (*Coronilla varia*). Total organic matter disappearance was not significantly affected. The digestion process resulted in an increase in McDougall's buffer soluble phenolic compounds in the PVP-treated samples. The apparent source of this material was insoluble material prior to digestion. Lignin did not appear to be responsible for the increase in concentration of soluble phenolic compounds.

Phenolic compounds are ubiquitous in their distribution among vascular plants and are well-known for their ability to complex with protein (Van Sumere et al., 1975). The polymerized phenolics known as "tannins" are particularly well-known for this phenomenon (Loomis and Battaile, 1966; Feeny and Bostock, 1968) and are often blamed for the poor performance of animals fed tanniferous plants (Hawkins, 1955; Tamir and Alumot, 1970; Marquardt and Ward, 1979; Griffiths and Moseley, 1980). Monomeric phenolics are also capable of complexing with proteins and reducing proteolysis (Loomis and Battaile, 1966; Rhoades, 1977).

Since many agriculturally important legumes contain tannins and other phenolics (Sarkar et al., 1976; Milic, 1972), and since tannins depress digestibility of cellulose and protein in in vitro rumen fermentations (Tagari et al., 1965; Smart et al., 1961; Lyford et al., 1967), the nutritional effects of forage phenolics on ruminant digestion may be significant. In this study, we have examined the effect of removal of phenolic material from a high-quality forage, alfalfa (*Medicago sativa*), and a forage of disputed quality, crown vetch (*Coronilla varia*) (Hawkins, 1955; Reynolds et al., 1969; Burns et al., 1972), on in vitro nutrient digestibilities. It was expected that removal of phenolic compounds from these forages would result in increases in digestibilities of total organic matter, cellulose, and protein. The concentration of phenolic material was monitored in both the soluble and insoluble fractions of the digestion to determine if removal of phenolics from the soluble fraction affected insoluble forms of phenolic compounds.

Department of Animal Science, Animal Sciences Laboratory, University of Illinois, Urbana, Illinois 61801.

Table I. Composition of McDougall's Buffer Solution

constituent	mM/L
sodium bicarbonate	117
sodium phosphate dibasic	26
potassium chloride	8
sodium chloride	8
magnesium sulfate	0.3
calcium chloride	0.2

MATERIALS AND METHODS

Plant Material. The alfalfa was first-cut, early bloom material preserved as hay. The crown vetch was collected at Dixon Springs Agricultural Center, Simpson, IL, in late Nov 1979 and was lyophilized before grinding. Both forages were ground to pass a 2-mm screen and dried again at 45 °C. No information on varieties of the forages was available.

Removal of Forage Phenolics. Prior to in vitro digestion experiments, both forages were extracted for 24 h on a metabolic shaker, in the dark, with 30 mL of McDougall's (1948) buffer (pH 6.8) per g of plant material. The buffer composition is given in Table I. Forage phenolics were removed from a portion of each extract (after filtration) by using 1 g of polyvinylpolypyrrolidone (PVP) per 30 mL of extract and shaking for 1 h. Phenolics bind preferentially to PVP (Loomis and Battaile, 1966; Anderson and Sowers, 1968). The insoluble PVP was then removed by filtration and the PVP extraction was repeated 5 times. The change in phenolic concentration was monitored by the Folin–Denis assay (Swain and Hillis, 1959). The PVP was washed twice with 95% ethanol, 3 times with

0021-8561/81/1429-0817$01.25/0

Table II. Effect of Phenolic Level on in Vitro Organic Matter Disappearance (OMD), Cellulose Digestion (CD), and Residual Crude Protein (CP) Content of Alfalfa and Crown Vetch

		type of digestion[a]											
		fermentation			two stage			pepsin			treatment mean[b]		
forage	treatment	OMD, %	CD, %	CP, mg/g of OM	OMD, %	CD, %	CP, mg/g of OM	OMD, %	CD, %	CP, mg/g of OM	OMD, %	CD, %	CP, mg/g of OM
alfalfa	control	26.0	41.8	247.1	30.9	39.3	181.2	10.3	11.5	124.6	22.4	30.8[a]	184.3[a]
	±1 SE	1.5	0.4	4.1	0.2	0.3	9.3	1.8	1.5	4.7	3.2	6.2	18.0
	PVP treated	27.0	42.1	232.8	32.4	42.6	162.3	13.5	17.6	111.5	24.3	34.1[b]	168.9[b]
	±1 SE	1.3	1.3	1.4	1.8	2.4	5.0	2.4	1.6	4.6	3.0	5.3	17.7
crown vetch	control	20.4	32.9	257.7	23.6	36.8	206.7	11.4	15.5	158.7	18.5	28.4	204.4
	±1 SE	0.8	4.1	6.0	2.4	5.7	12.2	2.7	9.3	8.5	2.1	5.1	13.7
	PVP treated	17.8	32.1	246.1	21.1	30.5	207.3	14.3	11.4	165.9	17.8	24.7	206.4
	±1 SE	1.6	0.9	2.1	1.5	5.2	10.8	3.2	3.4	9.5	1.5	4.5	12.3

[a] The type of digestion was significant for all three parameters in both forages ($P < 0.05$). [b] Values with different letter superscripts for individual parameters, within each column and each forage, are different ($P < 0.05$).

distilled water, and twice with McDougall's buffer prior to use, as preliminary work had shown a depression in digestion due to some unknown factor in the PVP. Also, for further correction for any remaining inhibition due to PVP, the buffer solution used to extract the control fractions of the forages (control treatment) was treated with 1 g of PVP/30 mL of solvent 5 times prior to use for extraction of the forages.

In Vitro Procedure. A modified Tilley and Terry (1963) procedure was utilized for the digestion studies. The residues from the extractions were dried at 45 °C, and the equivalent of 500 mg of the unextracted forages (taking into consideration the percent lost by solubilization) was added to 50-mL plastic centrifuge tubes. A small amount of casein was added to the PVP-treated extracts to replace the soluble protein lost during the PVP treatment. Fifteen milliliters of extract from either the control or PVP-treated extracts was added to each tube such that the extract and sample were from the same treatment. This replaced the material removed by buffer extraction, except for the phenolic material removed by PVP treatment. All tubes received an additional 9 mL of McDougall's buffer and 6 mL of rumen fluid. The rumen fluid was obtained from a fistulated Angus steer maintained on alfalfa hay. One-third of the tubes from each treatment were allowed to undergo a 24-h anaerobic fermentation. Another third of the tubes underwent a two-stage digestion where the fermentation was followed by the addition of 1 N HCl to reduce the pH to ~2, and then 6000 units of pepsin was added. The pepsin digestion was allowed to continue for 24 h. The last third of the tubes were subjected to only the 24-h pepsin digestion. All tubes were capped with stoppers equipped with one-way valves and incubated in a 39 °C shaking water bath. Digestion was terminated by rapid chilling in a −10 °C freezer and storage in a 4 °C refrigerator. Rumen fluid blanks were chilled immediately.

Analysis of Digestion Residues. All samples were centrifuged at 20000*g* for 20 min. Supernatant samples were saved for phenolic assay. All residues were dried at 45 °C except those to be analyzed for insoluble phenolics. Sample residues were dried at 100 °C and ashed at 500 °C for calculation of organic matter disappearance. Residue samples were analyzed for Kjeldahl nitrogen to determine residual crude protein (Association of Official Agricultural Chemists, 1975).

Insoluble phenolics were determined from samples which were refrigerated after centrifugation. The samples were extracted with 30 mL of 50% ethanol for 24 h on a metabolic shaker. After centrifugation at 20000*g* for 20 min, the supernatants were assayed for Folin–Denis phenolics. The residual insoluble material was analyzed for permanganate lignin and cellulose (Goering and Van Soest, 1970). The lignin and cellulose analyses were only conducted on samples from the second and third replications of the experiment.

Statistical Analysis. The experiment was repeated 3 times and analyzed as a randomized complete block design (Steel and Torrie, 1960).

RESULTS

The buffer extractions removed ~27% of the organic matter (OM) from both forages. The alfalfa extract was dark green in color, and after removal of phenolics with PVP, turned light green in color. In contrast, the crown vetch was a dark brown extract which became light green in color after PVP treatment. The alfalfa control extract contained 6.65 mg of gallic acid equivalents of Folin–Denis phenolics per g of OM, and the PVP-treated extract was reduced in concentration to 2.88 mg of gallic acid equivalents/g of OM. The crown vetch control extract contained 6.69 mg of gallic acid equivalents/g of OM, and the PVP-treated extract was reduced to 2.45 mg of gallic acid equivalents/g of OM. The Folin–Denis phenolic levels of the two forages are not comparable as the assay measures total phenolics and does not differentiate between the various groups of phenolic compounds. For this reason, all statistical analyses were done separately for each forage.

Data on organic matter disappearance (OMD), cellulose digestion (CD), and residual crude protein (CP) levels for both forages are presented in Table II. Type of digestion significantly affected OMD, CD, and CP values, but there were no statistical interactions between phenolic level (control vs. PVP treated) and type of digestion for these parameters (Table II). This indicated that the effects of phenolics are independent of type of digestion in this in vitro system. For neither forage did the removal of phenolics result in a significant change in OMD, although there was a trend for increased OMD of alfalfa with the PVP treatment. Alfalfa exhibited a significantly higher CD with phenolic removal. The large effect of phenolic removal on acid–pepsin digestion of cellulose was unexpected. The phenolics may increase cross-linkage of cellulose fibers (Morrison, 1974) and decrease acid hydrolysis of amorphous cellulose. No effect of phenolic level on CD was observed for crown vetch. Protein digestibility of the forages cannot be calculated because the samples are contaminated with microbial cells. The microbes convert soluble and insoluble nitrogen-containing compounds to microbial protein and nucleic acids such that the crude protein of the insoluble digestion residues is actually

Table III. Levels of Folin-Denis Phenolics in the Digestion Supernatants and Their Changes Relative to Initial (Expressed as Gallic Acid Equivalents)

			type of digestion[a]							
			fermentation		two stage		pepsin		treatment mean[b]	
forage	treatment	initial concn	μg/mL	% change	μg/mL	% change	μg/mL	% change	μg/mL	% change
alfalfa	control	175.5	124.3	−29.3	136.6	−19.0	167.1	−4.9	142.7[a]	−17.8[a]
	±1 SE	5.0	6.7	2.0	9.3	4.1	9.6	2.8	7.7	3.9
	PVP treated	112.7	106.8	−4.6	134.1	+19.7	135.7	+21.5	125.5[b]	+12.2[b]
	±1 SE	5.4	7.1	8.8	6.5	9.5	9.4	12.8	6.1	6.7
crown vetch	control	176.2	149.1	−15.6	129.7	−26.5	144.9	−17.7	141.2[a]	−19.9[a]
	±1 SE	7.0	10.0	2.6	9.6	3.6	8.7	4.5	5.6	2.5
	PVP treated	105.5	112.8	+7.7	105.3	+1.2	124.3	+19.5	114.2[b]	+9.5[b]
	±1 SE	5.9	3.1	7.6	8.9	13.8	12.8	16.9	5.4	7.2

[a] Type of digestion was significant for the concentration of phenolics in both forages and for percentage change in alfalfa ($P < 0.05$). [b] Values with different letter superscripts for individual parameters, within each column and each forage, are different ($P < 0.05$).

Table IV. Percentage Change in Lignin Content of Forages after Digestion

		% change			
		type of digestion[a]			
forage	treatment	fermentation	two stage	pepsin	treatment mean[b]
alfalfa	control	−8.8	+3.9	+21.7	+5.6
	±1 SE	2.9	4.6	3.4	10.1
	PVP treated	+3.6	−3.6	+23.9	+8.0
	±1 SE	0.4	5.2	13.3	6.4
crown vetch	control	+10.0	+15.0	+32.2	+19.1
	±1 SE	0.3	0.1	2.2	4.3
	PVP treated	+12.3	+17.2	+25.2	+18.2
	±1 SE	9.8	12.4	7.0	5.1

[a] The type of digestion was significantly different for both forages ($P < 0.05$). [b] No significant differences were noted ($P > 0.05$).

greater than the amount of crude protein added as insoluble sample. However, since total OMD was not significantly affected by phenolic level, the amount of microbial mass should be approximately the same for both control and PVP-treated systems, and any differences in total residual crude protein should be attributable to plant protein levels. Alfalfa PVP-treated digestion residues contained significantly less CP than the control residues (Table II), indicating greater digestion of buffer-insoluble plant protein. No effect was seen for crown vetch.

Phenolic analyses of supernatants from the in vitro digestions showed significantly more Folin–Denis positive material in the control solutions (Table III). This was expected as the PVP treatment prior to digestion established such a difference. However, when the relative changes in concentrations of phenolics were evaluated as compared to their predigestion levels, a different pattern emerged. For both alfalfa and crown vetch, the controls generally lost phenolics relative to initial levels while an increase in soluble phenolics in PVP-treated samples was noted. These differences were statistically significant ($P < 0.05$). Type of digestion did not affect the change in soluble phenolics of crown vetch but significantly affected the actual concentration in both forages. There was a statistical interaction between phenolic level and type of digestion for the relative change in total alfalfa phenolics. However, this is probably due to the signs (+ and −), thus making it more of a mathematical rather than a biological phenomena. The general trend is from the lowest level of phenolics in the fermentation to the highest level of phenolics as a result of the pepsin digestion.

Lignin is generally the largest source of phenolic material in forages. The permanganate lignin content of the digestion residues was compared to levels in the predigestion samples, and data are presented as the percentage change (Table IV). Variability among replicates was great and the only significant difference was due to type of digestion for alfalfa. The only obvious trend is a large increase in lignin content during the pepsin digestion.

An estimate of buffer-insoluble phenolic material, other than lignin, was obtained by ethanol extraction of the forages and digestion residues. Concentration of Folin–Denis phenolics in the residues was not significantly different for either forage relative to treatment (Table V), although, for alfalfa, the PVP treatment tended to result in decreased levels of ethanol-soluble phenolics in the residues ($P < 0.06$). The percentage change in ethanol-soluble phenolics between pre- and postdigestion samples showed a significantly greater decline for the PVP-treated samples than the alfalfa control; crown vetch exhibited no difference for phenolic level. Type of digestion significantly affected both total ethanol-soluble phenolics and relative changes.

DISCUSSION

The data presented in this report lead to three primary conclusions. First, for alfalfa, the removal of buffer-soluble phenolic material appeared to increase digestibility of the forage. The trend toward increased OM disappearance, the significant elevation in CD, and the reduced level of residual CP associated with the PVP treatment all suggest that phenolics inhibit forage utilization by ruminants. Second, the lack of any beneficial effect from phenolic removal on crown vetch digestion indicates that the use of PVP to remove phenolics from forages is very species dependent, as Burns and Cope (1974) have previously demonstrated a significant negative correlation between in vitro dry matter disappearance and total phenolic content of crown vetch clones. We attribute this species effect to the apparent differences in the ability of PVP to

Table V. Quantitative and Relative Changes in 50% Ethanol Extractable Forage Phenolics

		type of digestion[a]							
		fermentation		two stage		pepsin		treatment mean[b]	
forage	treatment	mg/g of OM	% change	mg/g of OM	% change	mg/g of OM	% change	mg/g of OM	% change
alfalfa	control	4.51	−23.4	5.26	−15.9	5.23	+7.8	5.00	−10.5[a]
	±1 SE	0.27	1.6	0.12	5.3	0.38	4.4	0.19	5.1
	PVP treated	4.14	−33.3	4.86	−27.3	4.63	−11.2	4.54	−23.9[b]
	±1 SE	0.56	7.9	0.29	2.3	0.18	2.0	0.22	4.1
crown vetch	control	3.98	−46.5	6.29	−14.9	6.44	+1.1	5.57	−20.1
	±1 SE	1.00	7.0	0.61	9.8	0.27	7.2	0.53	8.1
	PVP treated	3.75	−40.2	4.85	−29.4	5.83	−4.1	4.81	−24.6
	±1 SE	0.62	9.3	0.49	5.4	0.41	4.6	0.39	6.3

[a] The type of digestion was significant for both parameters in both forages ($P < 0.05$). [b] Values with different letter superscripts for individual parameters, within each column and each forage, are different ($P < 0.05$).

remove various forms of phenolics from solution. Loomis and Battaile (1966) stated that different groups of phenolic compounds have extremely variable affinities for binding to PVP. Alfalfa and crown vetch contain different groups and quantities of phenolics compounds (Milic, 1972; Sarker et al., 1976); therefore, the failure of crown vetch digestibility to increase with phenolic removal may be due to a lack of removal of its phenolic compounds. The method of drying may have contributed to the differential response of the phenolics to PVP extraction. Also, extraction of the phenolics with PVP at a more acidic pH could have resulted in more efficient phenolic removal (Anderson and Sowers, 1968), but we were concerned primarily with those phenolics soluble in the rumen on a high-forage diet (pH 6.8). The third important point raised by these results is that phenolic concentration is not static during the digestive process. The overall increase in phenolic concentration observed in the PVP-treated samples during digestion makes the detection of enhanced digestion due to removal of soluble phenolics very difficult to measure. The difference in alfalfa soluble phenolic concentration between control and PVP-treated samples was reduced from 36% prior to digestion to 12% after digestion. Similar results were seen for crown vetch. This additional phenolic material could be potentially inhibitory to digestion.

The source of these additional phenolics is not clear. Lignin is the most abundant phenolic material in forages, and lignin disappearance during digestion varies from over 30% to a net appearance of more than 30% after digestion (Allinson and Osbourn, 1970; Fahey et al., 1980). The values for lignin disappearance in this study were equally variable, with a trend toward lignin appearance in an acid medium. The changes in lignin during the digestion do not appear to explain the observed shift in soluble phenolics. The changes in ethanol-soluble phenolics were such that this fraction could account for the additional phenolics in solution after digestion. The chemical nature of this solubilized material is unknown. These phenolic compounds may be similar to the soluble lignin–carbohydrate complexes described by Gaillard and Richards (1975), as this material is only produced after a rumen fermentation or acid hydrolysis. The carbohydrate portion of this complex is protected from ruminal degradation, and it is conceivable that protein could be similarly protected by phenolic complexes. The release of phenolics into solution during digestion may be similar to the "compartmentation phenomenon" of Monties and Rambourg (1978) in their work with phenolic extraction from alfalfa.

The study indicates that phenolics may indeed potentially inhibit digestion of forages by ruminants. The use of PVP in this work appears limited due to differences between plant species in the ability of PVP to remove various classes of phenolics. The release of phenolic material during digestion suggests a dynamic pool of phenolic material which must be considered when the effects of phenolics on digestion are discussed.

LITERATURE CITED

Allinson, D. W.; Osbourn, D. F. *J. Agric. Sci.* **1970**, *74*, 23.
Anderson, R. A.; Sowers, J. A. *Phytochemistry* **1968**, *7*, 293.
Association of Official Agricultural Chemists "Official Methods of Analysis", 12th ed.; AOAC: Washington, DC, 1975.
Burns, J. C.; Cope, W. A. *Agron. J.* **1974**, *66*, 195.
Burns, J. C.; Mochrie, R. D.; Cope, W. A. *Agron. J.* **1972**, *64*, 193.
Fahey, G. C., Jr.; Al-Haydari, S. Y.; Hinds, F. C.; Short, D. E. *J. Anim. Sci.* **1980**, *50*, 1165.
Feeny, P. P.; Bostock, H. *Phytochemistry* **1968**, *7*, 871.
Gaillard, B. D. E.; Richards, G. N. *Carbohydr. Res.* **1975**, *42*, 135.
Goering, H. K.; Van Soest, P. J. *U.S., Res. Serv., ARS* **1970**, *No. 379*.
Griffiths, D. W.; Moseley, G. *J. Sci. Food Agric.* **1980**, *31*, 255.
Hawkins, G. E., Jr. *J. Dairy Sci.* **1955**, *38*, 237.
Loomis, W. D.; Battaile, J. *Phytochemistry* **1966**, *5*, 423.
Lyford, S. J.; Smart, W. W. G., Jr.; Bell, T. A. *J. Anim. Sci.* **1967**, *26*, 632.
Marquardt, R. R.; Ward, A. T. *Can. J. Anim. Sci.* **1979**, *59*, 781.
McDougall, E. I. *Biochem. J.* **1948**, *42*, 99.
Milic, B. L. *J. Sci. Food Agric.* **1972**, *23*, 1157.
Monties, B.; Rambourg, J. S. *Ann. Technol. Agric.* **1978**, *27*, 629.
Morrison, I. M. *Biochem. J.* **1974**, *139*, 197.
Reynolds, P. J.; Jackson, C., Jr.; Henson, P. R. *Agron. J.* **1969**, *61*, 187.
Rhoades, D. F. In "Creosote Bush. Biology and Chemistry of *Larrea* in New World Deserts"; Mabry, T. J.; Hunziker, J. H.; Difeo, D. R., Jr., Eds.; Dowden, Hutchinson, Ross, Inc.: Stroudsberg, PA, 1977.
Sarkar, S. K.; Howarth, R. E.; Goplen, B. P. *Crop Sci.* **1976**, *16*, 543.
Smart, W. W. G., Jr.; Bell, T. A.; Stanley, N. W.; Cope, W. A. *J. Dairy Sci.* **1961**, *44*, 1945.
Steel, R. G. D.; Torrie, J. H. "Principles and Procedures of Statistics"; McGraw-Hill: New York, 1960.
Swain, T.; Hillis, W. E. *J. Sci. Food Agric.* **1959**, *10*, 63.
Tagari, H.; Henis, Y.; Tamir, M.; Volcani, R. *Appl. Microbiol.* **1965**, *13*, 437.
Tamir, M.; Alumot, E. *J. Nutr.* **1970**, *100*, 573.
Tilley, J. M. A.; Terry, R. A. *J. Br. Grassl. Soc.* **1963**, *18*, 104.
Van Sumere, C. F.; Albrecht, J.; Dedonder, A.; DePooter, H.; Pe, I. In "The Chemistry and Biochemistry of Plant Proteins"; Harborne, J. B.; Van Sumere, C. F., Eds.; Academic Press: New York, 1975.

Received for review November 18, 1980. Revised April 10, 1981. Accepted April 20, 1981.