Inhibitory Potential of Phenolic–Carbohydrate Complexes Released during Ruminal Fermentation

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Forage cell walls were fermented with ruminal inocula to release phenolic–carbohydrate complexes. Fluid portions of the fermentations were centrifuged to collect a small-particle fraction. Solutions were separated on a Sephadex G50 molecular sieve column into five fractions. Original solutions and the six fractions of each forage solution were used to replace 50 or 100% of the buffer in a ruminal fermentation of cellulose or hemicellulose. Alkaline-extractable phenolic composition differed among forages for the solutions and among the fractions. All solutions depressed fermentation of both carbohydrates, and volatile fatty acid proportions were different from controls. A very low molecular weight fraction inhibited cellulose fermentation, but a slightly higher molecular weight fraction was associated with reduced hemicellulose degradation. Proportions of volatile fatty acids were affected by the fractions. Fermentability was correlated negatively with phenolics and varied among fractions, with *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde most commonly associated with inhibition of fermentation.

The plant cell wall esters of *p*-coumaric and ferulic acids have been shown to be correlated negatively with forage digestibility (Hartley, 1972; Burritt et al., 1984). During fermentation of forage cell walls by ruminal microorganisms, phenolic–carbohydrate complexes are solubilized both in vitro and in vivo (Gaillard and Richards, 1975; Neilson and Richards, 1978; Jung et al., 1983a,b). Similar results have been obtained when forage cell walls are hydrolyzed by purified cellulases (Hartley, 1973; Tanner and Morrison, 1983). It has been shown repeatedly that when the phenolic acids found in forages are added to in vitro ruminal fermentations, cellulose degradation and microbial growth are suppressed (Akin, 1982; Chesson et al., 1982; Jung and Fahey, 1983; Jung, 1985; Borneman et al., 1986). This inhibition may result from interference with attachment by fibrolytic bacteria to cell walls (Varel and Jung, 1986). In mixed cultures alteration of the fermentation end products was observed when free phenolic acids were present (Jung, 1985). However, the phenolic concentrations at which these effects were observed were approximately 10× greater than observed rumen concentrations of soluble phenolics (Jung et al., 1983b). When cinnamic acids were esterified to cellulose, inhibition of fermentation was seen at much lower concentrations (Jung and Sahlu, 1986). It is not known whether the soluble phenolic–carbohydrate esters released from forage cell walls during fermentation have any inhibitory potential.

This study was designed to determine whether the presence of soluble phenolic–carbohydrate complexes released from forage cell walls during rumen microbial fermentation will inhibit further fermentation of structural carbohydrates. Three different types of forages were studied to provide a diversity of phenolic–carbohydrate complexes.

MATERIALS AND METHODS

Alfalfa (*Medicago sativa*) hay, smooth bromegrass (*Bromus inermis*) hay, and corn (*Zea mays*) silage were chosen as forage sources. These plants represent the three major taxa of forages (C_3 legume, C_3 grass, and C_4 grass, respectively) fed normally to ruminants and differ in cell wall chemistry (Akin et al., 1984). Cell wall material was prepared from these forages by extraction with neutral detergent minus sodium sulfite (Goering and Van Soest, 1970). Cell wall preparations were extensively washed with hot water to remove detergent. Previous work has shown that normal amounts of fiber are fermented from neutral detergent cell walls (Jung and Varel, 1988).

Preparation of Phenolic–Carbohydrate Complexes. Thirty gram portions of the cell wall preparations were fermented in vitro with 1440 mL of buffer (McDougall, 1948) and 360 mL of ruminal fluid collected from a rumen-fistulated steer fed alfalfa hay. After incubation at 39 °C for 48 h the samples were strained through four layers of cheesecloth. Each forage cell wall preparation was fermented in triplicate. The fermentation supernatant solutions were lyophilized to remove a portion of the volatile fatty acid fermentation end products. The samples were redissolved in distilled water to their original volumes and assayed for NH_4^+ (Chaney and Marbach, 1962), reducing sugars (Dubois et al., 1956), and volatile fatty acids (Varel and Hashimoto, 1981). Ammonium chloride, glucose, acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, and caproate were added to each of the fermentation supernatants and a control buffer solution to equalize these constituents among treatments. These solutions were then used in the subsequent experiments. The pH values of all solutions were within the range 6.6–6.8 units.

Experiment One. Each of the replicate fermentation supernatant solutions was used to replace 50 or 100% of the control buffer solution in an in vitro fermentation of cellulose and hemicellulose (α-cellulose and larchwood xylan, respectively; Sigma, St. Louis, MO). Five-hundred milligram portions of cellulose or hemicellulose were placed in a 50-mL screw-cap culture tube. The control treatment received 24 mL of control buffer solution. The fermentation supernatant treatments included either 12 mL of supernatant solution plus 12 mL of control buffer (50% treatment) or 24 mL of supernatant solution (100% treatment). Six milliliters of ruminal fluid from an alfalfa fed steer was added to all treatments. The samples were fermented for 48 h at 39 °C with occasional shaking. After fermentation, the samples were centrifuged at 2000*g* for 20 min. The residual carbohydrate was dried at 100 °C for 48 h, and in vitro dry matter disappearance was calculated. The supernatant solutions from the carbohydrate fermentations were analyzed for volatile fatty acids. Each treatment was conducted in triplicate, and the experiment was repeated on three separate occasions. Rumen fluid blanks were included to correct for bacterial dry matter and volatile fatty acids.

Experiment Two. The three replicate fermentation supernatants were combined for each forage. The com-

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Table I. Concentrations of Alkali-Labile Phenolics and Nitrobenzene Oxidation Products of Forage Cell Wall Preparations

	compounds,[a] g·kg^{-1} cell wall									
	cinnamic acids			benzoic acids				benzaldehydes		
procedure: forage	CA	PCA	FA	PHBA	PA	VA	SYA	PHBAL	VAN	SYAL
alkaline extraction:										
alfalfa hay	0	0.12	0.08	0.02	0.05	0.01	0.04	0.01	0.01	0.01
smooth bromegrass hay	0.03	2.49	2.15	0.01	0.05	0.04	0.49	0.03	0.14	0.03
corn silage	0.01	10.27	5.67	0.01	0.05	0.03	0.42	0.11	0.22	0.00
SEM[b]	0.01	0.11	0.11	0.002	0.01	.002	0.02	0.01	0.002	0.001
nitrobenzene oxidation:										
alfalfa hay	0.30	0.09	0.17	0.05	nd[c]	0.74	nd	0.28	6.41	4.53
smooth bromegrass hay	0.36	0.86	2.71	0.06	nd	0.61	nd	0.16	2.91	3.34
corn silage	0.09	2.52	2.14	0.04	nd	0.11	nd	0.26	1.34	2.02
SEM	0.10	0.09	0.09	0.01		0.23		0.03	1.09	0.47

[a] Key: CA, cinnamic acid; PCA, *p*-coumaric acid; FA, ferulic acid; PHBA, *p*-hydroxybenzoic acid; PA, protocatechuic acid; VA, vanillic acid; SYA, syringic acid; PHBAL, *p*-hydroxybenzaldehyde; VAN, vanillin; SYAL, syringaldehyde. [b] Standard error of the mean (SEM) for duplicate analysis of each sample. [c] Compound not detectable (nd) by the nitrobenzene oxidation procedure.

bined supernatant solutions were centrifuged at 16000*g* for 60 min to recover the small-particle fraction. The supernatant solutions were eluded through a molecular sieve (Sephadex G50, Pharmacia; 90 × 3 cm (i.d.)) with 0.02 M NaCl, pH 5.8, at a flow rate of 1.5 mL·min^{-1}. Forage supernatants were collected as eighty 8-mL fractions. The phenolic content of the fractions was monitored as absorbance at 280 and 320 nm. Five composite fractions of each forage supernatant solution were made by combining five individual 8-mL fractions corresponding to specific areas on the elution curve (Figure 1). Column exclusion and inclusion volumes were determined with blue dextran and glucose, respectively. The particulate and five soluble composite fractions from each forage fermentation supernatant solution were used to replace 50 or 100% of a buffer control solution, which had also been fractionated on the molecular sieve, in an in vitro fermentation trial similar to that described in experiment 1. This fermentation experiment was conducted with three replicates for each treatment combination. Volatile fatty acid concentrations and composition also were measured after carbohydrate fermentations.

Phenolic Analysis. The forage cell wall preparations, fermentation supernatant solutions, and molecular sieve fractions were assayed for alkaline-extractable phenolic esters and nitrobenzene oxidation products. The methods of extraction and quantification by high-pressure liquid chromatography were those of Jung et al. (1983a). Compounds were determined at 254 or 313 nm, depending upon individual extinction coefficients. The liquid samples were lyophilized before extraction and analysis. Determination of nitrobenzene oxidation products of core lignin was done on the insoluble residues of the alkaline extraction of esterified phenolics.

Statistical Analysis. Experiment 1 was analyzed as a randomized complete-block (RCB) design with replication of in vitro fermentations as the blocking factor. Forage species and treatment level (50 or 100%) were arranged as a 2 × 3 factorial within the RCB. In experiment 2, data were analyzed as a 3 × 6 × 2 factorial of forage species, fraction, and treatment level. Comparisons between controls and treatments were tested as a one-way analysis of variance for all treatments. Individual means were tested by the F-protected least significant difference method. All computations were done on the Statistical Analysis System (SAS, 1982).

RESULTS AND DISCUSSION

The phenolic compositions of the forage cell wall preparations are given in Table I. As expected, the alfalfa contained very low concentrations of ester-linked, alkaline-extractable phenolic compounds (Jung et al., 1983a). *p*-Coumaric (PCA) and ferulic (FA) acids were the predominant phenolics present in both grasses, with the PCA to FA ratio being much greater in the corn silage than in the smooth bromegrass sample (1.81 vs 1.16). There were also appreciable levels of PCA and FA in the nitrobenzene oxidation products of the grasses (Table I). These components may be similar to the ether-linked FA found in wheat (*Triticum aestrivium*) straw (Scalbert et al., 1985). The benzaldehydes were the primary products of core lignin oxidation, and alfalfa hay cell wall contained the highest concentrations because it had the greatest core lignin content (17.3, 9.9, and 7.8% Klason lignin in alfalfa, smooth bromegrass, and corn silage cell walls, respectively). It should be noted that the vanillin to syringaldehyde ratio of alfalfa was different from that observed for smooth bromegrass or corn silage (1.42 vs 0.87 and 0.66, respectively). These data suggest that legume core lignin is different in composition from that present in grasses.

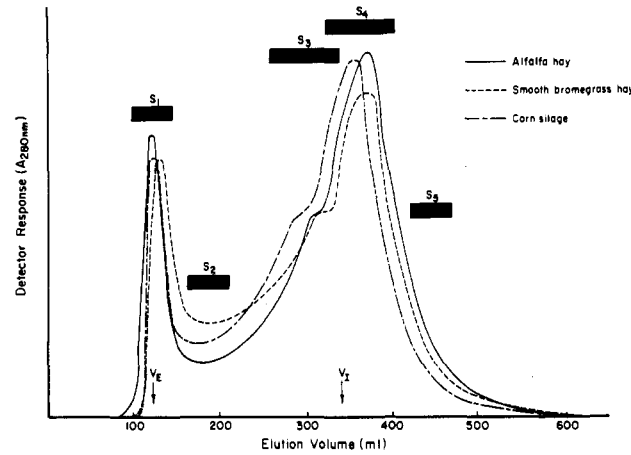

Figure 1. Elution profiles of the forage fermentation supernatants through a Sephadex G50 molecular sieve. V_E and V_I correspond to the exclusion and inclusion volumes, respectively. S_1–S_5 indicate fractions that were collected.

Although the forage cell wall preparations appeared to be very different in their phenolic composition, the phenolic components found in the fermentation fluid phases were not as different among the forages as expected (Table II). Supernatant solutions derived from corn silage fermentations contained more ($P < 0.05$) esterified PCA and FA than did the other forages. This relationship also was observed for vanillic and syringic acids, but corn silage was intermediate in *p*-hydroxybenzoic acid levels between alfalfa and smooth bromegrass. Among the nitrobenzene oxidation products in the fermentation supernatant solu-

Table II. Concentrations of Alkali-Labile Phenolics and Nitrobenzene Oxidation Products in the Liquid Phase after Fermentation of Forage Cell Walls in Vitro (Means of Three Fermentations)

	compounds,[a] mg·L^{-1}									
	cinnamic acids			benzoic acids				benzaldehydes		
procedure: forage	CA	PCA	FA	PHBA	PA	VA	SYA	PHBAL	VAN	SYAL
alkaline extraction:										
alfalfa hay	0.01	0.40[b]	0.18[b]	0.32[b]	2.91	0.24[b]	0.24[b]	0.54	0.72	0.05
smooth bromegrass hay	0.004	0.40[b]	0.16[b]	0.43[c]	2.13	0.10[b]	0.66[b]	0.83	0.31	0.04
corn silage	0.11	1.61[c]	0.81[c]	0.39[bc]	2.50	0.54[c]	1.80[c]	0.66	0.74	0.09
SEM	0.06	0.09	0.06	0.03	0.30	0.07	0.24	0.08	0.24	0.02
nitrobenzene extraction:										
alfalfa hay	0.04	0.98[b]	0.77[b]	0.30	nd	3.10	nd	1.63	2.37	0.58
smooth bromegrass hay	0.12	0.37[c]	0.29[c]	0.36	nd	3.07	nd	1.69	2.54	0.36
corn silage	0.05	0.08[c]	0.14[c]	0.34	nd	2.92	nd	1.48	2.66	0.33
SEM	0.04	0.14	0.16	0.05		0.38		0.30	0.64	0.07

[a] Key to abbreviations is given in Table I. Means in the same column, for the same procedure, not sharing a common superscript are different ($P < 0.05$).

Table III. In Vitro Dry Matter Disappearance (IVDMD) of Structural Carbohydrates Fermented by Ruminal Microorganisms with Supernatants from Previous Fermentation of Forage Cell Walls

	IVDMD,[a] %			
	cellulose		hemicellulose	
treatment	50%	100%	50%	100%
control[b]	61.8		78.3	
alfalfa hay[c,d]	42.4	28.1	67.9	59.3
smooth bromegrass hay[c,d]	39.3	27.9	68.5	58.4
corn silage[c]	47.5	33.8	71.3	64.5
SEM	0.5	0.5	1.1	1.3

[a] Either 50 or 100% of the buffer was replaced by fermentation supernatants from these forages. [b] All treatment means are lower ($P < 0.05$) than the appropriate control. [c] The 100% replacement treatment resulted in a lower cellulose IVDMD than the corresponding 50% treatment ($P < 0.05$). [d] The 100% replacement treatment resulted in a lower hemicellulose IVDMD than the corresponding 50% treatment ($P < 0.05$).

tions, the alfalfa fermentation had higher ($P < 0.05$) levels of PCA and FA than did the grasses. This observation and the values for esterified PCA and FA are unusual in that the original alfalfa cell walls contained very little of these cinnamic acids. It should be noted that while none of the forages contained much protocatechuic acid in their cell walls, this benzoic acid was the major esterified phenolic in all the fermentation supernatant solutions. This result has been seen in ruminal samples from sheep fed legume and grass hays, but other phenolics were quantitatively more important at different points in the digestive tract (Jung et al., 1983b). When the amount of phenolic material present in the fermentation supernatant solutions was compared to that present in the forages used for the fermentations, it was noted that most recoveries were greater than 100%. This also has been observed for total tract digestion of phenolics by sheep (Jung et al., 1983b). This relates to incomplete extraction of the esterified phenolics from intact forage cell walls and increased extractability of phenolics from digesta. Reduction in particle size would be an obvious effect of fermentation that can increase extractability. Recent results indicate that the 1 N concentration of NaOH normally used extracts less phenolic material than can be removed with 4 N NaOH (Jung, H. G., unpublished observations). Presumably, the ability of ruminal bacteria to partially metabolize phenolic compounds accounts for a portion of these differences between substrate and solubilized phenolic concentrations (Chesson et al., 1982; Jung and Fahey, 1983; Varel and Jung, 1986).

Experiment One. When the fermentation supernatant solutions from these forage cell wall preparations were included in a subsequent in vitro fermentation of cellulose or hemicellulose, significant depressions in degradability of the carbohydrates were noted (Table III). All supernatant treatments reduced ($P < 0.05$) in vitro dry matter disappearance (IVDMD) relative to the appropriate carbohydrate control. The 100% substitution of the supernatant treatments resulted in lower ($P < 0.05$) IVDMD values for both carbohydrate substrates than seen with the 50% substitution, except for the corn silage supernatant solution where the 50 and 100% levels were not significantly different for hemicellulose IVDMD (Table III). No significant differences were noted among the forages.

Evidence that phenolic compounds in the forage fermentation supernatant solutions may be responsible for reduced carbohydrate IVDMD is provided by observed negative correlations between phenolic concentrations and IVDMD (Table IV). Among the alkaline-extractable esterified phenolics, *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde were correlated negatively ($P < 0.05$) with cellulose and hemicellulose IVDMD; protocatechuic acid was also correlated negatively ($P < 0.05$) with hemicellulose IVDMD. The cinnamic acids reported to be correlated negatively with forage IVDMD when expressed as a proportion of the original forage dry matter or cell wall (Hartley, 1972; Burritt et al., 1984) were not correlated with

Table IV. Correlation Coefficients for Phenolic Compounds Released from Forage Cell Walls by Microbial Fermentation with in Vitro Fermentation of Structural Carbohydrates

	compounds[a]									
	cinnamic acids			benzoic acids				benzaldehydes		
substrate: procedure	CA	PCA	FA	PHBA	PA	VA	SYA	PHBAL	VAN	SYAL
cellulose:										
alkaline extractn	0.02	0.03	0.01	−0.51[b]	−0.46	0.02	0.12	−0.69[b]	−0.27	−0.41
nitrobenzene oxidn	−0.36	−0.40	−0.49[b]	−0.60[b]		−0.79[b]		−0.71[b]	−0.58[b]	−0.51[b]
hemicellulose:										
alkaline extractn	0.31	0.05	0.07	−0.55[b]	−0.59[b]	0.08	0.15	−0.71[b]	−0.37	−0.43
nitrobenzene oxidn	−0.49[b]	−0.40	−0.40	−0.83[b]		−0.92[b]		−0.86[b]	−0.74[b]	−0.63[b]

[a] Key to abbreviations is given in Table I. [b] $P < 0.05$.

Table V. Volatile Fatty Acid (VFA) Concentrations and Molar Proportions of Acetate (Ac) and Propionate (Pr) from Fermentation of Structural Carbohydrates with Supernatants from Fermented Forage Cell Walls (Means across Forage Species)[a]

	cellulose			hemicellulose		
treatment	VFA, μmol·mL^{-1}	Ac	Pr	VFA, μmol·mL^{-1}	Ac	Pr
control	71.5^{ab}	0.606^{ab}	0.349^{a}	110.5^{a}	0.570^{a}	0.288^{a}
50% replacement	72.7^{a}	0.596^{a}	0.353^{a}	103.7^{a}	0.542^{a}	0.331^{b}
100% replacement	62.8^{b}	0.655^{b}	0.300^{b}	96.2^{b}	0.506^{b}	0.340^{b}
SEM	3.1	0.016	0.013	2.6	0.011	0.009

[a] Means in the same column not sharing a common superscript are different ($P < 0.05$).

IVDMD when examined as solubilized components (Table IV). Ferulic and *p*-hydroxybenzoic acids in the nitrobenzene oxidation products were correlated negatively ($P < 0.05$) with cellulose IVDMD. Cinnamic, *p*-hydroxybenzoic, and vanillic acids were correlated negatively ($P < 0.05$) with hemicellulose IVDMD. All the nitrobenzene oxidation benzaldehydes were correlated negatively with both cellulose and hemicellulose IVDMD. Caution must be exercised in evaluating the importance of these correlations as the experimental design partially contributes to the observed correlations due to the use of two levels of each supernatant solution.

The volatile fatty acid (VFA) concentrations and proportions from these carbohydrate fermentations were not affected by forage species, but the overall 50 and 100% supernatant treatments were different from the controls (Table V). Total VFA concentrations were greater ($P < 0.05$) for the 50% level than the 100% level in the case of cellulose fermentation, but neither level was different ($P > 0.05$) from that of the control. For hemicellulose fermentation, VFA concentrations were lower ($P < 0.05$) for the 100% supernatant level than for the control, corresponding to the reduction in IVDMD observed (Table III). Molar proportions of acetate and propionate were altered when cellulose was fermented in the presence of the treatment supernatants (Table V). Acetate tended to be higher for the 100% level, and propionate was significantly depressed at this level. The same effects were observed for proportions of acetate and propionate when cellulose was fermented with free cinnamic acids (Jung, 1985), but when these same phenolic acids were esterified to cellulose, the VFA proportions were not affected (Jung and Sahlu, 1986). Even though both free and esterified cinnamic acids can inhibit cellulose fermentation, the modes of action appear to differ, based upon VFA response, and the supernatant solutions involved in the current study appear to be more similar to free phenolics in their effects on cellulose fermentation. Molar proportions of acetate were reduced ($P < 0.05$) and propionate increased ($P < 0.05$) when the 100% levels of supernatant solutions were included in hemicellulose fermentations, which is the opposite observed for cellulose fermentation (Table V). This suggests that the ruminal populations of fibrolytic organisms that degrade hemicellulose respond differently from those fermenting cellulose to the presence of phenolic containing supernatant solutions from forage cell wall fermentation. It has been observed previously that hemicellulose degradation in vitro was less susceptible to phenolic compound inhibition than was cellulose fermentation (Jung, 1985).

Experiment Two. The elution profiles at 280 nm of the forage cell wall fermentation supernatant solutions are shown in Figure 1. The profiles were similar at 320 nm. All three solutions gave similar profiles with a peak UV-absorbing fraction corresponding to the exclusion volume and another much larger peak eluding at the small-molecule inclusion volume. The two peaks were collected as fractions S_1 and S_4. The elution profile was different from that found by Tanner and Morrison (1983) when phenolic–carbohydrate complexes released by fungal cellulase were fractionated on a Sephadex G50 column. These workers found only a single large peak between the exclusion and inclusion volumes. The small shoulder on the upstream side of the S_4 peak was also collected (S_3). Fractions S_2 and S_5 were collected as low UV absorbance fractions (Figure 1). Although Figure 1 shows some overlap between the S_3 and S_4 fractions, this is only because these fractions had slightly different elution volumes among the forage supernatant solutions. The profile for carbohydrate content of the supernatant fractions was very similar to the elution profile as measured by UV absorbance. These five soluble fractions and the small-particle fraction (P) collected by centrifugation were used for experiment 2.

The alkaline-extractable phenolic composition of the various supernatant fractions was quite different (Table VI). Virtually all of the cinnamic acids were associated with the P fraction, and the differences mimicked those seen for the original forage cell walls (Table I). This indicates that the cinnamic acids are not released as true soluble complexes, but rather remain as part of the insoluble cell wall matrix. The other compounds, benzoic acids and aldehydes, generally were present in moderate concentration in the S_1 fraction, and the S_4 fraction contained the greatest levels. All other fractions contained very little phenolic material, suggesting that the phenolic–carbohydrate complexes released from forage cell walls during fermentation contain some high molecular weight material (P), but the majority of the phenolics are released as low molecular weight complexes with carbohydrate (S_4). Recoveries of phenolics found in the original supernatants in the six fractions were 40, 50, and 35% for alfalfa, smooth bromegrass, and corn silage, respectively. These recoveries are relatively low because the elution profiles of the supernatant solutions showed a very broad peak (Figure 1) and only a small portion of this major peak was collected in fractions S_3, S_4, and S_5. All of the nitrobenzene oxidation products were confined to the P fraction (data not shown). If there was any core lignin in the soluble fractions, it must have been alkali soluble.

Because VFA, NH_4^+, and glucose eluded in the S_4 fraction, the control buffer was subjected to the same fractionation procedure as the supernatant solutions in order that appropriate controls were available. In the case of cellulose degradation, the buffer controls were different ($P < 0.05$) among the fractions (Table VII). In order to be able to compare the various treatments and fractions, all supernatant treatments are presented as a proportion of their appropriate control. Only the alfalfa hay and corn silage derived S_4 fractions at the 100% replacement level were significantly different from the control fraction. When the six fractions were compared at the 50 and 100% levels, only for the S_4 fraction was the 100% treatment level lower ($P < 0.05$) in IVDMD than the 50% level. This effect was consistent across forages (Table VI). The other

Table VI. Concentrations of Alkali-Labile Phenolics in Forage Cell Wall Fermentation Supernatants Fractionated by Molecule Size

		compounds,[a] $\mu g \cdot L^{-1}$									
		cinnamic acids			benzoic acids				benzaldehydes		
fraction[b]	forage[c]	CA	PCA	FA	PHBA	PA	VA	SYA	PHBAL	VAN	SYAL
P	alf	3	120	14	17	208	6	4	16	18	3
	sbg	4	450	101	34	193	4	57	22	46	11
	cs	4	888	143	22	186	0	17	6	32	6
	SEM	1	144	24	4	14	2	11	4	9	3
S_1	alf	4	1	2	3	76	0	0	6	4	0
	sbg	0	23	8	9	174	4	0	4	11	2
	cs	0	16	4	8	144	6	11	0	53	5
	SEM	1	5	2	1	23	2	4	2	16	2
S_2	alf	4	1	1	4	80	2	21	2	11	9
	sbg	2	0	1	8	170	0	0	0	11	6
	cs	4	3	5	17	208	4	0	2	25	0
	SEM	1	1	2	3	25	1	5	1	5	2
S_3	alf	2	4	2	11	174	2	15	2	4	0
	sbg	2	3	5	8	148	0	8	2	25	6
	cs	2	1	0	14	174	2	0	2	7	6
	SEM	1	1	1	1	19	1	4	1	7	2
S_4	alf	4	4	1	158	761	55	19	55	49	8
	sbg	4	3	2	138	417	57	11	20	18	8
	cs	2	9	1	101	564	149	11	59	35	8
	SEM	1	1	1	18	64	21	4	13	10	1
S_5	alf	3	6	1	17	246	2	6	0	14	0
	sbg	4	4	8	23	197	10	4	0	56	6
	cs	2	16	0	18	205	6	0	2	7	5
	SEM	1	4	2	2	32	2	2	1	15	13

[a] Key to abbreviations is given in Table I. [b] Fractions are defined in Figure 1. [c] Key: alf, alfalfa hay; sbg, smooth bromegrass hay; cs, corn silage.

Table VII. In Vitro Dry Matter Disappearance (IVDMD) of Cellulose Fermented by Ruminal Microorganisms in the Presence of Phenolic Compounds Released from Forage Cell Walls Fractionated by Molecular Size

	IVDMD,[a] %											
	P[b]		S_1		S_2		S_3		S_4[c]		S_5	
treatment	50%	100%	50%	100%	%	100%	50%	100%	50%	100%	50%	100%
control	26.4[b]		27.0[de]		30.2[ef]		26.7[d]		32.1[f]		31.2[f]	
alfalfa hay	1.08	1.09	1.02	1.05	0.95	0.84	0.97	0.87	0.94	0.71[g]	0.95	0.91
smooth bromegrass hay	1.01	1.03	0.95	1.12	0.80	0.89	1.00	0.96	0.95	0.91	0.88	1.04
corn silage	1.05	1.07	1.09	0.99	1.00	0.93	1.04	1.09	0.92	0.77[g]	0.91	0.96
SEM	0.02	0.01	0.04	0.05	0.05	0.02	0.03	0.05	0.02	0.04	0.04	0.04

[a] Forage treatment IVDMD expressed as a proportion of appropriate control. [b] Fractions are defined in Figure 1. [c] Across all forages, IVDMD of the 50% concentration of fraction S_4 was greater than of the 100% concentration ($P < 0.05$). Control fractions not sharing a common superscript (d–f) differ ($P < 0.05$). Superscript g indicates a difference from the appropriate control ($P < 0.05$).

Table VIII. In Vitro Dry Matter Disappearance (IVDMD) of Hemicellulose Fermented by Ruminal Microorganisms in the Presence of Phenolic Compounds Released from Forage Cell Walls Fractionated by Molecular Size

	IVDMD,[a] %											
	P[cd]		S_1[de]		S_2[cd]		S_3[f]		S_4[c]		S_5[e]	
treatment	50%	100%	50%	100%	50%	100%	50%	100%	50%	100%	50%	100%
control	56.7		55.0		54.6		58.0		55.4		56.1	
alfalfa hay	1.06[g]	0.99	1.01	1.02	1.00	0.84	0.91	0.92	0.97	1.01	1.04	1.03
smooth bromegrass hay	0.98	0.93[g]	1.01	1.05	0.94	1.02	0.96	0.95	0.96	0.97	1.02	1.04
corn silage	1.04	0.98	0.97	1.03	1.00	1.00	0.93	0.96	1.00	1.03	1.00	1.03
SEM	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01

[a] Forage treatment IVDMD expressed as a proportion of appropriate control. [b] Fractions are defined in Figure 1. Fractions not sharing a common superscript (c–f) are different ($P < 0.05$). Superscript g indicates a difference from the appropriate control ($P < 0.05$).

fractions did not significantly depress cellulose IVDMD. The degree of cellulose fermentation inhibition seen in experiment 2 was less than in experiment 1, which is probably expected as only a portion of the total phenolics were recovered in the S_4 fraction. Although IVDMD of hemicellulose was variable among control fractions, the fractions did not differ significantly (Table VIII). The only significant difference for forage supernatant treatments from a control was the increased ($P < 0.05$) IVDMD seen when fraction P of the alfalfa supernatant solution was added at the 50% level and the decrease ($P < 0.05$) when smooth bromegrass fraction P was added at the 100% level. The forage supernatant fractions differed ($P < 0.05$), with the S_3 fraction having the lowest ($P < 0.05$) IVDMD for all forages. There was no consistent effect of treatment level on hemicellulose IVDMD (Table VIII). Inhibition of hemicellulose IVDMD was much less than that seen in experiment 1 (Table III).

Correlations of alkaline-extractable phenolic compounds and IVDMD of carbohydrates are shown in Table IX. Without regard to fraction, the overall relationships between cellulose IVDMD and phenolics were significant for *p*-hydroxybenzoic, protocatechuic, and vanillic acids and *p*-hydroxybenzaldehyde. All of these compounds were

Table IX. Correlation Coefficients for Phenolic Compounds in Fermentation Supernatants with in Vitro Fermentation of Structural Carbohydrates in Experiment 2

		compounds[a]									
		cinnamic acids			benzoic acids				benzaldehydes		
substrate	fraction[b]	CA	PCA	FA	PHBA	PA	VA	SYA	PHBAL	VAN	SYAL
cellulose	P	0.01	−0.10	−0.29	−0.38	0.36	0.26	−0.72	−0.16	−0.46	−0.58
	S_1	0.04	0.22	0.32	0.23	0.28	0.05	−0.15	0.26	−0.10	−0.03
	S_2	0.08	0.46	0.45	0.36	0.11	0.42	−0.24	0.24	0.32	−0.55
	S_3	−0.22	−0.92[c]	−0.52	0.15	−0.19	0.00	−0.97[c]	−0.22	−0.05	0.61
	S_4	−0.45	−0.63	−0.07	−0.68	−0.94[c]	−0.54	−0.84[c]	−0.92[c]	−0.94[c]	−0.76
	S_5	0.63	0.10	0.57	0.70	0.40	0.69	0.16	0.02	0.61	0.60
	overall	−0.16	0.33	0.33	−0.50[c]	−0.54[c]	−0.50[c]	−0.09	−0.46[c]	−0.10	−0.24
hemicellulose	P	−0.81	−0.37	−0.53	−0.95[c]	−0.66	−0.27	−0.84[c]	−0.75	−0.92[c]	−0.91[c]
	S_1	0.04	0.54	0.70	0.62	0.71	0.27	−0.13	0.46	−0.01	0.11
	S_2	−0.39	0.10	0.30	0.40	0.40	−0.04	−0.78	−0.40	0.20	−0.63
	S_3	0.26	−0.32	0.24	0.17	0.14	−0.31	−0.50	0.26	0.60	0.84[c]
	S_4	−0.06	0.91[c]	−0.20	0.23	0.67	0.85[c]	0.44	0.93[c]	0.72	0.53
	S_5	0.61	0.16	0.37	0.51	0.51	0.24	0.57	−0.32	0.42	0.05
	overall	−0.13	−0.02	−0.05	0.04	0.15	0.14	−0.38[c]	0.08	0.17	−0.27

[a] Key to abbreviations is given in Table I. [b] Fractions are defined in Figure 1. [c] $P < 0.05$.

Table X. Molar Proportions of Volatile Fatty Acids (VFA) after Fermentation of Structural Carbohydrates with Various Phenolic Compound Fractions

			fraction[a]						
VFA	substrate	control	P	S_1	S_2	S_3	S_4	S_5	SEM
acetate	cellulose	0.547[b]	0.542[b]	0.530[c]	0.518[c]	0.545[b]	0.521[c]	0.540[b]	0.004
	hemicellulose	0.559[bc]	0.566[c]	0.562[cd]	0.539[e]	0.562[cd]	0.529[f]	0.555[bd]	0.003
propionate	cellulose	0.376[b]	0.397[c]	0.405[c]	0.432[d]	0.378[b]	0.407[c]	0.390[bc]	0.007
	hemicellulose	0.314[bc]	0.310[c]	0.318[b]	0.340[d]	0.316[bc]	0.326[e]	0.325[e]	0.002

[a] Fractions are defined in Figure 1. Means in the same row not sharing a common superscript (b–f) are different ($P < 0.05$).

negatively correlated with cellulose IVDMD, and each accounted for approximately 25% of the variation in IVDMD. For hemicellulose IVDMD, only overall syringic acid concentrations were negatively correlated ($P < 0.05$) with IVDMD, and this compound explained only 14% of the variation. The overall results for cellulose IVDMD in experiment 2 are similar to those of experiment 1 where *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde were negatively correlated with IVDMD (Table IV). However, hemicellulose IVDMD was negatively related with a different set of compounds in experiment 1 than experiment 2. For the individual supernatant fractions, S_4 was the fraction resulting in several negative correlations ($P < 0.05$) of individual phenolics with cellulose IVDMD (Table IX). Protocatechuic and syringic acids, *p*-hydroxybenzaldehyde, and vanillin each accounted for about 80% of the variation in IVDMD for fraction S_4. Syringic acid and PCA in fraction S_3 were also negatively correlated with cellulose IVDMD. Hemicellulose IVDMD was negatively correlated ($P < 0.05$) with several phenolics in fraction P even though IVDMD was not significantly lower in this fraction than the others. Fraction S_3, where a negative correlation was expected, had a positive correlation ($P < 0.05$) for IVDMD with syringaldehyde. The S_4 fraction also showed several positive correlations ($P < 0.05$) of hemicellulose IVDMD with phenolics.

Although not shown, concentrations of VFA after carbohydrate fermentation were not different ($P > 0.05$) among supernatant fractions. Molar proportions of VFA also were not affected ($P > 0.05$) by forage source of supernatants or treatment level, but fractions were significantly different (Table X). Fraction S_4 depressed ($P < 0.05$) the proportion of acetate and increased ($P < 0.05$) the proportion of propionate for cellulose fermentation. This was opposite to what was seen in experiment 1 and for free cinnamic acids (Jung, 1985). For hemicellulose fermentation, fraction S_3 did not influence molar proportions of acetate or propionate. However, fraction S_4 decreased ($P < 0.05$) acetate and increased ($P < 0.05$) propionate as in experiment 1, but without the associated depression in IVDMD. Other fractions for both cellulose and hemicellulose fermentation altered VFA proportions relative to the control, but these were not related to observed IVDMD changes.

The data presented are in agreement with the hypothesis that phenolic compounds released from forage cell walls during fermentation as carbohydrate complexes are capable of inhibiting further microbial degradation of structural carbohydrates. The most important inhibiting alkaline-extractable phenolic esters in solution appear to be the benzoic acids and aldehydes, rather than the cinnamic acids normally associated with reduced cell wall digestion (Hartley, 1972; Burritt et al., 1984). In vitro experiments with free benzoic acids have shown no inhibitory potential for this group of compounds, although the benzaldehydes have been found to be effective inhibitors of both cellulose and hemicellulose fermentation (Jung, 1985; Borneman et al., 1986). Possibly the carbohydrate ester complexes of these benzoic acids confer solubility and conformational properties that enhance inhibitory properties. Phenolic–carbohydrate complexes generated by cellulose hydrolysis have been shown to contain all of the major cell wall sugars: glucose, xylose, arabinose, galactose, mannose, rhamnose (Hartley, 1973; Tanner and Morrison, 1983). The core lignin nitrobenzene oxidation products do not appear to be involved in this inhibition. On the basis of changes in fermentation end products, the effects of soluble phenolic–carbohydrate complexes appear similar to those of free phenolics. However, the loss of much of the inhibitory potential and contradictory VFA data after molecular sieve fractionation indicate that a more refined separation will be needed to demonstrate whether solubilized cell wall phenolic–carbohydrate complexes are responsible for reduced ruminal fermentation of fiber. To this end, preparative reversed-phase high-pressure liquid chromatography is being used to fractionate individual

phenolic carbohydrate complexes from ruminal fluid. These more clearly defined fractions will then be used to address the hypothesis.

Registry No. CA, 621-82-9; PCA, 7400-08-0; FA, 1135-24-6; PHBA, 99-96-7; PA, 99-50-3; VA, 121-34-6; SYA, 530-57-4; PHBAL, 123-08-0; VAN, 121-33-5; SYAL, 134-96-3; cellulose, 9004-34-6; hemicellulose, 9034-32-6.

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Intestinal Absorption, Metabolism, and Nutritional Effects of Dietary Disteryl Ethers in Mice

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Intestinal absorption and metabolism of both di([4-^{14}C]cholesteryl) ether and di([4-^{14}C]sitosteryl) ether were studied after intragastric administration to mice. Neither of the two substrates were absorbed by the cells of the intestinal mucosa. Radioactive metabolites of the two disteryl ethers were not detected in significant quantities in the various tissues of the gastrointestinal tract and the contents of small intestine. The digesta of cecum and colon as well as feces, however, were found to contain large proportions of labeled metabolites of both substrates. Ingestion (approximately 400 mg/kg of body weight per day) of dicholesteryl ether and disitosteryl ether over a period of 4 weeks did not alter significantly either feed intake or body weight and organ weights of mice. No ill effects were observed in the animals that had received diets containing the disteryl ethers.

Refining of fats and oils consists of various technical processes, such as deacidification, deodorization, and bleaching. Disteryl ethers, e.g. dicholesteryl ether (I) and disitosteryl ether (II), that are found in small proportions in commercial fats and oils, are predominantly generated by dehydration of sterols of plant and animal origin during the bleaching process (Kaufmann et al., 1970; Homberg, 1975; Smith, 1981; Kochhar, 1983).

The aim of the present study was to investigate intestinal absorption, metabolism, and nutritional effects of these artifactual sterol derivatives that are ubiquitous constituents of commercial food and feedstuffs containing

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