Portal and Hepatic Fluxes in Sheep and Concentrations in Cattle Ruminal Fluid of 3-(4-Hydroxyphenyl)propionic, Benzoic, 3-Phenylpropionic, and trans-Cinnamic Acids


*Department of Animal Sciences, University of Illinois, Urbana 61801; †Department of Animal Sciences, University of Kentucky, Lexington 40546; and ‡Department of Animal and Poultry Sciences, University of Arkansas, Fayetteville 72701

ABSTRACT: Extraction methods and HPLC procedures were developed for analysis of potential ruminal metabolites of dietary phenolics (reduced phenolics). Hepatic portal venous blood from wethers fed bromegrass, bermudagrass, ryegrass-wheat, and alfalfa hays also was analyzed for hippuric (HA), 3-(4-hydroxyphenyl)propionic (40HPPA), benzoic (BA), 3-phenylpropionic (PPA), and t-cinnamic (CA) acids. Additionally, mesenteric arterial and hepatic venous blood was analyzed and, in conjunction with blood flow measurements, fluxes for portal-drained viscera (PDV) and liver were calculated. Ruminal fluid from four steers fed two levels of forage and two forage particle sizes in a Latin square design was analyzed for PPA and CA. 3-Phenylpropionic and benzoic acids were the most concentrated reduced phenolics identified in hepatic portal venous blood. Concentrations of PPA in ruminal fluid varied with ruminal disappearance of p-coumaric and ferulic acids. Additionally, hepatic portal venous concentrations of PPA were correlated (P < .05) with p-coumaric acid (r = .57) and ferulic acid (r = .67) intakes. Net release of PPA from PDV was observed, suggesting absorption of PPA from the gut. The liver removed PPA and BA with less efficiency. Given the relatively high concentrations of PPA in blood of ruminants, specific effects of this reduced phenolic on liver metabolism of ruminants should be assessed.

Key Words: Portal Vein, Liver, Phenolics, Ruminants

Introduction

The effects of phenolics on ruminal digestion of forage and on ruminal microbial activity have been investigated extensively (Fahey and Jung, 1989), but the postabsorptive effects of phenolics or their ruminal metabolites have been given little attention. From studies examining the appearance of potential metabolic products of phenolic monomers in urine, Martin (1969) estimated that ruminants absorb and metabolize approximately 10 times the amount of phenolic monomers that nonruminants do. Because many have been shown to have physiological effects when ingested (Fahey and Jung, 1989), it is important to determine physiological concentrations of phenolics in blood for interpretation of future studies of their effects in vivo.

Four potential ruminal metabolites of phenolic monomers (benzoic acid [BA], 3-phenylpropionic [PPA], t-cinnamic [CA], and 3-(4-hydroxyphenyl)propionic [40HPPA] acids), referred to as reduced phenolics, have been identified in ruminal fluid by GLC-mass spectroscopy (Daolio et al., 1989) and have been shown to be absorbed by sheep (Martin, 1982a,b) and inhibitory to metabolism in vitro (Cremin et al., 1994). Our objectives were to determine the concentrations of these reduced phenolics in hepatic portal venous blood and to determine the net fluxes of these compounds across the portal-drained viscera (PDV) and liver in sheep fed forage diets.

Materials and Methods

Reagents

Water used in this study was distilled deionized water purified by a Milli-Q water purification system.
A. L. Goetsch and C. L. Ferrell (Trial 2, unpublished)

At 2.5 h after feeding, a priming dose (15

Intakes were restricted to 1.9% of steer BW. The diets

were formulated to be isonitrogenous. Within each period,

50 mL of ruminal fluid was collected from each steer

for each hour of the day, acidified with 2.5 mL of

6 N HCl, and stored frozen for later analyses. The samples

were composited over sampling time for each steer

and period before analyses. The objective of this

experiment was to determine the effects of two factors

known to decrease forage cell wall digestibility (forage

particle size and level of starch in the diet) on

digestion of individual cell wall components.

Data were analyzed using the GLM procedures of

SAS (1985). Variance was partitioned using a model

including the effects of forage level, particle size,

forage level x particle size interaction, animal, and

period. The variance of the treatment effects was

tested using the F-test with $P < .05$ considered

significant. Further description of the animals used in

this study and forage composition can be found in

Bourquin et al. (1994).

Clay Center. Hepatic portal venous blood samples

collected from multi-catheterized sheep from two

experiments conducted by A. L. Goetsch, C. L. Ferrell,

and H. C. Freely (Trial 1, Goetsch et al., 1995) and

A. L. Goetsch and C. L. Ferrell (Trial 2, unpublished

data) at the Roman L. Hruska Meat Animal Research

Center, Clay Center, NE were analyzed for reduced

phenolics. The wethers in these trials were fitted with

chronic indwelling mesenteric arterial and venous,

known to decrease forage cell wall digestibility (forage

phenolics). The wethers in these trials were fitted with

mesenteric arterial and venous, intraduodenal infusions of starch hydrolysate

[.5 g·(kg BW$^{-0.75}$)·h$^{-1}$] and sodium caseinate [.2 g·(kg

BW$^{-0.75}$)·h$^{-1}$] were begun. Within each period,

wethers received a somatostatin control infusion

(physiological saline). Alfalfa hay was withheld for 12 h before and
during blood sampling. Two hours before blood sampling,

intraduodenal infusions of starch hydrolysate

were begun. Within each period, wethers received a somatostatin control infusion

(physiological saline with .2% ovine serum albumin)

beginning .5 h before blood sampling followed by

somatostatin repletion (5.0 μg·kg BW$^{-1}$·h$^{-1}$)

beginning 2 h before blood sampling. A sample of the alfalfa

hay was alkali-extracted and the extract analyzed for

p-coumaric and ferulic acids. Blood flows were deter-

mined using p-aminohippuric acid dilution. Fluxes and hepatic extraction ratios were calculated according to Krehbiel et al. (1992). Further descriptions of

University of Kentucky. Mesenteric arterial, hepatic

portal venous, and hepatic venous blood samples from

an experiment conducted at the University of Ken-

tucky, Lexington were analyzed for reduced phenolics. The samples were collected from eight Polypay

wethers (average BW = 36.5 kg) in a crossover experimental design, the objective of which was to
determine the effects of somatostatin depletion and

repletion on total splanchnic and PDV nutrient fluxes. The wethers were fed chopped alfalfa hay twice daily at 1.2 x NE$_{$r}$. Endogenous somatostatin was depleted in these animals by intrajugular administration of
cysteamine·HCl (50 mg/kg BW) 12 h before the first

blood sampling period. The control period consisted of

infusion of the cysteamine vehicle (physiological saline). Alfalfa hay was withheld for 12 h before and
during blood sampling. Two hours before blood sampling,

intraduodenal infusions of starch hydrolysate

were begun. Within each period, wethers received a somatostatin control infusion

(physiological saline with .2% ovine serum albumin)

beginning .5 h before blood sampling followed by

somatostatin repletion (5.0 μg·kg BW$^{-1}$·h$^{-1}$)

beginning 2 h before blood sampling. A sample of the alfalfa

hay was alkali-extracted and the extract analyzed for

p-coumaric and ferulic acids. Blood flows were deter-

mined using p-aminohippuric acid dilution. Fluxes and hepatic extraction ratios were calculated according to Krehbiel et al. (1992). Further descriptions of

Methanol (HPLC grade) and 1-butanol (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ). Glacial acetic acid

was obtained from Mallinckrodt (Paris, KY). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Samples

Animals used in these experiments were treated

according to procedures established at the institutions

at which the research was conducted.

Ruminal Fluid. Ruminal fluid samples from an

experiment conducted at the University of Illinois

were analyzed for reduced phenolics. The experimental

design was a 2 x 2 factorial. Ruminally cannulated steers were fed either 96% long orchardgrass hay:4%

protein supplement; 96% ground orchardgrass hay:4%

protein supplement; 60% long orchardgrass hay:40%

protein and starch supplement; or 60% ground or-

chardgrass hay:40% protein and starch supplement.

Intakes were restricted to 1.9% of steer BW. The diets

were formulated to be isonitrogenous. Within each period,

50 mL of ruminal fluid was collected from each steer

for each hour of the day, acidified with 2.5 mL of

6 N HCl, and stored frozen for later analyses. The samples

were composited over sampling time for each steer

and period before analyses. The objective of this

experiment was to determine the effects of two factors

known to decrease forage cell wall digestibility (forage

particle size and level of starch in the diet) on

digestion of individual cell wall components.

Data were analyzed using the GLM procedures of

SAS (1985). Variance was partitioned using a model

including the effects of forage level, particle size,

forage level x particle size interaction, animal, and

period. The variance of the treatment effects was

tested using the F-test with $P < .05$ considered

significant. Further description of the animals used in

this study and forage composition can be found in

Bourquin et al. (1994).

Clay Center. Hepatic portal venous blood samples

collected from multi-catheterized sheep from two

experiments conducted by A. L. Goetsch, C. L. Ferrell,

and H. C. Freely (Trial 1, Goetsch et al., 1995) and

A. L. Goetsch and C. L. Ferrell (Trial 2, unpublished

data) at the Roman L. Hruska Meat Animal Research

Center, Clay Center, NE were analyzed for reduced

phenolics. The wethers in these trials were fitted with

chronic indwelling mesenteric arterial and venous, hepatic portal venous, and hepatic venous catheters

according to procedures of Ferrell et al. (1992).

Wethers were individually maintained on slatted floor

pens with free access to water. Experiments were

begun 14 d or more after surgery. In both trials,

wethers were offered hay twice daily to ad libitum

intake for 20 d (Trial 1) or 18 d before sampling

(Trial 2). At 2.5 h after feeding, a priming dose (15

mL) of p-aminohippuric acid (.15 M) was infused into

the mesenteric vein followed by a continuous infusion

(.8 mL/min) for the course of blood sampling. In Trial

1, blood was sampled hourly from 3 to 9 h after

feeding and in Trial 2 every 30 min from 3 to 6 h after

feeding. Blood was collected in heparinized syringes

then placed on ice until frozen. Animals sampled were

six Romanov x Composite III (Meat Animal Research

Center, 25 Suffolk, 25 Hampshire, and .50 Columbia,

Trial 1) wethers (average BW = 53 kg) fed

bromegrass hay (Bromus inermis, no flowering

heads), four Finn x Composite III wethers (Trial 2),

and four Romanov x Composite III wethers (Trial

1) (average BW = 42 kg for both trials) fed

bermudagrass hay (Cynodon dactylon, 6 to 8 wk

regrowth, same hay source for both trials), and five

Finn x Composite III wethers (average BW = 35 kg)

fed ryegrass (Lolium multiflorum, early head emer-
gence)-wheat (Triticum aestivum, anthesis) hay

(Trial 2) for ad libitum intake. Proximate analysis of

forage samples is described in Goetsch et al. (1995).

Samples of the forages fed were alkali-extracted and

the extract analyzed for p-coumaric and ferulic acids.

Data were analyzed using the GLM procedures of

SAS (1985). Correlations of hepatic portal venous

blood concentrations of reduced phenolics with intakes

of phenolic monomers and reduced phenolics were

calculated and significant ($P < .05$) correlations ($r$)

presented.

University of Kentucky. Mesenteric arterial, hepatic

portal venous, and hepatic venous blood samples from

an experiment conducted at the University of Ken-
tucky, Lexington were analyzed for reduced phenolics. The samples were collected from eight Polypay

wethers (average BW = 36.5 kg) in a crossover experimental design, the objective of which was to
determine the effects of somatostatin depletion and

repletion on total splanchnic and PDV nutrient fluxes. The wethers were fed chopped alfalfa hay twice daily at 1.2 x NE$_{$r}$. Endogenous somatostatin was depleted in these animals by intrajugular administration of
cysteamine·HCl (50 mg/kg BW) 12 h before the first

blood sampling period. The control period consisted of

infusion of the cysteamine vehicle (physiological saline). Alfalfa hay was withheld for 12 h before and
during blood sampling. Two hours before blood sampling,

intraduodenal infusions of starch hydrolysate

were begun. Within each period, wethers received a somatostatin control infusion

(physiological saline with .2% ovine serum albumin)

beginning .5 h before blood sampling followed by

somatostatin repletion (5.0 μg·kg BW$^{-1}$·h$^{-1}$)

beginning 2 h before blood sampling. A sample of the alfalfa

hay was alkali-extracted and the extract analyzed for

p-coumaric and ferulic acids. Blood flows were deter-

mined using p-aminohippuric acid dilution. Fluxes and hepatic extraction ratios were calculated according to Krehbiel et al. (1992). Further descriptions of
the animals and blood flow and collection procedures can be found in McLeod (1995).

Data were analyzed using the GLM procedures of SAS (1985). Five sets of the samples collected were analyzed in this experiment. Two of these sets of samples were from wethers that received control infusion in the first period and cysteamine infusion in the second period. The other sets of samples were from wethers that received cysteamine infusion in the first period and control infusion in the second period. A hepatic venous blood sample from a wether in Period 1 on the cysteamine and somatostatin infusion had an unusually high concentration of PPA. It was 6.5 times greater than the average PPA concentration and two times greater than the next highest concentration. This resulted in the calculation of a large net positive flux of PPA (614 μmol/h) from the liver of this animal. Of the 20 animals tested, this was the only animal with a net positive liver flux of PPA. Given these observations, it was judged that the analysis of this sample was erroneous and observations from this animal were dropped from the study. Variance was partitioned using a model including the effects of cysteamine, somatostatin, cysteamine x somatostatin interaction, period, sequence of treatment administration, and animal within sequence of treatment administration. If an effect was significant according to the F-test (P < .05), differences among means were compared using the least significant difference test (P < .05).

**Extraction**

Trans-3,4-dimethoxycinnamic acid (DMCA) was used as an internal standard. It was added (.08 mL of .3 mM DMCA) to 4 mL of thawed whole blood that was subsequently acidified by addition of 16 mL of 400 mM phosphate buffer (pH 2.0). After thorough mixing, the sample was centrifuged for 20 min at 25,900 x g (0°C). The supernatant was decanted and subsequently extracted using a conditioned C18 solid phase extraction column (Fisher Scientific, Pittsburgh, PA; 300 mg of C18). This extraction procedure is a modification of the procedure described by Titgemeyer et al. (1991). The columns were conditioned by washing twice with 2 mL of methanol, then rinsing three times with 2 mL of 40 mM phosphate buffer (pH 2.0). The supernatant was added to the conditioned column and pulled through under vacuum. The column was washed three times with 2 mL of 40 mM phosphate buffer. The reduced phenolics were eluted from the column by extracting it three times with 1 mL of 50:50 (vol:vol) 40 mM phosphate buffer: methanol.

The internal standard was not added to blood samples from the Clay Center trial. Instead, each sample was spiked with reduced phenolic standards and recovery of each reduced phenolic was calculated. The concentrations of reduced phenolics detected in the samples were adjusted based on the average recoveries of the reduced phenolics.

The same extraction procedure was used on ruminant fluid samples except that ruminant fluid was centrifuged for 20 min at 25,900 x g (0°C), then 2 mL of the supernatant was acidified with 8 mL of 400 mM phosphate buffer. Alkali-labile p-coumaric and ferulic acid monomers were extracted from forage samples using the procedure of Titgemeyer et al. (1991).

**High Performance Liquid Chromatography Methods**

All methods were conducted using a Dionex (Sunnyvale, CA) Bio-LC HPLC automated with AI-450 software and fitted with a 250-mm x 4.6-mm column packed with Supelcosil-C18 (Supelco, Bellefonte, PA; 5-µm particle size) and a 10-mm x 4.6-mm guard column packed with Adsorbosphere-C18 (Alltech, Deerfield, IL; 5-µm particle size). The temperature of the column was maintained at 35°C. Fifty microliters of sample was injected onto the column. The separated compounds were detected spectrophotometrically with a variable wavelength detector.

The HPLC separation methods used in this study are modifications of the procedure developed by Jung et al. (1983a) for separation of phenolic monomers extracted from plant tissue. To determine an optimal wavelength for spectrophotometric detection, the absorbance spectra of the reduced phenolics solubilized in methanol were measured at .5-nm intervals from 200 to 350 nm using a Gilford Response II spectrophotometer (Ciba Corning Diagnostics, Oberlin, OH).

A separation method was developed in which the reduced phenolics were separated at a flow rate of 1.2 mL/min using a linear gradient formed by mixing methanol with phenolic solvent (350:7:1 [vol:vol:vol] water:1-butanol:glacial acetic acid). From the time of injection until .4 min after injection, 350:7:1 was pumped isocratically. From .4 to 34.1 min after injection, the proportion of methanol was increased linearly, reaching 60% by 34.1 min after injection. The stationary phase was washed with methanol from 34.2 to 40.3 min after injection, then equilibrated with the phenolic solvent for 17.9 min before the next injection. A .01 absorbance unit was set to equal the full detector output (1 V).

The reduced phenolics have absorption maxima at wavelengths below 254 nm. However, we observed that the phenolic solvent absorbed light to a different extent than methanol between 200 and 254 nm. Consequently, the baseline of the chromatogram changed dramatically as the proportion of methanol increased, precluding the use of a methanol gradient for separation of these compounds when using wavelengths below 254 nm for detection. Hippuric acid (HA), 4OHPPA, BA, and CA had an absorbance maximum at 273 nm; therefore, these compounds were
analyzed using the gradient described above and quantified using 273 nm.

3-Phenylpropionic acid did not significantly absorb light at 273 nm. It had an absorbance maximum at 201 nm. To prevent baseline drift caused by differences in absorbance between the two eluents at this wavelength, an isocratic separation method was developed for PPA. The flow rate was 1.2 mL/min. A mixture of 65% phenolic solvent and 35% methanol was pumped isocratically from injection to 24.9 min after injection. The stationary phase was washed with methanol from 25 to 34.5 min, then equilibrated with the initial eluent for 27.5 min before the next injection. A .2 absorbance unit was set to equal the full detector output (1 V).

Alkali-labile ferulic and p-coumaric acids were separated and detected using the procedures of Jung et al. (1983a) with a wavelength of 305 nm for detection. This procedure was modified to separate the internal standard (DMCA) by linearly increasing the percentage of methanol in the eluent beginning at 19 min after injection. Methanol reached 30% of the eluent by 25.9 min after injection. The stationary phase was washed with methanol from 26 to 34 min after injection then equilibrated with initial eluent for 8 min before the next injection.

Identity of peaks in chromatograms from the samples was determined by comparison to retention times of standards. Identification of peaks was aided by spiking of sample extract with standards to determine whether the standards co-eluted with the peaks tentatively identified on the basis of retention time. Extracts representative of each type of sample were spiked.

Reagent blank extracts were analyzed to determine background contamination. There was a peak with a retention time coinciding with that of 40HPPA in ruminal fluid and blood extraction reagent blanks. The area of this peak was subtracted from subsequent 40HPPA values. Additionally, a new column was used for analysis of blood samples from the University of Kentucky trial and alkali extracts of forages. This column resulted in a chromatogram that had a peak coinciding with the retention time of CA that was not observed with the previous column. The area of this peak was consistent so it was subtracted from sample values. There was no background observed for HA, BA, PPA, or DMCA.

Validation

High Performance Liquid Chromatography Methods. The most concentrated standard tested contained 40, 40, 80, 400, 2, and 10 μM HA, 40HPPA, BA, PPA, CA, and DMCA, respectively. The ratio between peak area and concentration from one standard was used to convert peak area to concentration. Linearity was tested by regressing the concentrations measured in standards made by serially diluting the most concentrated standard 2, 4, 8, 15, 20, 40, 80, 150, 200, 400, and 750-fold on the calculated concentrations of these standards (Table 1).

In the Clay Center and University of Kentucky trials, blood flow was determined by dilution of p-aminohippuric acid infused into a mesenteric vein. Because this compound is structurally related to HA, the retention time of p-aminohippuric acid was determined (5.02 min) to ensure that it did not coelute with HA (6.50 min).

Extraction Methods. Recovery of compounds from blood and ruminal fluid (Table 2) was evaluated by spiking samples of whole blood or ruminal fluid with standards then extracting them using the procedures described above. Recovery from blood was measured in five samples of jugular venous blood collected from five steers. Recovery from each sample was run in duplicate. Recoveries from ruminal fluid are averages of the extraction procedure repeated using the four treatment samples from period one of the University of Illinois samples. Concentrations in samples were adjusted for DMCA recovery then adjusted for recovery of reduced phenolic (Table 2) relative to DMCA.

Results

Peaks were not consistently detected at concentrations below .5, .5, 1, 1.33, .013, and .025 μM for HA, 40HPPA, BA, PPA, CA, and DMCA, respectively. There was a linear relationship between the measured and calculated concentration of all the compounds tested (Table 1). Examples of chromatograms of sample extracts analyzed in this study are presented in Figure 1. The retention times of the reduced phenolics were 6.50, 10.62, 21.45, 24.85, and 30.88 min for HA, 40HPPA, BA, DMCA, and CA, respectively, in the HPLC method developed for quantification of these compounds. The retention time of PPA was 28.48 min using this method. In the method developed for PPA quantification, the retention time was 15.57 min. Recovery of HA from ruminal fluid was low (Table 2). Recovery of BA and 40HPPA from ruminal fluid and HA from blood was relatively low with relatively large standard deviations; therefore, conclusions from these results were limited. The source of background 40HPPA in reagent blanks of blood extractions is not known. The concentrations of 40HPPA that the background values would have equated to are .62 and .18 μM in Clay Center blood samples and University of Kentucky blood samples, respectively. Background value for CA in the University of Kentucky trial analysis was equivalent to .02 μM.

The concentrations of PPA in ruminal fluid were 597, 524, 317, and 322 μM for the 96% ground forage, 96% long forage, 60% ground forage, and 60% long forage diets with a SEM of 26. Increasing orchardgrass hay in the diet increased concentration of PPA
in ruminal fluid \( (P < .05) \). Only trace amounts of CA \( (< .006 \mu M) \) were detected in ruminal fluid.

Concentrations of p-coumaric and ferulic acids observed in bromegrass, bermudagrass, and ryegrass-wheat hays fed in the Clay Center trial were 3.22 and 8.97, 14.14 and 6.43, and 6.82 and 4.79 g/kg DM, respectively (Table 3). Only trace amounts of CA \( (< .05 \mu M) \) were detected in hepatic portal venous blood of wethers fed bromegrass, bermudagrass, or a mixture of ryegrass and wheat hays (Table 4). Concentration of PPA in hepatic portal venous blood was positively correlated with p-coumaric acid \( (r = .57) \), ferulic acid \( (r = .67) \), or total phenolic monomer \( (r = .61) \) intakes.

Wethers in the University of Kentucky trial were fed 843 g of alfalfa hay DM per day. The concentrations of p-coumaric and ferulic acids observed in alfalfa hay were 2.53 and 2.48 g/kg of DM, respectively. This resulted in intakes of p-coumaric and ferulic acids of 13.52 and 10.77 mmol/d for a total phenolic monomer intake of 24.29 mmol/d. Recovery of the internal standard (DMCA) averaged 59.1% (SD = 6.7) over the 57 samples analyzed.

Cysteamine decreased arterial concentration of BA but increased arterial, portal venous, and hepatic venous concentration of PPA (Table 5). Only trace amounts of CA \( (< .022 \mu M) \) were detected in the mesenteric arterial and hepatic portal venous and hepatic venous blood in this trial. Exogenous somatostatin decreased arterial and hepatic venous concentrations of BA and PPA. The treatments did not affect arterial portal venous or hepatic venous concentrations of HA. The mean HA concentration across treatments was 5.13, 5.27, and 7.31 \( \mu M \) with a SEM of .33, .38, and .41 \( \mu M \) for mesenterial arterial and hepatic portal and hepatic venous blood, respectively.

The treatments did not affect fluxes of HA across the PDV, liver, or splanchnic bed. The mean HA fluxes across treatments for these organs were 10.1, 164.1, or 174.2 \( \mu mol/h \) with a SEM of 8.0, 19.3, or 18.0 \( \mu mol/h \), respectively. Net flux of HA across the PDV was not significant \( (P > .10) \). There was net release of PPA from the PDV for all treatments (Table 6). There was a net release of BA \( (P < .10) \) from PDV for the cysteamine plus somatostatin treatment.

Net uptake of PPA by the liver was significant for all treatments. There was a net uptake of BA for the cysteamine control treatment, regardless of somatostatin infusion. However, there was only a trend \( (P < .09) \) for the effect of cysteamine on hepatic flux of BA.

Benzoic acid was removed by the splanchnic bed in the absence of cysteamine infusion and released from the splanchnic bed for the cysteamine minus somatostatin treatment \( (P < .10) \). The F-test for effect of cysteamine infusion on net splanchnic BA uptake was significant.

Because the liver consistently removed BA and PPA from the blood, the hepatic extraction ratio was

### Table 1. Y-Intercept, slope, and correlation \( (r) \) derived from the regression of measured detector response \((\mu M)\) on concentration standard solution \((\mu M)\) over the detectable range

<table>
<thead>
<tr>
<th>Compound</th>
<th>Y-intercept</th>
<th>Slope</th>
<th>( r )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippuric acid</td>
<td>-0.0073</td>
<td>.956</td>
<td>.9996</td>
</tr>
<tr>
<td>3-(4-Hydroxyphenyl)propionic acid</td>
<td>.053</td>
<td>.946</td>
<td>.9998</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>.107</td>
<td>.951</td>
<td>.9996</td>
</tr>
<tr>
<td>Trans-3,4-dimethoxycinnamic acid</td>
<td>.00529</td>
<td>1.61</td>
<td>1.0000</td>
</tr>
<tr>
<td>3-Phenylpropionic acid</td>
<td>.94</td>
<td>.954</td>
<td>.9996</td>
</tr>
<tr>
<td>Trans-cinnamic acid</td>
<td>.00330</td>
<td>.964</td>
<td>.9998</td>
</tr>
</tbody>
</table>

\(^a\text{Concentrations of standard solutions are given in the Materials and Methods section. Only concentrations that were consistently detectable were included in the regression. Peaks were not consistently detected below .5, .5, 1, 1.33, .013, and .025 \( \mu M \) for hippuric, 3-(4-hydroxyphenyl)propionic, benzoic, 3-phenylpropionic, t-cinnamic, or \( t \)-3,4-dimethoxycinnamic acids, respectively. Detector response was converted to concentration \((\mu M)\) by calibration with a single standard.}\)

### Table 2. Recoveries \((%)\) of standards extracted from blood and ruminal fluid

<table>
<thead>
<tr>
<th>Item</th>
<th>Hippuric acid</th>
<th>Benzoic acid</th>
<th>3-Phenylpropionic acid</th>
<th>Cinnamic acid</th>
<th>3-(4-Hydroxyphenyl)propionic acid</th>
<th>3,4-Dimethoxycinnamic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood ((n = 5))</td>
<td>53.09</td>
<td>79.77</td>
<td>71.37</td>
<td>53.91</td>
<td>72.02</td>
<td>39.19</td>
</tr>
<tr>
<td>SD(^a)</td>
<td>21.64</td>
<td>3.55</td>
<td>3.72</td>
<td>4.00</td>
<td>7.13</td>
<td>2.03</td>
</tr>
<tr>
<td>Ruminal fluid ((n = 4))</td>
<td>14.17</td>
<td>24.45</td>
<td>98.46</td>
<td>75.79</td>
<td>52.55</td>
<td>82.88</td>
</tr>
<tr>
<td>SD(^a)</td>
<td>3.80</td>
<td>21.45</td>
<td>4.60</td>
<td>1.56</td>
<td>10.24</td>
<td>1.75</td>
</tr>
</tbody>
</table>

\(^a\text{Standard deviation of the mean.}\)
PORTAL AND HEPATIC REDUCED PHENOLIC FLUXES

Figure 1. Example chromatograms of extract of ruminal fluid (a,b) and extract of hepatic portal venous blood (c,d). Extraction and chromatographic methods are described in the Materials and Methods section. Chromatograms a and c were generated using the method for analysis of hippuric, 3-(4-hydroxyphenyl)propionic, benzoic, and t-cinnamic acids. Chromatograms b and d were generated using the method for analysis of 3-phenylpropionic acid. For all samples, a 50-μL volume of sample was injected. Samples analyzed in chromatograms a and b were extracts of ruminal fluid from a steer consuming a 96% ground orchardgrass diet (University of Illinois trial), and c and d were extracts of hepatic portal venous blood from a wether consuming ryegrass-wheat hay on an ad libitum basis (Clay Center trial). Chromatogram d was generated using a strip chart recorder that did not print the axes; however, retention times are the same as for chromatogram b. Extract analyzed in chromatogram b was diluted 5x. Peaks identified in the chromatograms are as follows. In panel a, 1 = 3-(4-hydroxyphenyl)propionic acid, 2 = benzoic acid, 3 = internal standard (3,4-dimethoxyphenylpropionic acid), and 4 = t-cinnamic acid; in panel b, 1 = 3-phenylpropionic acid; in panel c, 1 = hippuric acid, 2 = 3-(4-hydroxyphenyl)propionic acid, 3 = benzoic acid, and 4 = t-cinnamic acid; in panel d, 1 = 3-phenylpropionic acid.

calculated for these reduced phenolics. The hepatic extraction ratio for a reduced phenolic is the net hepatic uptake of the reduced phenolic expressed as a percentage of the total delivery of the reduced phenolic to the liver by the hepatic portal vein and hepatic artery. Cysteamine decreased BA extraction (hepatic extraction ratios of 29.5 and 3.22% for control and cysteamine infusion, respectively; LSD = 18.14). There was no effect on hepatic extraction of PPA (average = 79.5%; LSD = 7.08).
Table 3. Dry matter (g/d) and phenolic monomer (mmol/d) intakes of wethers with ad libitum access to bromegrass hay, bermudagrass hay, or a mixture of ryegrass and wheat hays (Clay Center trial)\(^a\)

<table>
<thead>
<tr>
<th>Item</th>
<th>Bromegrass</th>
<th>Bermudagrass</th>
<th>Ryegrass-wheat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter intake</td>
<td>616 77 6</td>
<td>558 187 8</td>
<td>850 155 5</td>
</tr>
<tr>
<td>Phenolic monomer intake</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(p)-Coumaric acid</td>
<td>19.6 3.7 6</td>
<td>48.1 16.1 8</td>
<td>35.3 6.4 5</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>11.4 1.8 6</td>
<td>18.4 6.0 8</td>
<td>20.9 3.8 5</td>
</tr>
<tr>
<td>Total(^b)</td>
<td>31.1 5.4 6</td>
<td>66.5 22.1 8</td>
<td>56.2 10.2 5</td>
</tr>
</tbody>
</table>

\(^a\)Values are least squares means (LSM).
\(^b\)The total is the sum of \(p\)-coumaric and ferulic acid intakes.

Discussion

Martin (1982b) concluded that phenolic monomers are not absorbed directly by ruminants; rather, ruminal microorganisms metabolize them to more chemically reduced phenyl ring-containing acids (referred to as reduced phenolics in the current discussion) that subsequently are absorbed. Large ruminal disappearances (Bourquin et al., 1990), the presence of bacterial species in ruminal fluid that reduce phenolics (Krumholz and Bryant, 1986), conversion of phenolics to more reduced forms in other anaerobic environments (Young and Frazer, 1987), and relatively low concentrations of \(p\)-coumaric and ferulic acids and high concentrations of PPA and other reduced phenolics in ruminal fluid (Chesson et al., 1982) support this conclusion. Also, the observation of Chesson et al. (1982) that PPA is not detectable in alkali extracts of forages suggests that ruminal PPA is derived from ruminal metabolism.

We hypothesized that BA, PPA, and CA, and 4OHPPA would be present in hepatic portal venous blood because they were present in ruminal fluid (Chesson et al., 1982; Daolio et al., 1989) and, based on appearance of phenyl ring-containing compounds in urine, were readily absorbed when infused into the abomasum of sheep (Martin, 1982a). Additionally, based on the conclusions of Martin (1982a,b) that reduced phenolics are absorbed from the gastrointestinal tract, we hypothesized that there would be a net positive flux of reduced phenolics across the PDV. Scheliner (1978) and Martin (1982a) hypothesized that reduced phenolics are converted to BA and subsequently to HA by the liver. Therefore, we hypothesized that there would be a net negative flux of reduced phenolics across the liver.

In our study, BA, PPA, and trace amounts of CA, but not 4OHPPA, were identified in hepatic portal venous blood of wethers fed a variety of grass hays or alfalfa hay. Also, these compounds were detected in the arterial and hepatic venous blood of wethers fed alfalfa hay. Reports of concentrations of reduced phenolics in blood are rare and previous reports of concentrations of reduced phenolics in hepatic portal venous blood or of net PDV or hepatic fluxes of reduced phenolics in ruminants is not known. Scott et al. (1964), using GLC, measured concentrations of BA and PPA in peripheral blood of sheep fed 1 kg of hay and .2 kg of oats per day. They found concentrations of 10 \(\mu M\) BA and PPA in peripheral blood. We did not measure concentrations of reduced phenolics in peripheral blood but expect that peripheral blood concentrations would be similar to mesenteric arterial and hepatic venous blood concentrations of reduced phenolics. We observed similar concentrations of BA but lower concentrations of PPA in the mesenteric artery and hepatic venous blood compared to peripheral blood concentrations reported by Scott et al. (1964).

Table 4. Concentrations of 3-\((4\)-hydroxyphenyl\)propionic (4OHPPA), benzoic (BA), and 3-phenylpropionic (PPA) (\(\mu\)mol/L) acids in hepatic portal venous blood of wethers with ad libitum access to bromegrass hay, bermudagrass hay, or a mixture of ryegrass and wheat hays (Clay Center trial)\(^a\)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Bromegrass</th>
<th>Bermudagrass</th>
<th>Ryegrass-wheat</th>
</tr>
</thead>
<tbody>
<tr>
<td>4OHPPA(^b)</td>
<td>ND(^c) 6</td>
<td>ND 8</td>
<td>ND 5</td>
</tr>
<tr>
<td>BA</td>
<td>1.24 .30 6</td>
<td>1.11 .35 8</td>
<td>2.39 .21 5</td>
</tr>
<tr>
<td>PPA</td>
<td>7.00 1.83 6</td>
<td>16.66 4.90 8</td>
<td>19.06 4.37 5</td>
</tr>
</tbody>
</table>

\(^a\)Values are least squares means (LSM) adjusted based on recovery of standards from samples (recovery data not shown).
\(^b\)Observed background of .62 \(\mu M\) 4OHPPA was subtracted from the least squares means.
\(^c\)ND = not detectable.
Table 5. Arterial, portal, and hepatic venous concentrations (µM) of benzoic (BA), 3-phenylpropionic (PPA), and 3-(4-hydroxyphenyl)propionic (4OHPPA) acids in wethers treated with cysteamine and somatostatin (University of Kentucky trial)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Item</th>
<th>(-) Cysteamine</th>
<th>(+) Cysteamine</th>
<th>SEM</th>
<th>Effects\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Somatostatin</td>
<td>Somatostatin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(-)</td>
<td>(+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arterial concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA</td>
<td>2.94</td>
<td>2.35</td>
<td>2.37</td>
<td>1.80</td>
</tr>
<tr>
<td>PPA</td>
<td>.46</td>
<td>.32</td>
<td>1.20</td>
<td>.49</td>
</tr>
<tr>
<td>4OHPPA\textsuperscript{c}</td>
<td>ND\textsuperscript{d}</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Portal venous concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA</td>
<td>3.54</td>
<td>2.85</td>
<td>2.91</td>
<td>2.61</td>
</tr>
<tr>
<td>PPA</td>
<td>2.89</td>
<td>2.22</td>
<td>3.85</td>
<td>3.10</td>
</tr>
<tr>
<td>4OHPPA\textsuperscript{c}</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Hepatic venous concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA</td>
<td>2.47</td>
<td>1.98</td>
<td>2.80</td>
<td>1.98</td>
</tr>
<tr>
<td>PPA</td>
<td>.56</td>
<td>.20</td>
<td>1.13</td>
<td>.55</td>
</tr>
<tr>
<td>4OHPPA\textsuperscript{c}</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values are least squares means adjusted based on recovery of standards from blood (Table 2). There were five observations for each mean for all treatments except the (+) cysteamine, (+) somatostatin treatment, for which there were four observations.

\textsuperscript{b}Significant effects according to the \( F \)-test \((P < .05)\). C = cysteamine, S = somatostatin.

\textsuperscript{c}Observed background of .18 µM 4OHPPA was subtracted from the least squares means.

\textsuperscript{d}ND = not detectable.

Consistent with our hypothesis, we observed net release of PPA from PDV. Significant uptake of BA from PDV was not consistently detected (University of Kentucky trial). This may be the result of large variations in measurement of PDV fluxes. We detected only trace amounts and failed to detect net releases of CA and 4OHPPA, probably because of relatively low ruminal concentrations in the case of CA (Chesson \textit{et al.}, 1982) or poor absorption in the case of 4OHPPA (Martin, 1982b).

Consistent with our hypothesis concerning liver reduced phenolic metabolism, we observed net uptake of PPA and BA by the liver. Extraction of PPA by liver was almost quantitative \((79.5\%)\), resulting in virtually complete removal of PPA absorbed from the gut as evidenced by insignificant net release of PPA from the

Table 6. Blood flow and net portal-drained visceral (PDV) and hepatic flux of benzoic (BA), 3-phenylpropionic (PPA), and 3-(4-hydroxyphenyl)propionic (4OHPPA) acids in wethers treated with cysteamine and somatostatin (University of Kentucky trial)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Item</th>
<th>(-) Cysteamine</th>
<th>(+) Cysteamine</th>
<th>SEM</th>
<th>Effects\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Somatostatin</td>
<td>Somatostatin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(-)</td>
<td>(+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Portal blood flow, L/h</td>
<td>70</td>
<td>69</td>
<td>85</td>
<td>71</td>
</tr>
<tr>
<td>Hepatic blood flow, L/h</td>
<td>81</td>
<td>81</td>
<td>98</td>
<td>89</td>
</tr>
<tr>
<td>PDV flux, µmol/h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA</td>
<td>42.3</td>
<td>34.8</td>
<td>41.8</td>
<td>58.7\textsuperscript{f}</td>
</tr>
<tr>
<td>PPA</td>
<td>166.8\textsuperscript{*}</td>
<td>132.0\textsuperscript{*}</td>
<td>225.7\textsuperscript{*}</td>
<td>186.5\textsuperscript{*}</td>
</tr>
<tr>
<td>4OHPPA</td>
<td>ND\textsuperscript{c}</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Hepatic flux, µmol/h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA</td>
<td>-78.1\textsuperscript{*}</td>
<td>-64.9\textsuperscript{*}</td>
<td>-5.4</td>
<td>-41.1</td>
</tr>
<tr>
<td>PPA</td>
<td>-157.2\textsuperscript{*}</td>
<td>-141.5\textsuperscript{*}</td>
<td>-231.8\textsuperscript{*}</td>
<td>-180.2\textsuperscript{*}</td>
</tr>
<tr>
<td>4OHPPA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Total splanchnic flux, µmol/h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA</td>
<td>-35.8\textsuperscript{g}</td>
<td>-30.9\textsuperscript{g}</td>
<td>36.5\textsuperscript{g}</td>
<td>17.2</td>
</tr>
<tr>
<td>PPA</td>
<td>9.6</td>
<td>-9.4</td>
<td>-6.1</td>
<td>6.3</td>
</tr>
<tr>
<td>4OHPPA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values are least squares means adjusted based on recovery of standards from blood (Table 2). There were five observations for each mean for all treatments except the (+) cysteamine, (+) somatostatin treatment, for which there were four observations.

\textsuperscript{b}Significant effects according to the \( F \)-test \((P < .05)\). C = cysteamine, S = somatostatin, C × S = cysteamine × somatostatin interaction.

\textsuperscript{c}ND = not detectable.

\textsuperscript{*}Value is different from zero according to the \( F \)-test \((P < .05)\).

\textsuperscript{f}Value is different from zero according to the \( t \)-test \((P < .05)\).

\textsuperscript{g}Value is different from zero according to the \( t \)-test \((P < .01)\).
splanchnic bed. In contrast, extraction of BA by liver averaged only 29.5 and 3.2% for control and cysteamine treatments, respectively. This could explain why the arterial concentration of BA is approximately five times greater than that of PPA. The ability of cysteamine to decrease hepatic extraction of BA could be the result of cysteamine increasing hepatic portal venous blood flow, resulting in reduced time available for hepatocytes to remove BA.

Concentrations of p-coumaric and ferulic acids observed in the forages from the Clay Center and University of Kentucky trials are in general agreement with previously published data (Jung et al., 1983a,b). Because p-coumaric and ferulic acids could be metabolized to reduced phenolics in the rumen and because of their relatively large concentrations in forages (Fahey and Jung, 1989), we hypothesized that they could be significant precursors of ruminal reduced phenolics. We made some observations in our study consistent with this hypothesis. First, we observed that increasing the dietary forage level increased the concentration of PPA in ruminal fluid. In the study from which our ruminal fluid samples were obtained, Bourquin et al. (1994) observed that increasing the dietary forage level increased ruminal disappearance of p-coumaric and ferulic acids (31.8 and 42.8% of p-coumaric acid and 62.6 and 73.8% of ferulic acid for the 60 and 96% forage levels, respectively). This increased p-coumaric and ferulic acid disappearance correlates with the increased ruminal concentration of PPA observed in our study, suggesting that a significant amount of PPA in the rumen is derived from ruminal metabolism of p-coumaric and ferulic acids. Second, hepatic portal venous blood PPA concentrations correlated with intakes of p-coumaric or ferulic acids in the Clay Center trial, suggesting that blood PPA is derived from dietary p-coumaric and ferulic acids. These observations support the hypothesis that p-coumaric and ferulic acids are significant precursors of ruminal and hepatic portal venous PPA.

We hypothesized that because the ruminal epithelium is a highly metabolically active tissue and is constantly exposed to ruminal reduced phenolics, it might be a major site of HA production. This would result in a net positive flux of HA across the PDV. Contrary to this hypothesis, we did not observe any net flux of HA across PDV in our analysis of blood from the University of Kentucky trial. Our results show that the liver, not the PDV, is the major producer of HA released by splanchnic tissues.

In the absence of cysteamine infusion, there was a net uptake of BA by the splanchnic bed as a result of increased extraction by liver. This implies that BA is produced in peripheral tissues. It is unlikely that there is significant de novo production of BA by peripheral tissues. One possible source of this peripheral contribution to blood BA could be hydrolysis of HA by the extra-splanchnic tissues.

In summary, PPA and BA were the most concentrated reduced phenolics detected in hepatic portal venous blood. The concentration of PPA in ruminal fluid varied with ruminal disappearance of p-coumaric and ferulic acids from orchardgrass and the concentration in the hepatic portal venous blood varied with intake of p-coumaric and ferulic acids, suggesting that a significant amount of PPA is derived from ruminal metabolism of these acids. The only reduced phenolic for which there was consistent net release from PDV was PPA. The liver efficiently removed PPA but not BA from the blood.

Implications

Reduced phenolics were identified in ruminal fluid and blood perfusing liver. Of those identified, the most concentrated reduced phenolic in both ruminal fluid and blood perfusing liver was 3-phenylpropionic acid. The presence of this reduced phenolic in relatively high concentrations suggests that research concerning the effects of 3-phenylpropionic acid on the ruminant liver is needed.

Literature Cited


Krumholz, L. R., and M. P. Bryant. 1986. *Syntrophococcus suromutans*, sp. nov. gen. nov. uses carbohydrates as electron donors and formate, methoxymonomobenzenoids or *Methanobreuibacter* as electron acceptor systems. Arch. Microbiol. 143:313.


