APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1987, p. 1046–1050
0099-2240/87/051046-05$02.00/0

Vol. 53, No. 5

Effect of Phenolic Acids and Phenolics from Plant Cell Walls on Rumenlike Fermentation in Consecutive Batch Culture

M. K. THEODOROU,[1]* D. J. GASCOYNE,[1] D. E. AKIN,[2] AND R. D. HARTLEY[1]

The Animal and Grassland Research Institute, Hurley, Maidenhead, Berkshire SL6 5LR, United Kingdom,[1] and Richard B. Russell Agricultural Research Center, U.S. Department of Agriculture Agricultural Research Station, Athens, Georgia 30613[2]

Received 20 October 1986/Accepted 4 February 1987

Information on the interaction between mixed populations in the rumen and plant phenolics is required to fully elucidate the limitations of phenolic compounds on forage digestibility. The objective of this study was to examine the degradation of Italian ryegrass (*Lolium multiflorum* L.) hay incubated with mixed ruminal populations in consecutive batch culture (CBC) with or without phenolic acids or phenolic compounds extracted from plant cell walls. Each CBC consisted of a series of 10 cultures (3 replicates per culture) inoculated (10%, vol/vol) in sequence at 48-h intervals with microbial suspension from the previous set of cultures. All cultures were grown on a semidefined medium containing Italian ryegrass hay, and each CBC was initiated with an inoculum from the rumen. Rumenlike fermentation characteristics were maintained in control CBCs by repeated inoculum transfer. Treatment CBCs were transferred as described above, but cultures 5, 6, and 7 were incubated in the presence of *trans-p*-coumaric, *cis-p*-coumaric, or *trans*-ferulic acid or phenolics extracted from the cell walls of maize stem or barley straw. Mean apparent dry matter disappearance in control CBC cultures was 495 mg per g of hay, whereas the presence of phenolics reduced the initial dry matter disappearance by 6.3 to 25.6%. *trans-p*-Coumaric acid and, to a lesser extent, the phenolics from cell walls of maize stem were the most inhibitory compounds for dry matter disappearance and for the production of volatile fatty acids; *trans-p*-coumaric acid altered the molar ratio of acetate/propionate/butyrate. The CBC further showed variations in the ability of the rumen microbial population to adapt to phenolic compounds.

Cell walls of mature graminaceous plants such as maize and barley can contain up to 3% (wt/wt) of monomeric phenolic acids (15). Major acids are *trans-p*-coumaric acid (t-PCA) and *trans*-ferulic acid (t-FA), together with lesser amounts of *cis* isomers, *p*-hydroxybenzoic acid, *trans,trans*-diferulic acid, and a number of as yet unidentified compounds (18). It has been shown that some and possibly all of the major phenolic acids are ester linked to arabinoxylan constituents of the cell wall (26); these can be released by treatment with aqueous alkali (7, 15). The association of aromatic compounds, including lignin and phenolic acids, with plant cell walls may limit the digestibility of wall polysaccharides in the rumen (14, 16). With certain forages, there is a negative correlation between the aromatic constituents of cell walls and apparent digestibility (4, 14, 19). The nonwettable nature of lignified cell walls may confer resistance to microbial attack by inhibiting the attachment process (27) or by limiting access to microbial enzymes (29). Unlike the mechanisms associated with lignification, however, the mode of action of low-molecular-weight phenolics is generally related to their ability to combine with and disrupt cell membrane systems or their ability to inactivate essential enzymes or a combination of the two (10).

Bacteria, protozoa, fungi, and viruses can all be inhibited by phenolics of the type found in the Graminae (1, 2, 13). Phenolic acids have been shown to exert toxic effects on pure cultures of rumen bacteria representing a wide range of genera (8). Phenolic acids also have a negative effect on the extent of cellulose digestion by mixed populations of rumen microorganisms in vitro (1, 23). Phenolic acids and soluble lignin-fragments are consumed when alkali-treated straws are included in the feed (11). Since cellulolytic bacteria are closely associated with cell wall polysaccharides in the rumen, they may encounter high local concentrations of potentially toxic phenolic acid substituents as degradation proceeds.

The work presented in this publication demonstrates the effects of monomeric phenolic acids and phenolics extracted from the cell walls of maize stem and barley straw on the fermentation of high-digestibility hay by mixed populations of rumen microorganisms undergoing rumenlike fermentations in vitro.

MATERIALS AND METHODS

Phenolics. t-PCA, *cis-p*-coumaric acid (c-PCA), and t-FA were obtained from Koch Light (Colnbrook, Bucks, United Kingdom). Phenolics were isolated from the cell walls of maize stem and barley straw and quantified (Table 1) by the method of Hartley and Buchan (17). Stock solutions (1.5%, wt/vol) of phenolics were prepared in 0.001 M NaOH immediately before use; t-PCA was used as a reference compound to adjust the concentration of plant phenolics.

Microbial inoculum. Fistulated sheep were fed twice daily on a diet of Italian ryegrass hay (1,100 g [fresh weight] day^{-1}). Rumen contents, removed by aspiration from four animals, were combined in equal amounts and strained through four layers of muslin, and the rumen fluid was collected in a CO_2-filled flask. The residual digesta solids were comminuted under CO_2 for 60 s in a Kenwood electronic blender after the addition of anaerobic buffer (24) equal in volume to the rumen fluid removed. Fluid from the comminuted digesta was strained through muslin as above and combined in equal volumes with the rumen fluid. This procedure ensured that the resultant microbial suspensions contained free-floating as well as fiber-associated rumen

* Corresponding author.

TABLE 1. Phenolic acid content of cell walls (dry matter basis)

Phenolic acid	Phenolic acid content (mg g^{-1})	
	From maize stem cell walls	From barley straw cell walls
t-PCA	36.42	6.36
c-PCA	1.79	0.43
t-FA	6.59	3.32
cis-Ferulic	0.59	0.06
p-Hydroxybenzoic	0.15	0.10

microorganisms. Samples of 1 ml of microbial suspension were used to inoculate tubes of culture medium.

Media and culture conditions. Media were prepared and inoculated by aseptic and anaerobic techniques (3, 21, 25). To ensure representative sampling of particulate suspensions, pipettes were modified by removing the tip and recalibrating the stem. Cultures were grown on medium B, a semidefined, rumen fluid-free medium (24), which contained Italian ryegrass hay ground to pass through a 1-mm dry mesh screen. Hay (0.1 g [fresh weight] ± 5%) was weighed into anaerobic culture tubes (18 by 142 mm; Bellco Glass, Vineland, N.J.) and autoclaved at 121°C for 15 min, and medium was added aseptically. For fermentations in the absence of phenolics, culture tubes contained 9 ml of medium. For fermentations in the presence of phenolics (1 ml of phenolic acid stock solution), or control solutions (1 ml), tubes contained 8 ml of medium in which the concentration of all components (except hay) had been increased by 10%. Culture medium was dispensed 24 h before inoculation, and tubes were sealed with no. 1 butyl rubber stoppers (Bellco Glass). Inoculated tubes were incubated at 39°C without agitation.

CBC. A brief account of consecutive batch culture (CBC), a technique developed during research by Gascoyne (12; D. J. Gascoyne, Ph.D. thesis, University of London, 1986) and Theodorou et al. (28) has been reported previously. A more complete description is given here. Each CBC was initiated by inoculating three replicate culture tubes of prewarmed (39°C) semidefined medium B (9 ml) plus hay (0.1 g) with 1 ml of microbial inoculum prepared from rumen contents. After 48 h of incubation, 10% (vol/vol) of each culture was combined, and 1-ml samples were used to inoculate three tubes of fresh medium. Up to 10 consecutive incubations were conducted in this way, by sequential transfer of combined inoculum from the previous set of cultures, over a period of 20 days. During incubations 1 to 4 (preperturbed period) and 8 to 10 (postperturbed period), tubes containing 9 ml of medium were inoculated. During incubations 5, 6, and 7 (perturbed period), cultures were inoculated as above, but tubes contained 1 ml of phenolics stock solution (added 2 h after inoculation) and 8 ml of medium (in which the concentration of all components except hay had been increased by 10%). Two controls were required. In the CBC control for cultures treated with t-PCA, c-PCA, or t-FA, water replaced the phenolic acids during the perturbed period. In the CBC control for cultures treated with phenolics from maize stem or barley straw, an aqueous extract obtained from the phenolics extraction procedure (17) in the absence of cell walls replaced phenolics during the perturbed period.

Chemical analysis. Spent medium (9 ml) from each consecutive culture was centrifuged at 26,000 × *g* for 15 min at 4°C. The supernatant was removed and acidified for volatile fatty acid (VFA) analysis as described previously (24), and the pellet was suspended in an equal volume of water. After centrifugation and washing twice more, the pellet was suspended in a small volume of water and transferred to a predried and weighed aluminium dish. Samples were dried to constant weight at 104°C and weighed in the presence of silica gel. In calculations involving apparent dry matter disappearance (DMD) of hay (milligrams per gram), all fresh weight values were converted to dry weights by determination of the hay dry matter content.

Statistical analysis. Data from cultures 2 to 10 were subjected to analysis of variance, and the F test was used to determine least significant difference. Each group of three cultures, from which the combined inoculum for the next three cultures was obtained, was regarded as an experimental unit. Thus, there were no true replicates, and treatment effects were determined by using data from preperturbed cultures to estimate the standard error of the difference for comparisons between means of preperturbed, perturbed, and postperturbed cultures. Comparisons were also made after subtraction of values for treatment cultures from corresponding control cultures. In these cases the standard error of the difference was increased by a factor of 2 (i.e., for comparisons involving any two means).

RESULTS

Since data from each control CBC were not significantly different, values for the control incubations refer to cultures in which water replaced phenolics during the perturbed period. The mean apparent DMD of hay in control incubations was 495 (±0.008) mg g^{-1}. When the apparent DMDs in consecutive cultures of the control were averaged according to incubation period, however, values for the perturbed and postperturbed cultures were significantly different from those obtained in preperturbed cultures (Table 2). Apart from the CBC series in which cultures were perturbed with t-FA, the apparent DMD values from preperturbed and postperturbed periods in cultures treated with phenolics were significantly different from each other, but not significantly different from those obtained in corresponding periods in control incubations (Table 2). All cultures treated with phenolics during the perturbed period showed a significant reduction in apparent DMD in comparison with controls (Table 2). Cultures incubated in the presence of t-PCA demonstrated a mean reduction in apparent DMD of 130 mg g^{-1} relative to the corresponding control cultures. Similar reductions of 82, 50, 47, and 32 mg g^{-1} were observed in cultures perturbed with phenolics from maize cell walls,

TABLE 2. Apparent DMD in CBC during preperturbed, perturbed, and postperturbed periods

Treatment[a]	$\bar{x}$ apparent DMD (mg g^{-1})[b] in cultures		
	Preperturbed	Perturbed	Postperturbed
t-PCA	468cd	377a	493efg
c-PCA	471cd	460cd	506g
t-FA	477cde	457c	479de
Maize cell wall phenolics	480def	425b	509g
Barley cell wall phenolics	479de	475cde	500fg
Water (control)	475cde	507g	502g

[a] Phenolic compounds or water (control) were added to cultures during perturbed incubations (cultures 5, 6, and 7).

[b] Data followed by the same letter in the rows and columns were not significantly different ($P < 0.05$); standard error of the mean was 6.96.

t-FA, c-PCA, and phenolics from barley cell walls, respectively.

Since the same treatment was applied during three consecutive incubations, observations on microbial adaptation could be made. All cultures exhibited a decrease in apparent DMD upon initial exposure to phenolic compounds (Fig. 1). Analysis of variance of the difference in apparent DMD from individual consecutive cultures treated with phenolics relative to the corresponding control cultures showed that significant differences ($P < 0.05$) occurred between the last preperturbed culture (no. 4) and the first perturbed culture (no. 5) in the presence of all phenolics (Fig. 1). During subsequent incubations, however, significant differences were also observed between cultures 6 and 7 in the presence of t-PCA and between the last perturbed culture (no. 7) and the first postperturbed culture (no. 8) in the presence of t-PCA, c-PCA, or phenolics from maize cell walls (Fig. 1). Although t-PCA caused a significant reduction in apparent DMD in all perturbed cultures, the apparent DMD increased from 63.4% of the control value in culture no. 5 to 85.1% in culture no. 7. In cultures treated with phenolics from barley cell walls, apparent DMD values also increased in consecutive incubations. However, the magnitude of the initial reduction in apparent DMD (in culture no. 5) and the subsequent increases (in cultures 6 and 7) were small in comparison to those from cultures treated with t-PCA (Fig. 1). In cultures perturbed with c-PCA or t-FA, apparent DMD values decreased in consecutive incubations relative to the corresponding control cultures (Fig. 1). During consecutive incubations in the presence of phenolics from maize stem,

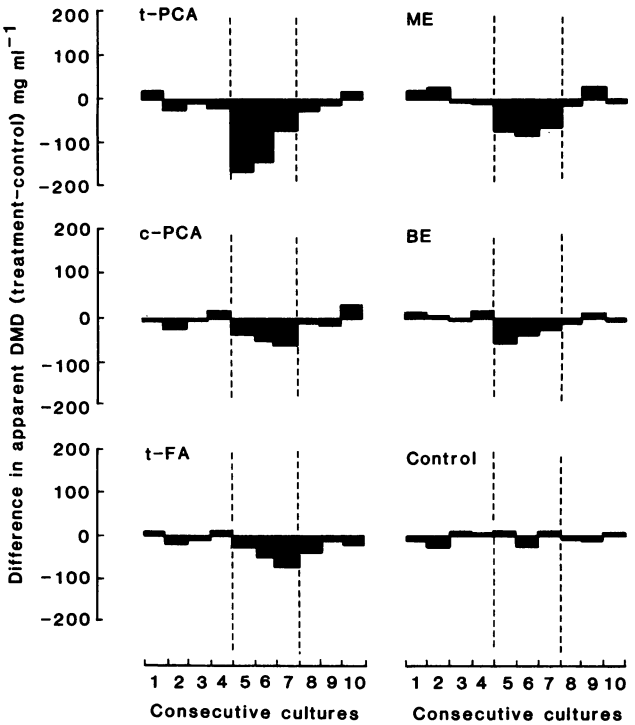

FIG. 1. Difference in apparent DMD between corresponding treatment and control (water) consecutive batch cultures. Microbial suspensions were incubated in the presence of phenolics in perturbed cultures 5, 6, and 7 (these are represented by the areas within the dotted lines). Abbreviations: ME, phenolics extracted from maize cell walls; BE, phenolics extracted from barley cell walls; control, treatment CBC represented by the aqueous extract control.

TABLE 3. VFA (acetate-propionate-butyrate) concentrations in CBC during preperturbed, perturbed, and postperturbed periods[a]

Treatment	$\bar{x}$ Total VFA (mM) in cultures		
	Preperturbed	Perturbed	Postperturbed
t-PCA	39.51b	32.14a	42.43bcdef
c-PCA	41.66bcdef	42.95defg	46.47ij
t-FA	40.99bcde	43.37efgh	46.25hij
Maize cell wall phenolics	41.92bcdef	39.93bc	47.11j
Barley cell wall phenolics	40.30bcd	44.41fghij	43.50efghi
Water (control)	40.33bcd	42.93cdefg	45.67ghij

[a] See footnotes *a* and *b* of Table 2; standard error of the mean was 1.05.

however, apparent DMD values remained relatively unchanged (Fig. 1). A comparison of the differences in apparent DMD in the two CBC controls during consecutive incubations is also presented in Fig. 1.

More dry matter (0.4 to 6.9%) was lost in postperturbed cultures as compared with preperturbed cultures (Table 2). A similar trend was also observed with respect to VFA concentrations; 8 to 13% more VFA (acetate-propionate-butyrate) accumulated during the postperturbed period as compared with the preperturbed period (Table 3). However, apart from incubations in the presence of t-PCA and, to a lesser extent, the phenolics from maize stem, the acetate, propionate, and butyrate concentrations in cultures perturbed with all other phenolics were similar to the values for corresponding controls (Table 3). During incubations in the presence of t-PCA, cultures accumulated 25% less VFA than corresponding control cultures (Table 3). The VFA which accumulated during consecutive incubations in control cultures were associated with an acetate/propionate/butyrate molar ratio of 0.63:0.28:0.09; these values are not unlike those normally encountered in the rumen. Apart from incubations in the presence of t-PCA, molar ratios from all other incubations were similar to the control values. However, in perturbed and postperturbed cultures acetate and butyrate proportions were slightly higher and propionate was slightly lower (0.64:0:24:0.12). The molar ratios in cultures perturbed with t-PCA showed a significant decrease in propionate and a significant increase in butyrate, whereas the value for acetate remained unaltered (0.66:0.13:0.21) ($P < 0.05$).

DISCUSSION

Previous studies with CBC (12, 28; Gascoyne, thesis) and the results presented here demonstrate that relatively straightforward culture techniques can be used to maintain mixed rumen populations in vitro which demonstrate rumenlike fermentation characteristics. Although certain microbial subpopulations such as the protozoa do not survive, others such as the fibrolytic and saccharolytic bacteria and methanogenic microorganisms have been detected (Gascoyne, thesis). Experiments involving phenolics and CBC cultures (Akin et al., submitted for publication) also reveal that microbial suspensions from CBC cultures adhere to and disrupt leaf blade tissues in much the same way as microbial suspensions obtained directly from the rumen (1). The main fiber-digesting organisms in these cultures have been tentatively identified by electron microscopy and are the same as those normally encountered in the rumen, viz., *Bacteroides* spp. and *Ruminococcus* spp. Thus, it appears that species diversity can be maintained in CBC cultures

because of the habitat-simulating growth environment which incorporates heterogeneous, particulate substrates of the type normally encountered in the rumen.

The inhibitory nature of phenolic acids has been reported in several studies (1, 8, 23). Comparative research has shown that t-PCA is more toxic to rumen microorganisms than other phenolic acids (2), and the presence of t-PCA in plant cell walls has also been correlated with the poor nutritive value of certain forages (5, 6). In the present work, t-PCA was more effective than other phenolics in causing inhibition of apparent DMD and reduced VFA accumulation. This confirms earlier studies (1) and further indicates the ability of free phenolic acids to reduce in vitro digestibility. Phenolics extracted from cell walls of maize stem, although less effective than t-PCA (particularly in reducing VFA concentrations), also caused significant reductions in apparent DMD. In comparison with barley straw, maize stems released greater concentrations of PCA and had a higher PCA/FA ratio when treated with NaOH (19); this was confirmed in the present study (Table 1). Since phenolics from maize contained significantly higher amounts of t-PCA than corresponding tissues from barley (Table 1), this could account for the greater toxicity of maize extracts. The *cis* isomer of PCA, however, which is a relatively minor constituent of maize stem and barley straw (Table 1), was less toxic in perturbed cultures than t-PCA.

Reduced apparent DMD during consecutive incubations in the presence of phenolics could be correlated to reduced VFA production for only two of the five treatments (Tables 2 and 3). In comparison to control cultures, t-PCA and phenolics from maize stem cell walls reduced VFA concentrations by 25 and 7%, respectively (Table 3). t-PCA also altered the VFA molar ratio by causing an increase in butyrate and a reduction in propionate. It is uncertain why reduced solubilization of particulate substrates in the presence of phenolics was not associated with corresponding reductions in VFA concentrations in other treatments.

The breakdown and modification of phenolic acids and other aromatic compounds under strictly anaerobic conditions has been demonstrated (9, 20). Although some of these reactions occur very slowly and are unlikely to be of significance in the rumen, microbial consortia are usually necessary to bring about these changes, particularly with higher-molecular-weight aromatics. Certain phenolic acids, however, do invoke detoxification systems in some pure cultures of rumen bacteria (8, 23). Since the same treatment was applied during three consecutive incubations in perturbed cultures, it was possible to make observations on microbial adaptation. The decrease in apparent DMD relative to control cultures, upon initial exposure of microbial suspensions to t-PCA, was 37.6% (Fig. 1). However, the apparent DMD increased in subsequent cultures as microbial populations adapted to the presence of t-PCA (Fig. 1). Results similar to these were also recorded for apparent DMD in the presence of phenolics from the cell walls of barley straw (Fig. 1) and for VFA production in the presence of t-PCA (data not presented). Cultures treated with c-PCA or t-FA demonstrated the opposite effect, and phenolic acid toxicity increased in consecutive incubations (Fig. 1). In the presence of phenolics from maize stem, however, apparent DMD values remained unaltered during consecutive incubations (Fig. 1). Results with t-PCA and, to a lesser extent, the barley phenolics suggest that mixed populations of rumen bacteria can adapt, at least in part, to the presence of these compounds. Some microorganisms do have the ability to modify the chemical structure of t-PCA in vitro, making it less toxic (8). If these microorganisms proliferate in successive cultures, the efficiency of the toxin will become reduced. Chemical transformations which result in reduced toxicity have also been demonstrated in the rumen for the conversion of 3-hydroxy-4 (1*H*)-pyridone (22). The apparent increases in toxicity which were observed in consecutive incubations in the presence of c-PCA or t-FA could arise where microbial populations were less able to adapt to the presence of the toxin. Thus, the toxicity of c-PCA and t-FA increased in consecutive incubations as microbial populations were progressively inhibited.

ACKNOWLEDGMENTS

We acknowledge the technical assistance of S. Hitching and A. S. Keene and the statistical advice of J. M. Cobby and M. S. Dhanoa.

The Animal and Grassland Research Institute is financed through the Agricultural and Food Research Council. D.J.G. was the recipient of a Farmos Ltd. (Finland) postgraduate grant during this research.

LITERATURE CITED

1. **Akin, D. E.** 1982. Forage cell wall degradation and p-coumaric, ferulic and sinapic acids. Agron. J. **74**:424–428.
2. **Akin, D. E., and L. L. Rigsby.** 1985. Influence of phenolic acids on rumen fungi. Agron. J. **77**:180–182.
3. **Bryant, M. P.** 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. **25**:1324–1328.
4. **Burns, J. C., and W. A. Cope.** 1974. Nutritive value of crownvetch forage as influenced by structural constituents and phenolic and tannin compounds. Agron. J. **66**:195–200.
5. **Burritt, E. A., A. S. Nittner, J. C. Street, and M. J. Anderson.** 1984. Correlations of phenolic acids and xylose content of cell wall with in vitro dry matter digestibility of three maturing grasses. J. Dairy Sci. **67**:1209–1213.
6. **Chaves, C. M., J. E. Moore, H. A. Moye, and W. R. Ocumpaugh.** 1982. Separation, identification and quantification of lignin saponification products extracted from digitgrass and their relation to forage quality. J. Anim. Sci. **54**:196–203.
7. **Chesson, A.** 1981. Effect of sodium hydroxide on cereal straw in relation to enhanced degradation of structural polysaccharides by rumen microorganisms. J. Sci. Food Agric. **32**:745–758.
8. **Chesson, A., C. S. Stewart, and R. J. Wallace.** 1982. Influence of plant phenolic acids on growth and cellulolytic activity of rumen bacteria. Appl. Environ. Microbiol. **44**:597–603.
9. **Colberg, P. J., and L. Y. Young.** 1985. Anaerobic degradation of soluble fractions of [^{14}C-lignin]lignocellulose. Appl. Environ. Microbiol. **49**:345–349.
10. **Davidson, P. M., and A. L. Branden.** 1981. Antimicrobial activity of non-halogenated phenolic compounds. J. Food Prot. **44**:623–632.
11. **Gaillard, B. D. E., and G. N. Richards.** 1975. Presence of soluble lignin-carbohydrate complexes in the bovine rumen. Carbohydr. Res. **42**:135–145.
12. **Gascoyne, D. J.** 1986. Consecutive batch culture—an *in vitro* technique for studying potential rumen manipulants. J. Sci. Food Agric. **37**:262–263.
13. **Grodzinska-Zachwieja, Z., Z. Zgorniak-Nowosielska, M. Marciszewska, and A. Gatkiewiez.** 1976. Antiviral activity of caffeic acid *in vitro* studies. Acta Biol. Crac. Ser. Bot. **19**:29–37.
14. **Hartley, R. D.** 1972. p-Coumaric and ferulic acid components of cell walls of ryegrass and their relationships with lignin and digestibility. J. Sci. Food Agric. **23**:1347–1354.
15. **Hartley, R. D.** 1981. Chemical constitution, properties and processing of lignocellulose wastes in relation to nutritional quality for animals. Agric. Environ. **6**:91–113.
16. **Hartley, R. D.** 1985. Chemistry of lignocellulosic plant materials and non-microbial processes for increasing their food value for the ruminant, p. 10–30. *In* Improved utilisation of lignocellulosic materials in animal feed. Organization for Economic Co-operation and Development, Paris.

17. **Hartley, R. D., and H. Buchan.** 1979. High performance liquid chromatography of phenolic acids and aldehydes derived from plants from the decomposition of organic matter in soil. J. Chromatogr. **180**:139–143.
18. **Hartley, R. D., and E. C. Jones.** 1976. Diferulic acid as a component of cell walls of *Lolium multiflorum*. Phytochemistry **15**:1157–1160.
19. **Hartley, R. D., and E. C. Jones.** 1978. Phenolic components and degradability of the cell walls of the brown midrib mutant, bmz, of Zea mays. J. Sci. Food Agric. **29**:777–789.
20. **Healy, J. B., Jr., and L. Y. Young.** 1979. Anaerobic biodegradation of eleven aromatic compounds to methane. Appl. Environ. Microbiol. **38**:84–89.
21. **Hungate, R. E.** 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. **14**:1–49.
22. **Jones, R. J., and J. B. Lowry.** 1984. Australian goats detoxicify the goitrogen 3-hydroxy-4(1H)-pyridone (DHP) after rumen infusion from an Indonesian goat. Experientia **40**:1435–1436.
23. **Jung, H. G., and G. C. Fahey, Jr.** 1983. Interactions among phenolic monomers and *in vitro* fermentation. J. Dairy Sci. **66**:1255–1263.
24. **Lowe, S. E., M. K. Theodorou, A. P. J. Trinci, and R. B. Hespell.** 1985. Growth of anaerobic rumen fungi on defined and semi-defined media lacking rumen fluid. J. Gen. Microbiol. **131**:2225–2229.
25. **Miller, T. L., and M. J. Wolin.** 1974. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl. Microbiol. **27**:985–987.
26. **Mueller-Harvey, I., R. D. Hartley, P. J. Harris, and E. H. Curzon.** 1986. Linkage of p-coumaroyl and feruloyl groups to cell wall polysaccharides of barley straw. Carbohydr. Res. **148**:71–85.
27. **Richards, G. N.** 1976. Search for factors other than "lignin-shielding" in protection of cell wall polysaccharides from digestion in the rumen, p. 129–135. *In* Carbohydrate research in plants and animals. Miscellaneous Paper no. 12. Landbouwhogesch, Wageningen, The Netherlands.
28. **Theodorou, M. K., D. J. Gascoyne, and D. E. Beever.** 1984. The role of consecutive batch culture in rumen microbiology. Can. J. Anim. Sci. **64**(Suppl.):47–48.
29. **Van Soest, P. J.** 1975. Physio-chemical aspects of fibre digestion, p. 351–365. *In* I. W. McDonald and A. C. I. Warner (ed.), Digestion and metabolism in the ruminant. University of New England Publishing Unit, Armidale, Australia.