Effect of Phenolic Monomers on Ruminal Bacteria

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Ruminal bacteria were subjected to a series of phenolic compounds in various concentrations to acquire fundamental information on the influence on growth and the potential limits to forage utilization by phenolic monomers. *Ruminococcus albus* 7, *Ruminococcus flavefaciens* FD-1, *Butyrivibrio fibrisolvens* 49, and *Lachnospira multiparus* D-32 were tested against 1, 5, and 10 mM concentrations of sinapic acid, syringaldehyde, syringic acid, ferulic acid, vanillin, vanillic acid, p-coumaric acid, p-hydroxybenzaldehyde, p-hydroxybenzoic acid, and hydrocinnamic acid. Responses were variable and dependent on the phenolic compound and microbial species. Compounds especially toxic (i.e., resulting in poor growth, effect on several species, dose-related response) were p-coumaric acid and p-hydroxybenzaldehyde, and adaptation to the toxins did not occur after three 24-h periods. Syringic, p-hydroxybenzoic, and hydrocinnamic acids stimulated growth of all four species and also stimulated filter paper degradation by *R. flavefaciens*. None of the stimulatory compounds supported microbial growth in the absence of carbohydrates. In vitro dry matter digestibility of cellulose (Solka-Floc) was not stimulated by any of the phenolic compounds (10 mM), but the cinnamic acids and benzoic aldehydes (10 mM) reduced (*P* < 0.05) digestion by the mixed population in ruminal fluid. Growth of *R. flavefaciens* in the presence of p-hydroxybenzoic acid (10 mM) or p-coumaric acid (5 mM) resulted in recognizable alterations in cell ultrastructure. Both phenolics caused a reduction in cell size (*P* < 0.05), and p-coumaric acid caused a reduction in capsular size (*P* < 0.05) and produced occasional pleomorphic cells.

Lignin is a complex, three-dimensional aromatic polymer consisting of basic phenylpropane units believed to arise from the precursor (p-coumaryl, coniferyl [ferulic], and sinapyl) alcohols (31). There is evidence that in addition to lignin there are low-molecular-weight phenolic compounds prevalent in the cell wall structure. Phenolic acid complexes consisting of ferulic (4-hydroxy-3-methoxycinnamic) acid (FA) and p-coumaric (4-hydroxycinnamic) acid (PCA) covalently bound to xylans and gluans have been isolated from forage cell walls (14, 27, 32). Phenolic acids have been reported to be released from cell walls by C1 cellulases (15) and by alkali treatment that is used to upgrade cereal straws (30).

It has been suggested (16) that, upon anaerobic degradation of these lignin building blocks or other phenolic constituents of the plant cell wall, the cinnamic acids and benzoic aldehydes are present as intermediary metabolites. Chen et al. (9) analyzed intermediates from a culture of ruminal microflora acclimated to dehydrovanillin and identified both carboxyvaloin and vanillic acid (VA). p-Hydroxybenzoic acid (HBA), VA, and syringic acid (SYA), along with p-hydroxybenzaldehyde (HBZ), vanillin (VN), and syringaldehyde (SYD), have been detected in forage cell walls (20), and PCA, HBA, and HBZ have been identified in the ruminal fluid of sheep (21). It is apparent, therefore, that the milieu of ruminal bacteria contains a vast assortment of phenolic compounds released from plants, with some compounds perhaps partially metabolized to other forms.

Certain of the phenolic monomers have been reported to be toxic to microorganisms. It has been reported that inhibitory effects are exerted on bacteria, yeasts, and filamentous fungi by guaiacyllyl and syringlyllyl compounds, representing the structure of native lignin (39). Natural and related phenolic compounds have shown antimicrobial properties against bacteria and fungi (22), and phenolics have long been used as food preservatives to inhibit microbial growth (12). Phenolic monomeric constituents of lignin have also been implicated as inhibitors of growth and cellulose degradation by ruminal microorganisms (11, 38). Although most investigators have reported inhibition by phenolic acids, Hungate and Stack (17) showed that a related compound, phenylpropanoic acid, stimulates growth of and cellulose digestion by *Ruminococcus albus* 7.

Evidence is accumulating that the simple phenolic monomers, which exist both in cell solubles and as constituents of lignin or plant cell walls, have a significant influence on animal nutrition (20). Yet reports of the effect of many of the phenolic monomers and the concentration at which they affect pure strains of ruminal microorganisms are few (11, 38) and reflect few feeding regimens. Because of the potential limitations of phenolic compounds on forage digestion by ruminal microflora, this study was undertaken to elucidate the effects of 10 phenolic monomers on the growth and activity of pure cultures and on the cellulytic activity of the mixed ruminal microflora from bovines fed Bermuda grass hay.

**MATERIALS AND METHODS**

**Cultural procedures.** *Ruminococcus flavefaciens* FD-1, *R. albus* 7, *Butyrivibrio fibrisolvens* 49, *Lachnospira multiparus* D-32, and *Bacteroides succinogenes* S-85 were acquired from M. P. Bryant and R. B. Hespell, Department of Dairy Science, University of Illinois, Urbana. The cultures of *B. succinogenes* could not be maintained and were not subsequently used for testing. Media were prepared with the semisynthetic anaerobic basal medium of Caldwell and Bryant (8), modified to contain 1% Na2CO3. Ruminal fluid obtained from a fistulated steer fed Bermuda grass (*Cynodon dactylon* L. Pers.) hay and pasture was clarified by autoclaving and centrifugation at 20,000 × g and added to the media at a 5% (vol/vol) concentration to replace an equal amount of...
All cultures were maintained on cellobiose (Eastman Kodak Co.). Additionally, *B. fibrisolvens* was maintained on xylan (larch sawdust; Koch-Light Laboratories, Ltd.). *L. multipes* was maintained on purified citrus pectin (Eastman Kodak Co.), and both ruminococci were maintained on basal medium plus a strip (12.5 by 114 mm) of Whatman no. 1 filter paper. Anaerobic conditions were obtained by bringing the media to a boil for 2 min, adding sodium sulfide-cysteine hydrochloride reducing agent (7), and gassing with CO2 for 15 min in an ice bath. The media were dispensed under CO2 into tubes and closed with caps containing black butyl rubber septa (Hungate type, catalog no. 2047; Belco Glass, Inc.). The pH after autoclaving was 6.5 to 6.7 and did not change with the addition of phenolic monomers. The phenolic monomers were soluble at all concentrations incorporated. Phenolic compounds were purchased from Aldrich Chemical Co., Inc., and K & K Laboratories, Inc. Their names and structures are shown in Fig. 1. The acids were incorporated into the cellobiose medium at 1, 5, or 10 mM to test for their effects on growth of the ruminal bacteria.

**Growth studies.** For turbidimetric growth studies, 7 ml of phenolic media was dispensed under CO2 into screw-cap tubes (16 by 125 mm) previously matched for identical absorbance with a Bausch and Lomb Spectronic 20 spectrophotometer. The *A*230 was read against a blank of uninoculated medium. Inoculum absorbance values (0 h) were subtracted from subsequent absorbance readings. All phenolic compounds were tested for their effects on each bacterium twice, with each trial consisting of duplicate tubes per treatment. The absorbances were recorded at approximately 2-h intervals until stationary growth was reached. The absorbances used to plot data represent the averages of the two trials. Slopes of exponential growth determined by linear regression analysis are reported as the growth rates. The growth rates were statistically analyzed by using analysis of variance for organisms by phenolic concentration interactions. The growth rates of each pure culture in control tubes within a series of treatments varied due to different inocula, and therefore each treatment was statistically evaluated with the control values for the same trial. In addition, Gram stains of each bacterial culture in the presence of the various concentrations of all the phenolic compounds were prepared from 14-h cultures.

**Filter paper degradation.** The cellulolytic activity of *R. flavefaciens* was evaluated by its ability to break filter paper strips (2). Quadruplicate tubes of anaerobic basal medium incorporating 10 mM concentrations of the phenolics found to stimulate growth rates of cellobiose-grown ruminococci were tested. The time (days) for complete breakage of the filter paper strip was used to compare the cellulolytic activity of cultures grown in tubes containing phenolic compounds and in control tubes.

**Adaptation.** To test for the ability of the microorganisms to adapt to growth in the presence of phenolic compounds, *R. flavefaciens* and *B. fibrisolvens* were inoculated into duplicate, optically matched tubes containing cellobiose (control) and cellobiose plus the following phenolic monomers: 5 mM PCA, 10 mM HBZ, and 10 mM HBA. For inoculation, 0.2-ml portions from cultures of *R. flavefaciens* actively growing on filter paper medium (96 h) and *B. fibrisolvens* grown 24 h on cellobiose medium were inoculated into tubes containing test compounds and cellobiose medium alone, and the absorbances were recorded at 2-h intervals to constitute the data for day 1. After 24 h of growth, 0.2 ml from each of the first cultures was inoculated into a second set of tubes containing the test media or cellobiose, and absorbances were recorded for day 2. For day 3, a similar procedure was followed with 0.1 ml from cultures of day 2 used to inoculate a third set of test and control media. Therefore, the adaptation for three periods of 24 h each was evaluated.

**In vitro cellulolytic activity of whole ruminal population.** A modified Tilley and Terry (37) procedure was used to test the effect of the phenolic monomers on the cellulolytic activity of the whole ruminal population. Each of the phenolic monomers (10 mM) was added to 50-ml plastic centrifuge tubes containing 400 mg of isolated cellulose (Solka Floc; Brown Co., Des Plaines, Ill.). Ruminal digesta was collected from a fistulated steer maintained on Coastal Bermuda grass hay and pasture plus a concentrate (12.3 kg of 74% oats, 10% cracked corn, 15% soybean meal, trace minerals, and 0.5% defluorinated phosphate per day). Strained ruminal fluid was added to McDougall buffer (26) equilibrated at 39°C with CO2 in a 1:2 ratio (fluid:buffer). The inoculum was continuously gassed with CO2, and 30 ml was dispensed into each tube, which was then sealed with a bunsen valve. Fermentations in duplicate tubes of each treatment were halted at 24 and 48 h, and the residue was centrifuged at 18,000 x g. The residual pellet was dried at 75°C for 48 h and then 110°C for 1.5 h, and the in vitro dry matter disappearance (IVDMD) was determined by calculating the dry weight loss. The experiment was repeated three times, and the results of each treatment were compared to the digestibility in control tubes (no phenolics) by using Student’s *t* test.

**Preparation for TEM.** Preparation for transmission electron microscopy (TEM) was done as follows. *R. flavefaciens* from an actively growing culture in filter paper medium was inoculated into media containing cellobiose, cellobiose plus 10 mM HBA, and cellobiose plus 5 mM PCA. Cultures were grown at 39°C in optically matched tubes. At mid-logarithmic growth (approximately 10 h), 2% glutaraldehyde was added to the tubes and the cells were fixed for 60 min. The cells were then allowed to cool to room temperature and were centrifuged at 2,000 x g for 10 min. The pellet was suspended twice in 3 ml of 0.1 M phosphate buffer (pH 7.0). After the last centrifugation, the pellet was suspended in 0.2 mg/ml phosphate buffer (pH 7.0) and incubated for 2 h.
ml of 0.1 M phosphate buffer to which 10% melted (60°C) gelatin was added. After solidifying, the gelatin was cut into approximately 0.5-mm cubes and postfixed for 60 min with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) containing 0.15% ruthenium red. The cubes were dehydrated in 25, 50, 70, 90, and 95% ethanol and in two changes of 100% ethanol at room temperature. The cubes were then infiltrated with Spurr low-viscosity embedding resin (33) and ethanol in ratios of 1:2 and 2:1 and with 100% resin for 45 min each. The blocks were placed in fresh 100% Spurr resin and polymerized at 70°C for 24 h. Thin sections were cut to 100 to 150 nm by a diamond knife on a Reichert Ultracut E microtome and placed on 200-mesh copper grids coated with a Formvar film. The sections were stained with 2% aqueous uranyl acetate (wt/vol) for 25 min followed by Reynolds (29) lead citrate for 7 min. The sections were then viewed with a JEOL 100CXII transmission electron microscope. Cell and capsule sizes were determined for 50 bacteria of each treatment by measuring micrographs of cell cross sections on a Bausch and Lomb Hi-Pad Digitizer. Only cells which showed a distinct cross section of the cell wall were measured (Fig. 2); cells cut at an oblique angle were not measured. The results of each treatment were compared to that of the control (no phenolics) by using Student's t test.

RESULTS

The effects of the phenolic monomers were mixed, depending on the compound and the microbial species. However, certain monomers were particularly influential, i.e., resulted in a significant alteration in growth rate, an effect on all species, or a dose-related response. Compounds that stimulated growth by the above-described criteria were (in increasing order of effect) hydrocinnamic acid (HCA), HBA, and SYA. Compounds that inhibited growth were (in increasing order of effect) HBZ and PCA. The rumenococci were more susceptible to both stimulatory and inhibitory effects by the phenolic monomers than were the noncellulolytic species.

Growth of ruminal bacteria in the presence of phenolic acids. The growth rates of pure cultures of the ruminal microorganisms in the presence of the three concentrations of phenolic monomers are shown in Table 1. No significant effects on R. albus occurred with sinapic acid (SA), SYD, VAN, or VA. Yet, there was an appreciable increase in the lag phase (not shown) with 10 mM VAN (3 h) and VA (5 h). SYA increased the rate by 14.9, 22.8, and 37.7% at the 1, 5, and 10 mM concentrations, respectively. Further, HBA showed a dose response for growth rates, with increases of 9.6, 14.3, and 22.4%, respectively, for the three concentrations. HCA was also found to increase the growth rate at the 5 and 10 mM concentrations, with the increase at 10 mM being significant. HCA shortened the lag phase by 1 h at the 5 and 10 mM concentrations.

Compounds found to markedly inhibit R. albus were PCA, HBZ, and FA. HBZ decreased the growth rate 12.9 and 37.1% at the 5 and 10 mM concentrations, respectively, and FA significantly slowed the rate at 10 mM. PCA exhibited the greatest effect on R. albus. At all concentrations there was a decrease in growth rate and an increase in lag time. The lag time was 1, 8, and 48 h greater than that for the control with 1, 5, and 10 mM concentrations of PCA, respectively. At times there was no growth observed with 10 mM PCA.

With R. flavefaciens, SYA, HBA, and HCA also stimulated bacterial growth. HCA shortened the lag phase at all concentrations by 2 h. VA tended to stimulate growth at the 1 and 10 mM levels but did not change the growth rate at 10 mM. PCA and HBZ significantly inhibited the growth of R. flavefaciens; there was a dose response for both of these phenolic compounds. There was an increase in lag time of 7 h with 10 mM PCA and 4 h with 10 mM HBZ over that of the control. Occasionally, no growth occurred at the 10 mM concentration of PCA and HBZ.

With B. fibrisolvens, stimulation of the growth rate occurred with SYA and HBA, and a nonsignificant increase occurred with HCA. Inhibitory phenolics were the same as those found for the cellulolytic species R. flavefaciens. VA tended to decrease the growth rate at the 5 and 10 mM concentrations, and PCA decreased the rate significantly at 10 mM. With HBZ, a dose-related response occurred with respect to the growth rates. There was no difference in the lag time for growth of B. fibrisolvens with any of the phenolics at the three concentrations tested compared with that in the control medium.

Vigorous growth of L. multiparus was observed with all phenolic monomers at the three concentrations tested, except for PCA and HBZ. SYA increased growth significantly at 5 and 10 mM. There was a small and insignificant increase in the growth rate with HBA, which indicated a general trend for this acid to stimulate bacterial growth.

Influence of phenolic monomers on cellulolytic activity. R. flavefaciens was tested for the time required to break filter paper in the presence of 10 mM concentrations of the phenolic monomers that had stimulated growth in cellobiose medium (Table 2). SYA and HBA both significantly increased the ability of R. flavefaciens to degrade the filter paper. The other monomers tested showed no significant effect on cellulolysis.

Ability to utilize phenolic monomers. All four bacteria were tested for their ability to utilize phenolic monomers (that were found to stimulate growth with cellobiose) as their sole carbon and energy source. Basal medium (negative control),
TABLE 1. Effects of phenolic monomers on growth of ruminal microorganisms

| Phenolic monomer | R. albus | R. flavefaciens | B. fibrisolvens | L. mutatus
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Growth rate (10^{-2} with indicated conc (mM) of phenolic monomer)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>SA</td>
<td>8.2</td>
<td>8.8</td>
<td>8.3</td>
<td>6.6</td>
</tr>
<tr>
<td>SYD</td>
<td>10.4</td>
<td>10.3</td>
<td>11.6</td>
<td>10.3</td>
</tr>
<tr>
<td>SYA</td>
<td>9.1</td>
<td>10.7</td>
<td>11.8</td>
<td>14.6</td>
</tr>
<tr>
<td>FA</td>
<td>8.2</td>
<td>7.7</td>
<td>8.3</td>
<td>6.4</td>
</tr>
<tr>
<td>VAN</td>
<td>9.9</td>
<td>10.7</td>
<td>9.7</td>
<td>8.4</td>
</tr>
<tr>
<td>VA</td>
<td>6.5</td>
<td>6.4</td>
<td>8.6</td>
<td>5.5</td>
</tr>
<tr>
<td>PCA</td>
<td>8.3</td>
<td>7.8</td>
<td>3.5</td>
<td>0.6</td>
</tr>
<tr>
<td>HBZ</td>
<td>6.2</td>
<td>5.5</td>
<td>5.4</td>
<td>3.9</td>
</tr>
<tr>
<td>HBA</td>
<td>6.6</td>
<td>7.3</td>
<td>7.7</td>
<td>8.5</td>
</tr>
<tr>
<td>HCA</td>
<td>8.1</td>
<td>7.4</td>
<td>8.5</td>
<td>9.1</td>
</tr>
</tbody>
</table>

* Slope of exponential growth.

**Treatment differs from control (P < 0.05).

**Treatment differs from control (P < 0.08).

cellobiose medium (positive control), and basal medium plus 10 mM SA, SYA, VA, HBA, or HCA were inoculated with actively growing cultures of each of the four species. Normal growth occurred in cellobiose medium, but bacteria did not grow even after 72 h in basal medium or in basal medium plus the phenolic compounds. Therefore, these bacteria do not appear to be able to derive carbon and energy solely from growth-promoting phenolic compounds.

Adaptation to phenolic monomers. Cellulolytic R. flavefaciens and hemicellulolytic B. fibrisolvens were tested for their ability to adapt to 10 mM HBA (a stimulator), 10 mM HBZ (an inhibitor), or 5 mM PCA (an inhibitor). PCA was used at a lower concentration because of lack of growth at the 10 mM level. R. flavefaciens growth for three successive 24-h periods (days 1 through 3) is shown in Fig. 3a to c. HBA stimulated the growth rate and maximal absorbance on the first transfer (14 and 12%, respectively). On days 2 and 3 there was no difference in the growth rate, but the maximal absorbance was 11% greater with HBA on both days. Apparently no adaptation occurred with HBA. PCA decreased microbial growth, and no adaptation occurred on either of the successive days. HBZ decreased growth on day 1, and a substantial increase in lag time occurred on day 2 and 3, although at day 3 the maximal absorbance was above that of the control (4%). Overall, no adaptation occurred in R. flavefaciens with stimulating or inhibiting phenolic monomers.

B. fibrisolvens was tested for its ability to adapt to the same concentrations of phenolic compounds (Fig. 4a to c). No adaptation occurred with any of the three phenolic monomers. There was a difference in growth at day 1 compared with growth in the presence of these monomers in other experiments (Table 1). PCA showed a small stimulation and HBA was not stimulatory at the same concentrations as those reported in Table 1; this variation apparently was due to the differences in the ages of the inocula. The inoculum for day 1 was a 24-h culture, whereas initial growth studies (Table 1) used mid-logarithmic growth cultures (12 h).

Morphology and ultrastructure of R. flavefaciens grown in the presence of phenolic compounds. R. flavefaciens grown until mid-logarithmic phase in cellobiose medium (control) and cellobiose medium plus 5 mM PCA or 10 mM HBA was prepared for TEM. Cross sections of about 50 cells from each treatment were measured to determine variations in cell diameter and in capsular width (Fig. 2). The capsules of the cellobiose-grown and HBA-grown cells were of approximately the same thickness (Table 3), whereas those of the PCA-grown cells were significantly smaller (P < 0.05). The ultrastructural study also revealed that HBA- and PCA-grown cells were smaller in diameter (P < 0.05) than the control cells (Table 3). A few PCA-grown cells had pleomorphic forms and irregular cell division (Fig. 5), although most cells appeared normal. Gram stains of R. flavefaciens and B. fibrisolvens grown in 10 mM PCA also revealed morphological changes. R. flavefaciens showed gram variability, some cell lysis, and pleomorphism, whereas B. fibrisolvens formed long chains of cells, with the length increasing with increasing concentrations of PCA.

Influence of phenolic monomers on cellulose digestion by whole ruminal populations. The average of three 24- and 48-h IVDM trials with isolated cellulose (Solka-Floc) with incorporation of a 10 mM concentration of each of the phenolic monomers is presented in Table 4. There was no stimulation of cellulose digestion with any of the monomers at 24 or 48 h. The most marked influence on digestion was inhibition with the benzoic aldehydes. At 24 h, there was a decrease of cellulose digestion with SYD (64%), VAN (56%), and HBZ (61%) from that in the control tubes. At 48 h, cellulose digestion was decreased by these same benzoic aldehydes by 40, 42, and 27%, respectively. The 48-h fermentation data revealed no statistical difference with the benzoic acid derivatives but did reveal a decrease (P < 0.05) in digestion with the cinnamic acid derivatives.

**DISCUSSION**

The levels of phenolic monomers tested in these experiments were higher than those that have been reported in the

| Table 2. Effect of phenolic monomers (10 mM) on cellulose (filter paper) degradation |
|-----------------------------------|-----------------------------------|
| Treatment                        | Time (days) to breakage by R. flavefaciens FD-1* |
| Control                          | 7.2 ± 0.64                        |
| HCA                              | 7.8 ± 0.94                        |
| SYA                              | 5.4 ± 1.38                        |
| HBA                              | 4.0 ± 0.24                        |
| VA                               | 6.0 ± 1.34                        |
| SA                               | 7.0 ± 0.94                        |

* Mean ± standard deviation for four tubes.

**Treatment differs from control (P < 0.05).
FIG. 3. Growth curves of *R. flavefaciens* FD-1 grown in cellobiose (□), cellobiose with 5 mM PCA (△), cellobiose with 10 mM HBZ (○), and cellobiose with 10 mM HBA (X). Three consecutive 24-h periods are shown in order (a, b, and c).
FIG. 4. Growth curves of *B. fibrisolvens* 49 grown in cellobiose (□), cellobiose with 5 mM PCA (△), cellobiose with 10 mM HBZ (○), and cellobiose with 10 mM HBA (X). Three consecutive 24-h periods are shown in order (a, b, and c).

rumen (19, 21). Chesson et al. (11) suggested that fibrolytic ruminal microorganisms would encounter levels of phenolic compounds in a microenvironment higher than those found in the ruminal fluid. Data are not available to indicate specific concentrations of phenolic monomers which may be present in such an environment. Indeed, different tissues have markedly different levels of phenolics, and the levels released in a microenvironment could vary with chemical treatment, plant stress, and type of feed.

The cellulolytic bacteria *R. albus*, *R. flavefaciens*, and *B.*
sucinogenes adhere to or are found in close proximity to plant cell wall material undergoing degradation in the rumen (5, 10). Chesson et al. (11) found that these cellulolytic species were no more tolerant of added phenolic acids than were ruminal bacteria normally not associated with plant particles. Further, the strains of cellulolytic bacteria used in this study, i.e., R. albus 7 and R. flavefaciens FD-1 (B. succinogenes S-85 did not grow with the cultural methods used), were found to be more susceptible to added phenolic monomers than were the noncellulolytic B. fibrisolvens 49 and L. multivar D-32. Similar results, showing a higher tolerance of the hemicellulolytic population to phenolic compounds, with a mixed ruminal population were reported by Jung (18). Recent research by Varel and Jung (38) further showed that B. succinogenes growth is inhibited by VAN and PCA.

The phenolics found to be most toxic to all the bacteria tested were PCA and its benzoic aldehyde, HBZ. Akin (4) and Chesson et al. (11) found the same effects exerted on ruminococci with PCA, but Chesson et al. found B. fibrisolvens B834 to be inhibited more by FA than by PCA. Varel and Jung (38) reported that B. succinogenes S-85 was slowed more by VAN than by FA or PCA.

Although there is much evidence supporting a toxic effect for phenolic monomers, work by Hungate and Stack (17) showed that a related compound, HCA, stimulated growth and cellulolytic activity of the ruminal bacterium R. albus 8. Further studies by Stack and Cotta (34) showed that 25 μM HCA stimulated the cellulolytic activity of R. albus 7 and 8 but not that of R. flavefaciens FD-1 or C94. Our studies confirmed that HCA at the 10 mM level does not stimulate cellulose degradation by R. flavefaciens FD-1, but it did significantly stimulate the growth rate on cellulose. HCA differs from the cinnamic acids by having a hydrogenated side chain and by having no hydroxyl on the aromatic ring. Of the phenolic monomers we tested, stimulation of the growth of R. albus occurred with (in order of increasing stimulatory effect) HCA, HBA, and SYA. As with inhibition, stimulation was greatest with the cellulolytic bacteria. Stimulation occurred with all the benzoic acids tested, except that there was a mixed reaction to VA. The general stimulatory effect of the benzoic acids was the only consistent relationship shown between growth and the chemical structure of the phenolic monomers, with SYA and HBA significantly enhancing filter paper breakdown by R. flavefaciens.

Despite enhanced growth and cellulolysis noted for pure cultures, none of the phenolics tested stimulated cellulose digestion by mixed populations from the rumen. Likewise, Gorosito et al. (13) reported that the addition of HCA did not improve the in vitro digestibility of plant cell walls. Possibly, the phenolic compounds are rapidly metabolized by noncellulolytic bacteria in the mixed microflora (9, 11, 19, 25) such that the compounds are no longer available as growth promoters. It is also possible that ruminal fluid supplies growth promoters (6) similar to the added phenolic compounds, such that the requirements for these or similar compounds are met. The benzoic aldehyde derivatives were found to be especially inhibitory to cellulose digestion, followed by the less toxic cinnamic acid derivatives. None of the benzoic acids significantly reduced cellulose degradation. Jung (18) also found the aldehyde derivatives to be toxic to cellulose digestion by a mixed ruminal population. Large standard deviations between our three trials occurred in fermentations with the phenolic compounds. These deviations may have been due in part to differences during ruminal digesta collections in microflora that responded differently to individual phenolic compounds. The deviations were especially large with the benzoic aldehydes, which further supports the concept that these compounds markedly influence ruminal microorganisms.

### Table 3. Ultrastructural effects of phenolic monomers on R. flavefaciens FD-1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n°</th>
<th>Cell size (μm)</th>
<th>Capsule size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose (control)</td>
<td>49</td>
<td>0.977 ± 0.11</td>
<td>0.104 ± 0.012</td>
</tr>
<tr>
<td>Cellulose + 10 mM HBA</td>
<td>50</td>
<td>0.913 ± 0.10c</td>
<td>0.106 ± 0.010</td>
</tr>
<tr>
<td>Cellulose + 5 mM PCA</td>
<td>50</td>
<td>0.915 ± 0.07c</td>
<td>0.094 ± 0.010c</td>
</tr>
</tbody>
</table>

* Number of cell cross sections measured.
* Mean ± standard deviation for measured cells.
* Treatment differs from control (P < 0.05).

### Table 4. Influence of phenolic compounds on IVDMD of isolated cellulose (Solka-Floc)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.4 ± 4.2</td>
<td>63.1 ± 4.9</td>
</tr>
<tr>
<td>SA</td>
<td>27.9 ± 9.9</td>
<td>50.8 ± 2.5</td>
</tr>
<tr>
<td>SYD</td>
<td>10.9 ± 5.7c</td>
<td>38.0 ± 11.5c</td>
</tr>
<tr>
<td>SYA</td>
<td>27.7 ± 3.7</td>
<td>57.3 ± 7.0</td>
</tr>
<tr>
<td>FA</td>
<td>22.0 ± 3.6c</td>
<td>52.3 ± 2.9c</td>
</tr>
<tr>
<td>VAN</td>
<td>13.4 ± 10.4c</td>
<td>36.4 ± 12.9c</td>
</tr>
<tr>
<td>VA</td>
<td>27.3 ± 4.5</td>
<td>57.4 ± 4.7</td>
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<tr>
<td>PCA</td>
<td>20.4 ± 4.7c</td>
<td>51.4 ± 3.7c</td>
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<tr>
<td>HBZ</td>
<td>11.9 ± 2.2c</td>
<td>46.2 ± 9.6c</td>
</tr>
<tr>
<td>HBA</td>
<td>27.8 ± 4.8</td>
<td>60.2 ± 6.1</td>
</tr>
<tr>
<td>HCA</td>
<td>28.9 ± 2.1</td>
<td>57.2 ± 4.2</td>
</tr>
</tbody>
</table>

* Mean ± standard deviation of three trials run in duplicate.
* Treatment differs from control (P < 0.05).
* Treatment differs from control (P < 0.0058).
Hundreds of isomers and combinations of alkyl, hydroxyl, and methoxyl derivatives of phenolics are possible. The addition of n-alkyl groups to the phenol nucleus has been found to increase its antimicrobial activity (23). Suter (36) reported that n-alkyl derivatives were more effective than branched-chain derivatives. He also reported that separation of alkyl groups from the phenol nucleus by oxygen (e.g., methoxyl groups) decreased their activity. The nonmethoxylated phenolics used in this study were found to be the most active in stimulation (HBA) and inhibition (PCA and HBZ) of ruminal microorganisms. Correlated with these results is a study (3) showing that a facultatively anaerobic ruminal microorganism grown in an aerobic environment better utilized the more methoxyl-substituted phenolic compounds than the less substituted ones. Jung (18) found no clear relationship between depression of IVDMD of cellulose or hemicellulose and changes in substitutions on the aromatic ring of phenolics. In the present study, substitutions on the ring did not consistently affect the action of the phenolic monomers on the ruminal bacteria.

The two ruminococci used in this study have been frequently reported to adhere to fibrous substrates (via capsular material) to mediate cell wall degradation (1, 5, 24, 28). Stack and Hungate (35) demonstrated that HCA-grown R. albus 8 cells show a surface structure different from that of cells grown without HCA. HCA appears to increase capsular size concomitant with increased cellulolytic activity. In our study, HBA increased the cellulolytic activity of R. flavefaciens (Table 2) but did not alter the capsule size or structure when compared with the capsule of the cellulose-grown control. Possibly, HBA as a growth promoter brings about an increase in the number of R. flavefaciens cells (Table 1) rather than inducing an increase in the ability of each individual cell to degrade cellulose. TEM of R. flavefaciens grown with PCA revealed a decreased capsular size and occasional cell pleomorphism compared with those for cells grown in the control medium and medium containing HBA. Further, R. flavefaciens cells grown with PCA or HBA were smaller than those grown in the control medium. These ultrastructural as well as morphological and cellular changes observed by light microscopy in this and other studies (11) suggest that phenolic monomers play a role in altering cell metabolism and that alterations vary among species. However, the specific effect at the biochemical level is not known.

LITERATURE CITED

RUMINAL BACTERIA AND PHENOLIC COMPOUNDS