

Effects of Glycine and Bovine Serum Albumin on Inhibition of Propionate Metabolism in Ovine Hepatocytes Caused by Reduced Phenolic Monomers¹

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ABSTRACT: Hepatocytes isolated from sheep were incubated in the presence of reduced phenolics and glycine to determine the effects of these compounds on hepatic propionate metabolism *in vitro*. 3-Phenylpropionic (PPA) or *t*-cinnamic (CA) acids, but not benzoic (BA) or 3-(4-hydroxyphenyl)propionic (4OHPPA) acids, decreased conversion of propionate to glucose at .05 mM in the absence of supplemental glycine. At 1.2 mM, all reduced phenolics decreased conversion of propionate to glucose in the absence of supplemental glycine. Addition of glycine to the incubation medium linearly alleviated the inhibition

by BA, PPA, or CA, suggesting that physiological glycine concentrations limited alleviation of inhibition of propionate metabolism. Hippuric acid production increased as glycine concentration increased in the presence of PPA, CA, or 4OHPPA. Bovine serum albumin did not alleviate inhibition of conversion of propionate to glucose caused by BA, PPA, or CA and slightly alleviated inhibition caused by 4OHPPA (.4 mM). Of the reduced phenolics tested, PPA is the most likely to inhibit gluconeogenesis from propionate in ovine liver *in vivo*.

Key Words: Propionates, Phenolic Acids, Liver Cells, Sheep, Gluconeogenesis

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Introduction

Evidence suggests that ruminal microorganisms chemically reduce substituents of the phenyl ring of dietary phenolic monomers (Martin, 1982a,b). Potential products of this reduction that have been identified in ruminal fluid are benzoic acid (BA), 3-phenylpropionic acid (PPA), *t*-cinnamic acid (CA), and 3-(4-hydroxyphenyl)propionic acid (4OHPPA) (Daolio et al., 1989; Chesson et al., 1982). Because these compounds are probably the products of chemical reduction of dietary phenolic monomers, we refer

to them as reduced phenolics. These compounds are likely to be absorbed (Martin, 1982b) and have been shown to inhibit metabolism of propionate and palmitate by bovine liver tissue *in vitro* (Cremin et al., 1994).

The proposed detoxification of reduced phenolics is β -oxidation of the side-chain and subsequent conjugation with glycine producing hippuric acid (HA) or, in the case of 4OHPPA, 4-hydroxybenzoic acid, in the liver (Scheline, 1978; Figure 1). The first step in β -oxidation is esterification with CoA. Evidence suggests that reduced phenolics inhibit metabolism by decreasing the concentration of free CoA as concentrations of reduced phenolic-CoA esters increase (Cyr et al., 1991). We hypothesized that providing hepatocytes that are exposed to reduced phenolics with glycine would result in conversion of reduced phenolic-CoA esters to HA and consequently free CoA, thereby alleviating inhibition of gluconeogenesis from propionate.

Krebs et al. (1974) suggested that BSA nonspecifically binds a variety of toxins; therefore, we hypothesized that BSA would alleviate the observed inhibition of gluconeogenesis.

Previous research (Cremin et al., 1994) tested the ability of reduced phenolics to inhibit gluconeogenesis

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when present individually. We tested whether there was an interaction among reduced phenolics on inhibition of gluconeogenesis.

Materials and Methods

Reagents

Validation Study. Sodium pentobarbital was obtained from Anpro Pharmaceutical (Arcadia, CA), the sodium salt of [2-¹⁴C]propionate was from ICN Radiochemicals (Irvine, CA), and [1-³H]L-glucose was from Du Pont NEN Products (Boston, MA). All other chemicals were cell-culture tested or were of the highest purity available from Sigma Chemical (St. Louis, MO). The perfusion and wash media were the same as those used by Donkin and Armentano (1993) with the exception that the BSA (bovine albumin Cohn fraction V, charcoal-treated, dialyzed) was not dialyzed further before use. The incubation medium was Krebs-Ringer bicarbonate (KRB) buffer (Laser, 1961) containing 5 mg/L of phenol red and 2.0 mM CaCl₂·H₂O. To the buffer (25 mM) HEPES (pH 7.4) was added, and an equimolar amount of NaCl was removed to maintain equivalent osmolality. All media were filter-sterilized through a sterile .22- μ m Millex[®]-GV filter (Millipore, Bedford, MA). A concentrated stock solution of 150 mM [2-¹⁴C]propionate (specific radioactivity .033 μ Ci/ μ mol) was prepared by the following method. The required amount of [2-¹⁴C]propionate was dissolved in ethanol, and an equal volume of .5 M HEPES (pH 7.4) was added. The ethanol and water were evaporated under a stream of air. The appropriate mass of nonradioactive sodium propionate was added to the flask, and the compounds then were dissolved in KRB in which an equimolar amount of HEPES (pH 7.4) was substituted for NaHCO₃ (**KRB-H**).

Experiment 1. The same reagents were used as in the validation study. Additionally, concentrated stock solutions of glycine (3.6, 18, 36, or 72 mM) and reduced phenolics (12 mM) were prepared for addition to the medium. The glycine stock solutions were prepared by dissolving glycine in the neutral zwitterionic form in KRB-H (pH 7.4). To eliminate effects caused by differences in osmolality resulting from added glycine, a control stock solution for glycine was prepared by dissolving NaCl (72 mM) in KRB-H (pH 7.4). Stock solutions of reduced phenolics were prepared by adding the reduced phenolic acid to KRB-H, adjusting the pH to 7.4 with 1 N NaOH, and stirring for 2 h in a water bath at 40°C. To eliminate effects caused by differences in osmolality resulting from addition of reduced phenolic acids and pH adjustment, a control stock solution was prepared using the same procedure, except that an equimolar amount of 1 N HCl was substituted for reduced phenolic. Hydrochloric acid was used as the control compound because the

reduced phenolics were added in their acid forms.

Experiment 2. Concentrated stock solutions of glycine (10.5 mM), the reduced phenolics (.5 and 4 mM), and HA (12 mM) were prepared according to the procedure used for Exp. 1. The reduced phenolic control stock solutions were .5 and 4 mM HCl prepared and neutralized as described in Exp. 1. The BSA (bovine albumin Cohn fraction V, essentially fatty acid free) added to the incubation medium was from Sigma Chemical.

Isolation of Hepatocytes

For the validation study, hepatocytes were isolated from the caudal process of livers from Dorset wethers (average BW 52 kg) 7 to 8 mo of age. After weaning (8 wk), wethers were allowed ad libitum access to a 20:80 mixture of alfalfa hay:corn. Similar wethers were used for Exp. 1 (average BW 52 kg, aged 6 to 9 mo) and Exp. 2 (average BW 45 kg, aged 4 to 6 mo). The experiments were replicated with three wethers in the validation study, four wethers in Exp. 1, and four wethers in Exp. 2.

Hepatocytes were isolated using the procedures of Donkin and Armentano (1993) with minor modifications. Procedures for anesthesia and euthanasia were approved by the University of Illinois Laboratory Animal Care Advisory Committee. Wethers were given heparin USP (30,000 units) and then anesthetized by intrajugular injection of sodium pentobarbital (30 mg/kg BW). The procedures of Donkin and Armentano (1993) were used for the removal of the caudate process from the anesthetized wether, perfusion of the caudate process, dispersion of the hepatocytes, and washing of the hepatocytes except that the final hepatocyte wash was conducted using the incubation medium. Hepatocytes were suspended in a volume of incubation medium that resulted in a packed cell volume of 1.7 to 2.6% (3.8 to 7.1 mg DM/mL cells). Viability of hepatocytes was estimated in Exp. 2 by staining with trypan blue and was found to be greater than 90%.

Incubations and Experimental Design

Validation Study. This study was conducted to determine the range of incubation times within which the rate of production of glucose and CO₂ from [2-¹⁴C]propionate was linear. An aliquot (.5 mL) of the hepatocyte suspension was pipetted into a 25-mL Erlenmeyer flask containing 2.4 mL of incubation medium, .1 mL of [2-¹⁴C]propionate stock solution (final concentration of 5 mM), and 30 mg of gelatin (type B, bovine skin, 225 bloom). Hepatocytes were kept in suspension during pipetting by stirring with a magnetic stir bar. The flasks were capped with rubber septa, with a glass well for CO₂ collection suspended from the cap. Flasks were placed in a shaking water bath (39°C), and gassed with O₂-CO₂ (95:5, vol/vol)

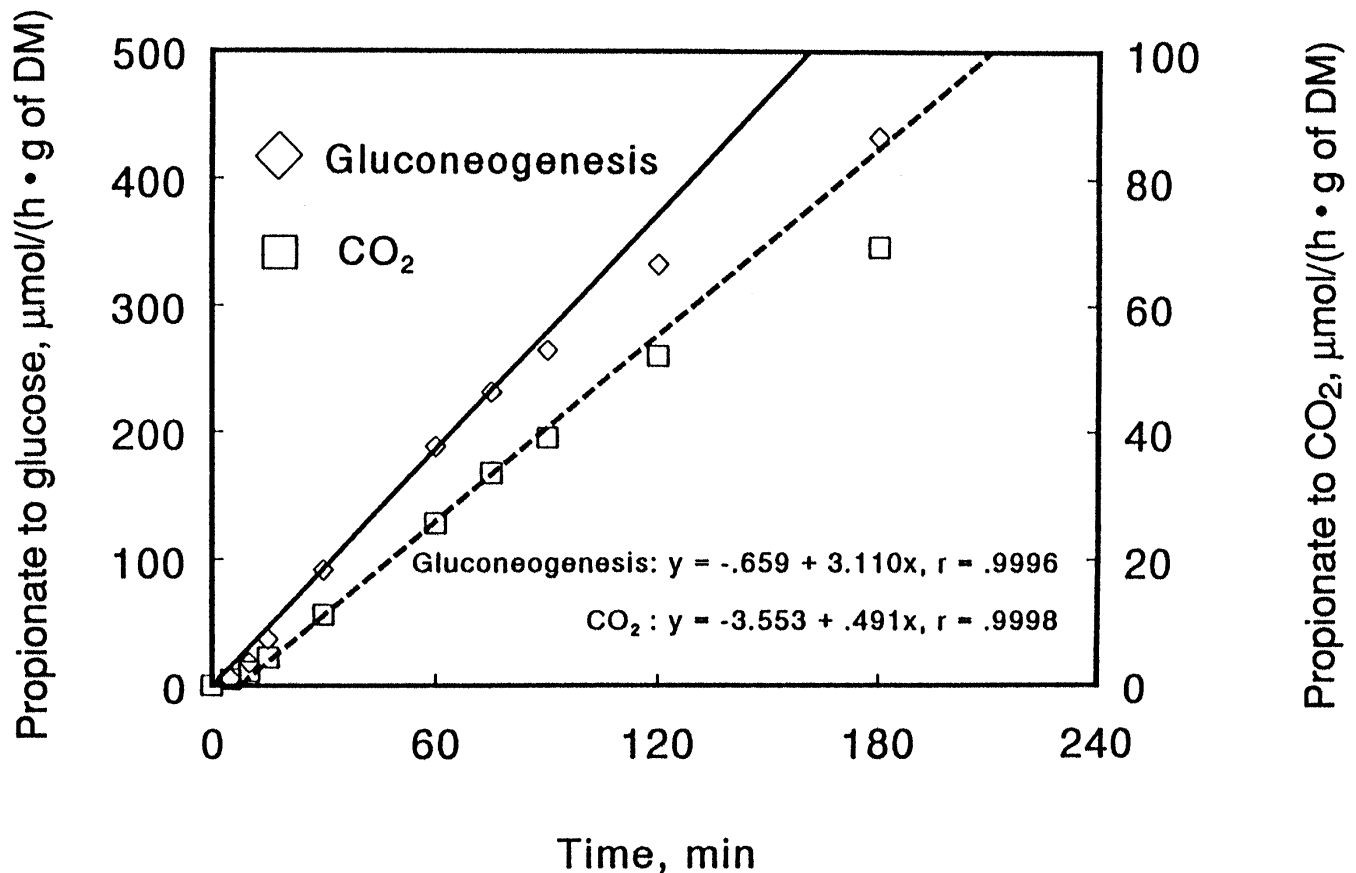


Figure 2. Results of the validation study which was conducted to determine the range of incubation time in which conversion of [2-¹⁴C]propionate to glucose and CO₂ was linear. Values used in the calculation of the regression equations presented are only those that were determined to be within the linear range (30, 60, and 75 min of incubation).

was 2.9% and .28% of the radiation found in flasks incubated for 75 min for the CO₂ and glucose measurements, respectively. In subsequent experiments, radiation from background flasks was subtracted from treatment values. The [¹⁴C]-glucose radioactivity in each sample was adjusted based on the recovery of radioactivity from the internal standard, which averaged 79.4% (6.5 SD). The DM content of triplicate .5-mL aliquots of hepatocyte suspension and incubation medium were determined by drying the aliquots in a forced-air oven at 105°C for 4 h. The DM in the incubation medium was subtracted from the DM in the hepatocyte suspension to determine the amount of hepatocyte DM added to the flasks. Rates of conversion of [2-¹⁴C]propionate to glucose and CO₂ were expressed as the micromoles of propionate converted per hour per gram of hepatocyte DM.

Results from this validation study showed that conversion of propionate to glucose and CO₂ was linear from 30 to 75 min of incubation (for this interval the regression equations were $y = -.659 + 3.110x$, $r = .9996$ and $y = -3.553 + .491x$, $r = .9998$ for

conversion of propionate to glucose and CO₂, respectively; Figure 2); therefore, in subsequent experiments, a pre-incubation of 30 min was conducted. After the pre-incubation period, treatment stock solutions were injected into the medium and the incubation continued for 45 min. To estimate the conversion of [2-¹⁴C]propionate to glucose and CO₂ during the 30-min pre-incubation, additional duplicate flasks from each replicate were killed at the end of the pre-incubation period and treated as described above. The radioactivity in these flasks was subtracted from the radioactivity in the treatment flasks before rates of conversion were calculated.

Experiment 1. This experiment tested the hypothesis that glycine would alleviate inhibition of [2-¹⁴C]propionate metabolism caused by BA, PPA, CA, or 4OHPPA and determined the relationship between glycine concentration and alleviation of inhibition. The procedures were the same as those described for the validation experiment with the following exceptions. Flasks contained 2.0 mL of incubation buffer and .1 mL of substrate stock solution at the start of the pre-incubation. After the pre-incubation period, .3 mL of

the appropriate reduced phenolic stock solution and .1 mL of the appropriate glycine stock solution were injected into the medium with 1-mL syringes, resulting in concentrations of 1.2 mM for the reduced phenolics and 0, .12, .6, 1.2, or 2.4 mM for glycine at the start of the incubation period. After incubation and addition of the internal standard, a .5-mL aliquot of medium was removed with a 1-mL syringe and stored at -70°C until analysis for concentrations of reduced phenolics. Subsequently, the remainder of the medium was acidified and analyzed for [^{14}C]-glucose according to the procedures used in the validation study. After removal of the .5-mL aliquot, recovery of [$1\text{-}^3\text{H}$]L-glucose averaged 66.5% (6.5 SD). Background radiation, expressed as a percentage of the radiation found in the control flasks after 75 min of incubation, averaged 1.8% for CO_2 and .27% for glucose measurements, respectively. Radiation in the pre-incubation flasks, expressed as a percentage of the radiation found in the control flasks after 75 min of incubation, averaged 33.1% for CO_2 and 29.9% for glucose measurements, respectively.

To determine the concentration of HA present at the end of the incubation, the .5-mL aliquot of medium was centrifuged at $12,400 \times g$ for 2 min. A .4-mL aliquot of the supernatant was removed and the internal standard *t*-3,4-dimethoxycinnamic acid (**DMCA**) was added (.8 mL of .3 mM DMCA), followed by 8 mL of 400 mM phosphate buffer (pH 2.0). After thorough mixing, the sample was centrifuged for 20 min at $25,900 \times g$ (0°C). Solid phase extraction was used to extract HA from the supernatant using a reverse-phase chromatography procedure modified from that described by Titgemeyer et al. (1991). Solid phase extraction columns containing .3 g of C-18 resin (Fisher Scientific, Pittsburgh, PA) were conditioned by washing twice with 2 mL of methanol and then rinsing three times with 2 mL of 40 mM phosphate buffer adjusted to pH 2.0 with concentrated HCl (buffer A). The supernatant was added to the conditioned column and pulled through under vacuum. The extraction column was washed three times with 2 mL of buffer A. Hippuric acid was eluted from the column by extracting it three times with 1 mL of buffer A-methanol (50:50, vol/vol).

The extract was analyzed using a reverse-phase HPLC separation and spectrophotometric detection described in Cremin et al. (1995). Identity of the HA peak was determined by comparison to the retention time of HA standard. Identification was aided by spiking of sample extract with authentic HA to determine whether this standard co-eluted with the peak tentatively identified as HA on the basis of retention time. Hippuric acid was found to have a retention time of 6.50 min and be detectable to a lower concentration of .5 μM . Detector response was linear through the range of concentrations detected. Recovery of the internal standard using the HA extraction

procedure averaged 93.8% (SD 4.7). After correction for internal standard recovery, results were expressed as micromoles of HA produced per hour of incubation per gram of cell DM.

The amount of added reduced phenolic remaining in the incubation medium after stopping metabolism with acid was determined by analyzing the HA extracts for BA, PPA, CA, and 4OHPPA using reverse-phase HPLC separation and spectrophotometric detection described in Cremin et al. (1995). To reduce the number of samples that needed to be analyzed, only the flasks with 0, .6, or 2.4 mM glycine were analyzed. Results were expressed as concentration (μM) in the medium.

Experiment 2. This experiment was conducted according to the procedures described in Exp. 1 with the same volumes of reduced phenolic and glycine stock solutions injected at the end of the pre-incubation period. Recovery of [$1\text{-}^3\text{H}$]L-glucose during glucose analysis averaged 70.1% (SD 10.6). Background radiation, expressed as a percentage of the radiation found in the control flasks after 75 min of incubation, averaged 2.6% for CO_2 and .45% for glucose measurements, respectively. Radiation in the pre-incubation flasks, expressed as a percentage of the radiation found in the control flasks after 75 min of incubation, averaged 37.9% and 42.4% for CO_2 and glucose measurements, respectively, when gelatin was present in the incubation medium and 38.4% and 38.8% for CO_2 for glucose measurements, respectively, when BSA was present in the incubation medium.

In this experiment we tested the hypotheses that 1) effects of reduced phenolics on propionate metabolism of hepatocytes varied with concentration of reduced phenolic and that different types of reduced phenolics differ in their ability to inhibit propionate metabolism; 2) substituting 3% (wt/vol) BSA for 1% (wt/vol) gelatin would alleviate inhibition of propionate metabolism; 3) a) the inhibition of propionate metabolism caused by the presence of all the reduced phenolics in the incubation medium would equal the sum of the inhibition of propionate metabolism caused by the individual reduced phenolics, and b) that glycine would alleviate inhibition caused by the combination of reduced phenolics; and 4) HA would not affect propionate metabolism.

To test hypothesis 1, we compared the rate of gluconeogenesis and CO_2 production from [$2\text{-}^{14}\text{C}$]propionate in flasks containing .05 or .4 mM BA, PPA, CA, or 4OHPPA. Additionally, we compared the reduced phenolic treatments to their respective control flasks. To test hypothesis 2, we compared the rate of gluconeogenesis and CO_2 production from [$2\text{-}^{14}\text{C}$]propionate in flasks containing .4 mM BA, PPA, CA, or 4OHPPA in the presence of either 1% (wt/vol) gelatin or 3% (wt/vol) BSA. To test hypothesis 3a, we compared rate of gluconeogenesis and CO_2 production from [$2\text{-}^{14}\text{C}$]propionate in flasks containing .05 mM

BA, PPA, CA, or 4OHPPA to flasks containing equimolar amounts of all four reduced phenolics (i.e., .2 mM total reduced phenolics). Additionally, we compared rate of gluconeogenesis and CO₂ production from propionate in the flasks containing combined reduced phenolics to flasks containing combined reduced phenolics and .35 mM glycine (hypothesis 3b). To test hypothesis 4, we compared rate of gluconeogenesis and CO₂ production from [2-¹⁴C]propionate in flasks containing 1.2 mM HA to .4 mM control flasks.

Statistical Analysis

In all experiments, incubations were conducted in duplicate. For Exp. 1 and the comparison of gelatin vs BSA in Exp. 2, rates for individual flasks were expressed as a percentage of the mean of control flasks (0 mM glycine and 0 mM reduced phenolic) within that duplicate (water bath). Statistical analysis was conducted on the duplicate averages using the GLM procedure of SAS (1985). A *P*-value equal to or less than .05 was considered significant.

Experiment 1. For data on conversion of [2-¹⁴C]propionate to glucose and CO₂ and production of HA, the significance of the effects of glycine concentration, reduced phenolic, and the interaction between glycine concentration and reduced phenolic was tested using the *F*-statistic calculated from the type I sums of squares (Freund et al., 1986). The data were blocked by wether. The effects of glycine concentration and glycine concentration × reduced phenolic interaction were partitioned into linear, quadratic, cubic, and quartic effects.

Data on concentration of reduced phenolic present in the medium at the end of incubation were analyzed as above, except that effects of glycine concentration were partitioned into linear and quadratic effects because only three levels of glycine concentration data were collected for this measurement.

If an effect was significant according to the *F*-test, differences among means were tested using the LSD statistic (Steel and Torrie, 1980). The LSD statistic and confidence intervals for the slopes and intercepts of the regression of dependent variables on glycine concentration were calculated according to the procedure of Steel and Torrie (1980). For HA production data, quadratic, cubic, and quartic effects of glycine and the interactions among quadratic, cubic, and quartic effects of glycine and types of reduced phenolic were highly nonsignificant (*P* > .25). These effects were eliminated from the model and the data re-analyzed.

Experiment 2. The four major hypotheses listed in the Incubations and Experimental Design section were individually analyzed as randomized complete block designs blocking by wether. Type III sums of squares were used to calculate the *F*-statistic. If an effect was significant according to the *F*-test, differences among means were tested using the LSD statistic (Steel and Torrie, 1980).

Results

Experiment 1

The interaction between the linear effect of glycine and the types of reduced phenolic was significant for conversion of [2-¹⁴C]propionate to glucose and CO₂. Coefficients for the regressions of rate of conversion of propionate to glucose or CO₂ or ratio of glucose:CO₂ on glycine concentration in the medium for each reduced phenolic are presented in Table 1. The slope of the regression of gluconeogenesis on concentration of glycine for the control treatment did not differ from zero (Table 1), as determined by comparison of the confidence interval to the difference between the slope and zero and the nonsignificant correlation coefficient (*P* > .05). From this observation, we infer that glycine did not affect gluconeogenesis from propionate within the range of concentrations tested in this study. All of the y-intercepts were negative, indicating that all reduced phenolics tested decreased gluconeogenesis in the absence of glycine (Table 1). The y-intercept for BA was greater than the y-intercept for the other reduced phenolics tested, suggesting that BA did not inhibit gluconeogenesis as much as the other reduced phenolics in the absence of glycine. The y-intercepts for PPA, CA, or 4OHPPA did not differ (Table 1). Slopes did not differ among the reduced phenolic treatments. The slope for 4OHPPA did not differ from the control or zero, suggesting that increasing glycine concentration in the medium did not affect gluconeogenesis in the presence of 4OHPPA. The slopes for BA, PPA, and CA were positive and differed from zero, suggesting that increasing glycine concentration increased gluconeogenesis in the presence of these reduced phenolics.

None of the slopes for conversion of [2-¹⁴C]propionate to CO₂ differed from zero. These observations suggest that the concentration of glycine did not affect the conversion of [2-¹⁴C]propionate to CO₂ in the presence or absence of the reduced phenolics. The y-intercepts for PPA, CA, and 4OHPPA differed from zero and were negative, suggesting that these reduced phenolics inhibited conversion of [2-¹⁴C]propionate to CO₂ in the absence of glycine. The y-intercepts for PPA and 4OHPPA were less than that for CA, indicating greater inhibition by PPA or 4OHPPA than CA.

Increasing glycine concentration increased the ratio of glucose to CO₂ in the presence of BA, PPA, or CA as demonstrated by slopes that were greater than zero. The control slope and 4OHPPA slope did not differ from zero, suggesting that glycine concentration did not affect the ratio of glucose to CO₂ in the absence of reduced phenolics or in the presence of 4OHPPA. The y-intercepts differed from zero and were negative, suggesting that all of the reduced phenolics decreased the ratio of glucose to CO₂. The y-intercept for BA was greater than the y-intercept for the other reduced

Table 1. y -Intercept, slope, and correlation coefficient (r) derived from the regression of gluconeogenesis and CO_2 production from $[2\text{-}^{14}\text{C}]$ propionate expressed as a percentage of the control value, and the ratio of these rates, on glycine concentration in the medium from hepatocyte incubations (Exp. 1) exposed to 1.2 mM benzoic acid (BA), 3-phenylpropionic acid (PPA), *t*-cinnamic acid (CA), or 3-(4-hydroxyphenyl)propionic acid (4OHPPA)^a

| Item | y -Intercept | CI ^b | Slope | CI | r |
|------------------------------|---------------------|-----------------|----------------------|-------|------|
| Gluconeogenesis | | | | | |
| Control | -1.07 ^x | 15.30 | -.22 ^y | 11.71 | .047 |
| BA | -43.27 ^y | | 15.71 ^x | | .951 |
| PPA | -78.82 ^z | | 12.89 ^x | | .990 |
| CA | -66.83 ^z | | 16.23 ^x | | .994 |
| 4OHPPA | -67.76 ^z | | 6.11 ^{xy} | | .936 |
| CO_2 production | | | | | |
| Control | 1.17 ^x | 11.07 | -5.17 ^y | 8.47 | .737 |
| BA | -4.53 ^{xy} | | 4.17 ^x | | .721 |
| PPA | -32.78 ^z | | 6.57 ^x | | .714 |
| CA | -11.87 ^y | | 2.64 ^{xy} | | .877 |
| 4OHPPA | -26.82 ^z | | 3.57 ^x | | .860 |
| Glucose: CO_2 ratio | | | | | |
| Control | 2.86 ^x | 15.20 | 2.93 ^z | 11.63 | .604 |
| BA | -41.73 ^y | | 13.08 ^{xy} | | .976 |
| PPA | -67.96 ^z | | 12.20 ^{xyz} | | .984 |
| CA | -62.89 ^z | | 15.24 ^x | | .990 |
| 4OHPPA | -57.37 ^z | | 5.44 ^{yz} | | .886 |

^aIntercepts are percentage change and slopes are percentage change/mM glycine.

^bConfidence interval ($P < .05$).

^{x,y,z}Means in the same column within each measurement with different superscript letters differ based on a protected LSD ($P < .05$).

phenolics, indicating that BA did not decrease the ratio of glucose to CO_2 as much as the other reduced phenolics in the absence of glycine.

Production of HA linearly increased as glycine concentration was increased in the presence of 1.2 mM BA, PPA, CA, or 4OHPPA (Table 2). The interaction between the linear effect of glycine and types of reduced phenolic was significant for HA production. The slope of the regression of HA production on glycine concentration differed from zero and was positive for PPA, CA, and 4OHPPA, suggesting that increasing glycine concentration increased HA production by ovine hepatocytes exposed to these reduced phenolics. None of the y -intercepts for the reduced

phenolics differed from zero, which indicates that there was no significant rate of HA production in the absence of glycine.

Effects of reduced phenolic, glycine, or their interaction on final concentrations of added reduced phenolic in the medium were not detected. Concentrations of reduced phenolic remaining at the end of the incubation were .97, .97, 1.30, and 1.16 (SEM .26, $n = 12$) for BA, PPA, CA, and 4OHPPA treatments, respectively.

Experiment 2

Addition of .05 mM PPA or CA to the medium inhibited conversion of $[2\text{-}^{14}\text{C}]$ propionate to glucose vs the .05 mM control (Table 3). All the reduced

Table 2. y -Intercept, slope, and correlation coefficient (r) derived from the regression of hippuric acid production ($\mu\text{mol}/[\text{h}\cdot\text{g}$ of DM]) on glycine concentration in the medium from hepatocyte incubations (Exp. 1) exposed to 1.2 mM benzoic acid (BA), 3-phenylpropionic acid (PPA), *t*-cinnamic acid (CA), or 3-(4-hydroxyphenyl)propionic acid (4OHPPA)^a

| Item | y -Intercept | CI ^b | Slope | CI | r |
|--------|--------------------|-----------------|--------------------|------|------|
| BA | 3.14 ^z | 12.58 | 3.79 ^z | 9.63 | .935 |
| PPA | 11.76 ^y | | 13.41 ^y | | .990 |
| CA | 8.13 ^{yz} | | 10.71 ^y | | .994 |
| 4OHPPA | 1.97 ^z | | 12.34 ^y | | .992 |

^aThe y -intercept is expressed as $\mu\text{mol}/(\text{h}\cdot\text{g}$ of DM) and the slope is expressed as $[\mu\text{mol}/(\text{h}\cdot\text{g}$ of DM)] $\cdot\text{mM}$ glycine⁻¹.

^bConfidence interval ($P < .05$).

^{y,z}Means in the same column within each measurement with different superscript letters differ based on a protected LSD ($P < .05$).

Table 3. Effects of concentration of benzoic, 3-phenylpropionic, *t*-cinnamic, or 3-(4-hydroxyphenyl)propionic acids on propionate metabolism by ovine hepatocytes^a

| Reduced phenolic and concentration, mM | [2- ¹⁴ C]Propionate to glucose, $\mu\text{mol}/(\text{h}\cdot\text{g of DM})^b$ | [2- ¹⁴ C]Propionate to CO ₂ , $\mu\text{mol}/(\text{h}\cdot\text{g of DM})^c$ | | |
|--|--|---|--------------------|--------------------|
| | | Means | Pooled means | Ratio ^d |
| Control ^e | | | | |
| .05 | 150.6 ^{VW} | 29.1 | 29.5 ^Z | 5.14 ^Z |
| .4 | 175.8 ^V | 30.0 | | 5.76 ^Z |
| Benzoic acid | | | | |
| .05 | 125.7 ^{WX} | 25.9 | 27.9 ^{YZ} | 5.03 ^Z |
| .4 | 83.1 ^{XY} | 29.8 | | 2.74 ^Y |
| 3-Phenylpropionic acid | | | | |
| .05 | 64.4 ^{YZ} | 22.1 | 20.8 ^X | 2.85 ^Y |
| .4 | 38.3 ^Z | 19.4 | | 1.95 ^Y |
| <i>t</i> -Cinnamic acid | | | | |
| .05 | 64.8 ^{YZ} | 25.0 | 25.8 ^Y | 2.44 ^Y |
| .4 | 62.3 ^{YZ} | 26.6 | | 2.33 ^Y |
| 3-(4-Hydroxyphenyl) propionic acid | | | | |
| .05 | 151.6 ^{VW} | 31.0 | 28.4 ^{YZ} | 4.97 ^Z |
| .4 | 70.4 ^{YZ} | 25.9 | | 2.74 ^Y |

^aValues are least squares means.

^bThe SEM for .4 mM control was 10.0 $\mu\text{mol}/(\text{h}\cdot\text{g of DM})$ (n = 9), and for all other values SEM was 14.9 $\mu\text{mol}/(\text{h}\cdot\text{g of DM})$ (n = 4).

^cOnly the effect of reduced phenolic was significant. The pooled means are the least squares means for this effect. The SEM for control was 1.1 $\mu\text{mol}/(\text{h}\cdot\text{g of DM})$ (n = 13), and for all other values SEM was 1.3 $\mu\text{mol}/(\text{h}\cdot\text{g of DM})$ (n = 8).

^dRatio = ratio of rate of conversion of [2-¹⁴C]propionate to glucose to rate of conversion of [2-¹⁴C]propionate to CO₂. The SEM for .4 mM control was .23 (n = 9), and for all other values SEM was .35 (n = 4).

^eThe control is a .4 mM HCl solution prepared and neutralized as described in Materials and Methods.

^{v,w,x,y,z}Means in the same column with different superscript letters differ based on a protected LSD ($P < .05$).

phenolics tested inhibited the conversion of [2-¹⁴C]propionate to glucose when concentrations were increased to .4 mM, but PPA was more inhibitory than BA. Increasing the concentration of reduced phenolics from .05 to .4 mM did not affect conversion of [2-¹⁴C]propionate to CO₂. 3-Phenylpropionic acid and CA inhibited conversion of [2-¹⁴C]propionate to CO₂, with PPA being more inhibitory than CA. The ratio of rate of conversion of [2-¹⁴C]propionate to glucose:CO₂ was decreased by .05 mM PPA or CA. When the concentration of reduced phenolics was increased to .4 mM, all reduced phenolics decreased the ratio of conversion of [2-¹⁴C]propionate to glucose:CO₂ compared with the .4 mM control.

Because substitution of 3% BSA for 1% gelatin increased the conversion of [2-¹⁴C]propionate to glucose, these data were converted to percentages of their respective controls to determine the effects of type of protein on inhibition of propionate metabolism caused by reduced phenolics (Table 4). All reduced phenolics inhibited conversion of [2-¹⁴C]propionate to glucose, but the type of protein did not have an effect on inhibition caused by PPA, CA, or 4OHPPA. In contrast, BA was more inhibitory in the presence of BSA vs gelatin. The interaction between type of protein and reduced phenolic was not significant for conversion of [2-¹⁴C]propionate to CO₂. The presence of BSA decreased conversion of [2-¹⁴C]propionate to CO₂ by 12%. The ratio of rate of conversion of [2-¹⁴C]propionate to glucose:CO₂ in control flasks was increased by BSA. However, all the reduced phenolics

decreased the ratio in the presence of either 1% gelatin or 3% BSA relative to their respective control values.

The inhibitory effect of the combination of .05 mM BA, PPA, CA, and 4OHPPA on conversion of [2-¹⁴C]propionate to glucose and on the ratio of rate of conversion of [2-¹⁴C]propionate to glucose:CO₂ was not different from the inhibition caused by either PPA or CA alone (Table 5). The effect of the combination of .05 mM BA, PPA, CA, and 4OHPPA on conversion of [2-¹⁴C]propionate to CO₂ was not different from the effect caused by BA, PPA, or CA individually. Although the rates of conversion of [2-¹⁴C]propionate to glucose and CO₂ were numerically increased when .35 mM glycine was added to flasks containing the combination of reduced phenolics, we did not detect a statistically significant change.

Values (\pm SE) of rate of conversion of [2-¹⁴C]propionate to glucose, CO₂, and the ratio of the rates of conversion of [2-¹⁴C]propionate to glucose:CO₂ in flasks containing 1.2 mM HA were 152.4 ± 3.5 $\mu\text{mol}/(\text{h}\cdot\text{g of DM})$, 28.9 ± 1.2 $\mu\text{mol}/(\text{h}\cdot\text{g of DM})$, and $5.08 \pm .23$, respectively. Values for control (.4 mM) were 174.1 ± 2.4 $\mu\text{mol}/(\text{h}\cdot\text{g of DM})$, $30.0 \pm .8$ $\mu\text{mol}/(\text{h}\cdot\text{g of DM})$, and $5.70 \pm .16$, respectively. Values for HA were averages of four observations; values for control were averages of nine observations. Hippuric acid decreased the conversion of [2-¹⁴C]propionate to glucose but had no effect on conversion of [2-¹⁴C]propionate to CO₂ or on the ratio of the rates of conversion of [2-¹⁴C]propionate to glucose:CO₂.

Table 4. Effects of type of protein in the incubation medium on inhibition of metabolism of propionate by ovine hepatocytes caused by .4 mM benzoic (BA), 3-phenylpropionic (PPA), *t*-cinnamic (CA), or 3-(4-hydroxyphenyl)propionic (4OHPPA) acids^a

| Reduced phenolic and protein in medium ^b | [2- ¹⁴ C]Propionate to glucose, % of control ^c | [2- ¹⁴ C]Propionate to CO ₂ , % of control | | Ratio ^e |
|---|--|--|--------------------------|--------------------|
| | | Mean | Pooled ^d | |
| Control | | | | |
| Gelatin (n = 9) | 100.9 ^Z | 99.3 | 99.6 ± 2.5 ^Z | 5.80 ^X |
| BSA (n = 8) | 100.0 ^Z | 100.0 | | 11.61 ^Z |
| BA | | | | |
| Gelatin (n = 4) | 54.1 ^{XY} | 100.3 | 91.8 ± 3.6 ^{YZ} | 2.74 ^{VW} |
| BSA (n = 4) | 35.3 ^{VW} | 83.2 | | 5.50 ^X |
| PPA | | | | |
| Gelatin (n = 4) | 22.8 ^{UV} | 67.0 | 59.7 ± 3.6 ^W | 1.95 ^V |
| BSA (n = 4) | 16.3 ^U | 52.5 | | 4.06 ^{WX} |
| CA | | | | |
| Gelatin (n = 4) | 38.5 ^{VWX} | 90.5 | 79.8 ± 3.6 ^X | 2.33 ^{VW} |
| BSA (n = 4) | 30.1 ^{UVW} | 69.2 | | 4.11 ^{WX} |
| 4OHPPA | | | | |
| Gelatin (n = 4) | 42.3 ^{WXY} | 87.6 | 86.1 ± 3.6 ^{XY} | 2.74 ^{VW} |
| BSA (n = 4) | 58.4 ^Y | 84.7 | | 8.30 ^Y |
| Pooled | | | | |
| Gelatin | | 88.9 ± 2.13 | | |
| BSA | | 77.9 ± 2.14 | | |

^aValues are least squares means.

^bMedium contained either 1% (wt/vol) gelatin or 3% (wt/vol) bovine serum albumin (BSA).

^cThe control is a .4 mM HCl solution prepared and neutralized as described in Materials and Methods. Values within a duplicate were expressed as a percentage of the mean control value within that duplicate. Duplicate values were averaged, and these means were statistically analyzed as described in Materials and Methods. The SEM was 3.8% for gelatin control, 4.0% for BSA control, and 5.6% for all other values. The least squares means (±SEM) for rates of conversion of [2-¹⁴C]propionate to glucose were 177.3 (±13.0) μmol/(h·g of DM) for gelatin control and 320.1 (±13.8) μmol/(h·g of DM) for BSA control.

^dEffects of reduced phenolic and protein were significant (*P* < .05), but the interaction was not. Pooled reduced phenolic values are the least squares means (±SEM) of 17 observations for control and eight observations for all other means. Pooled protein values are the least squares means (±SEM) of 25 observations for gelatin and 24 observations for BSA.

^eRatio = ratio of rate of conversion of [2-¹⁴C]propionate to glucose to rate of conversion of [2-¹⁴C]propionate to CO₂. The SEM = .48 for gelatin control, .51 for BSA control, and .72 for all other values.

^{v,w,x,y,z}Means in the same column with different superscript letters differ based on a protected LSD (*P* < .05).

Discussion

We chose to investigate effects of reduced phenolics on propionate metabolism by the liver because conversion of propionate to glucose by the liver is critical for maintenance of glucose homeostasis in ruminants. Additionally, gluconeogenesis requires a net input of

metabolic energy, transport of intermediates across intracellular membranes, and a number of enzymes. Consequently, gluconeogenic rate should be sensitive to a variety of perturbations of hepatocyte structure and metabolism, making it a good indicator of hepatocyte integrity. We added the treatments to the incubation medium after allowing a pre-incubation

Table 5. Test of interactive effects of .05 mM benzoic, 3-phenylpropionic, *t*-cinnamic, or 3-(4-hydroxyphenyl)-propionic acids on propionate metabolism by ovine hepatocytes^a

| Reduced phenolic | [2- ¹⁴ C]Propionate to glucose, μmol/(h·g of DM) | [2- ¹⁴ C]Propionate to CO ₂ , μmol/(h·g of DM) | Ratio ^b |
|---|---|--|--------------------|
| Benzoic acid | 125.7 ^Z | 25.94 ^{YZ} | 5.03 ^Y |
| 3-Phenylpropionic acid | 64.4 ^Y | 22.09 ^Z | 2.85 ^Z |
| <i>t</i> -Cinnamic acid | 64.8 ^Y | 24.96 ^Z | 2.44 ^Z |
| 3-(4-Hydroxyphenyl)propionic acid | 151.6 ^Z | 31.01 ^Y | 4.97 ^Y |
| Combined reduced phenolics, .2 mM total | 52.4 ^Y | 22.04 ^Z | 2.34 ^Z |
| Combined reduced phenolics + .35 mM glycine | 76.7 ^Y | 26.01 ^{YZ} | 2.98 ^Z |

^aValues are least squares means with a SEM of 12.7 μmol/(h·g of DM), 1.9 μmol/(h·g of DM), and .34 (n = 4) for rate of conversion of [2-¹⁴C]propionate to glucose, rate of conversion of [2-¹⁴C]propionate to CO₂, and ratio, respectively.

^bRatio = ratio of rate of conversion of [2-¹⁴C]propionate to glucose to rate of conversion of [2-¹⁴C]propionate to CO₂.

^{y,z}Means in the same column with different superscript letters differ, based on a protected LSD (*P* < .05).

period during which the hepatocytes, whose metabolism had been depressed by storage on ice, adjusted to the incubation conditions and reached steady-state metabolism. This was done to avoid introducing the treatments when the ability of the hepatocytes to defend against an insult could have been depressed.

The rates of conversion of [2-¹⁴C]propionate to [¹⁴C]-glucose and ¹⁴CO₂ in Exp. 1 and 2 were comparable to other published values (Faulkner and Pollock, 1986). In the absence of glycine, 1.2 mM reduced phenolics inhibited conversion of propionate to glucose and CO₂ (Table 1), except that BA did not inhibit conversion of propionate to CO₂. These results agree with observations on the effects of 1.2 mM concentrations of these reduced phenolics on conversion of propionate to glucose and CO₂ by bovine liver slices (Cremin et al., 1994).

The concentrations of glycine in the incubation medium probably did not change significantly over the course of the incubation in Exp. 1. On the basis of the regression equation for the highest HA production rate in Table 2 (PPA), the amounts of glycine utilized for HA production would be 25 and 82 nmol at glycine concentrations of .12 and 2.4 mM, respectively. Demigne et al. (1991) reported that ovine hepatocytes incubated in the presence of .5 mM glycine and 2.5 mM propionate removed glycine from the medium at a rate of 30 nmol/(min·g of DM) (assuming that hepatocytes are 30% dry matter). On the basis of this rate, the sum of glycine removed by hepatocytes and that used for HA synthesis would be 7.9% and 1.2% of the amount of glycine added to flasks when glycine concentrations were .12 and 2.4 mM, respectively. Consequently, glycine concentrations probably were essentially constant over the course of the incubation.

A saturating concentration of reduced phenolic probably was present throughout the incubation period. Measurement of the concentrations of the reduced phenolics present in the medium at the end of the incubation showed that reduced phenolic concentrations did not decrease below .95 mM. In Exp. 2, increasing the concentrations of PPA or CA from .05 to .4 mM did not further decrease conversion of propionate to glucose. Additionally, results of Cremin et al. (1994) with bovine liver tissue indicated that effects of BA, PPA, or CA, but not 4OHPPA, are not significantly different between .2 and .4 mM. These observations suggest that concentrations of BA, PPA, or CA greater than .4 mM are saturating. It is not known whether 1.16 mM 4OHPPA is saturating.

The current study did not determine the mechanism by which reduced phenolics inhibit propionate metabolism, but many of the results are consistent with a mechanism involving their proposed hepatic metabolism (Figure 1). The metabolism of BA by rat hepatocytes has been well studied and can serve as a model to explain the hypothesized mechanism of inhibition. The first step in the metabolism of BA is

activation by esterification to CoA in hepatocyte mitochondria, forming benzoyl-CoA (Killenberg et al., 1971); benzoyl-CoA then is conjugated with glycine, forming HA and releasing CoA (Dhirendra et al., 1979). In rat hepatocytes in vitro, addition of BA to incubation medium not supplemented with glycine will cause intracellular (Cyr et al., 1991) and intramitochondrial (Gatley and Sherratt, 1977) accumulation of benzoyl-CoA, decrease concentrations of free CoA, and decrease gluconeogenesis, ureagenesis, and fatty acid synthesis (McCune et al., 1982; Cyr et al., 1991). One explanation of the inhibition of metabolism caused by BA is that benzoyl-CoA inhibits enzymes in the affected pathways. Vessey et al. (1991) found that benzoyl-CoA, but not BA, competitively inhibited carnitine acetyltransferase from bovine liver at relatively low concentrations ($K_{\text{inhibition}} = .022 \text{ mM}$). An alternative mechanism of metabolic inhibition is that the concentration of free CoA in hepatocytes becomes limiting as a result of sequestration of free CoA as benzoyl-CoA when hepatocytes do not have glycine to cleave benzoyl-CoA through HA production (McCune et al., 1982). In either case, inhibition would be the result of an elevated concentration of benzoyl-CoA. Inhibition of vital cellular enzymes by benzoyl-CoA and sequestration of CoA as benzoyl-CoA could be mechanisms by which BA inhibited metabolism of propionate in the absence of added glycine in our study, because metabolism of propionate requires initial activation by esterification with CoA.

We hypothesized that these same mechanisms could account for the inhibition of propionate metabolism caused by PPA, CA, or 4OHPPA. Scheline (1978) proposed that CA is first esterified to cinnamoyl-CoA and subsequently undergoes β -oxidation in the liver, producing benzoyl-CoA (Figure 1). Similarly, PPA and 4OHPPA could be esterified with CoA and subsequently β -oxidized to benzoyl-CoA and 4-hydroxybenzoyl-CoA, respectively. Accumulation of reduced phenolic-CoA esters or subsequent metabolism to and accumulation of benzoyl-CoA in the absence of glycine could account for inhibition of propionate metabolism by the same mechanisms as proposed for BA. Other mechanisms are possible, but we will discuss our data in relation to this hypothesis because it is supported by the literature and many of our results are consistent with it.

For BA, PPA, or CA, alleviation of inhibition of propionate metabolism linearly increased as glycine concentration in the medium increased. On the basis of this observation, we conclude that glycine concentration limited the rate of alleviation within the range of glycine concentrations tested. The observation that HA production in the presence of PPA or CA increased as alleviation of inhibition of propionate metabolism increased supports the hypothesis that glycine alleviates this inhibition by serving as a substrate for

conversion of reduced phenolic-CoA esters to HA. For BA, HA production did not significantly increase as glycine concentration increased, but there was a trend for increased HA production. The observation that a high concentration of HA (1.2 mM) inhibited conversion of propionate to glucose suggests that conversion of reduced phenolics to HA would not alleviate inhibition of conversion of propionate to glucose. However, this inhibition was not large (12.5%), and we observed that concentrations of HA in blood perfusing the liver of sheep fed roughage diets were substantially lower than 1.2 mM (6 to 38 μ M, J. D. Cremin, Jr., unpublished data).

The results for 4OHPPA are inconsistent with the hypothesis that glycine alleviates inhibition of propionate metabolism by serving as a substrate for HA synthesis. Increasing glycine concentration did not alleviate inhibition of gluconeogenesis, but HA production did increase as glycine concentration increased. These results suggest that 4OHPPA could be inhibiting propionate metabolism by a mechanism other than discussed above.

The typical concentration of glycine in hepatic portal venous blood perfusing the ovine liver is .32 mM (Heitmann and Bergman, 1980), which is within the range of glycine concentrations tested in our study. Consequently, if reduced phenolic concentration is not limiting *in vivo* and assuming that the rates of glycine uptake by hepatocytes *in vitro* and *in vivo* are similar, the glycine concentration in blood perfusing the liver could limit alleviation of inhibition of conversion of propionate to glucose caused by reduced phenolics *in vivo*.

Conversion of BA, PPA, and CA to HA was expected based on previous work (Martin, 1982a,b). However, we did not expect production of HA from 4OHPPA (Table 2), because enzymatic dehydroxylation of phenyl rings is not a common activity in mammalian tissue. It is possible that the 4OHPPA used was contaminated with PPA, BA, or CA and that the HA observed in medium containing 4OHPPA was from these reduced phenolics. However, the purity of the reduced phenolics added to the incubation medium was checked with the HPLC method described in Materials and Methods. Contamination of 4OHPPA with BA or any cross-contamination among the other reduced phenolics was not detected. It is possible that 4-hydroxyhippuric acid co-eluted with HA.

Martin (1969) summarized values for excretion of HA in urine of sheep fed a variety of feeds and reported that the mean value for HA excretion was 2.96 mmol/(kg BW^{.75}.d). Using the regression equations given in Table 2, the rates of HA production from BA, PPA, CA, and 4OHPPA at a physiological glycine concentration (approximately .32 mM; Heitmann and Bergman, 1980) were calculated to be 4.35, 16.05, 11.56, and 5.92 μ mol/(h·g of hepatocyte DM), respectively. Assuming that 1) all HA excreted was

produced by the liver, 2) the liver of the sheep was 2% of BW, 3) hepatocytes constitute 80% of the weight of liver tissue (Clark et al., 1976), and 4) the DM content of hepatocytes is 30%, then the rates of HA production from sheep in our study would be 1.35, 4.97, 3.58, and 1.83 mmol/(kg BW^{.75}.d) for BA, PPA, CA, and 4OHPPA, respectively. These rates are in agreement with the rates observed in the study conducted by Martin (1969).

Serum albumin decreases the toxicity of fatty acids and bilirubin *in vitro* (Krebs et al., 1974). Both fatty acids and reduced phenolics have carboxylic acid functional groups attached to hydrophobic moieties; therefore, we hypothesized that serum albumin might bind the reduced phenolics and moderate any inhibitory effects on hepatic metabolism *in vivo*. We found that a physiological concentration of BSA (3%, Swenson, 1983) did not alleviate inhibition of propionate metabolism by BA, PPA, or CA in ovine hepatocytes *in vitro*. Some alleviation may have occurred for 4OHPPA. When gluconeogenesis was expressed as a percentage of the control, inhibition of gluconeogenesis caused by 4OHPPA was not different from that caused by BA or CA in the presence of gelatin. However, in the presence of BSA, 4OHPPA was less inhibitory than BA or CA. Despite the fact that 4OHPPA was still inhibitory relative to the control, these observations suggest that BSA may have partially alleviated inhibition of gluconeogenesis caused by 4OHPPA.

The effect of the combination of reduced phenolics did not conform to our hypothesis. We predicted greater inhibition from the combination of reduced phenolics than from any individual reduced phenolic. In the current study, we observed that inhibition caused by the combination was not different from inhibition caused by the most inhibitory individual reduced phenolic (i.e., PPA or CA). This may have occurred because inhibition caused by the most inhibitory reduced phenolics was equal to a hypothesized maximal inhibition caused by reduced phenolics. A plateau in reduced phenolic inhibition of propionate metabolism was observed in our previous work (Cremin et al., 1994) and is suggested by the observation in the current study that increasing the concentration of PPA and CA from .05 to .4 mM did not further decrease propionate metabolism. These conclusions also are supported by the observation that inhibition of hepatic metabolism by BA reached a plateau in rat hepatocytes (Cyr et al., 1991). We attempted to use concentrations of reduced phenolics that individually would cause slight inhibition but whose combined effects would cause moderate inhibition. Such a scheme would have allowed observation of positive or negative interactive effects. However, we chose these concentrations on the basis of the relationship between reduced phenolic concentration and inhibition observed in a previous study with bovine liver slices (Cremin et al., 1994). In the current study,

the individual reduced phenolics had much greater inhibitory effects at lower concentrations than in the previous study. This could be a result of differences between liver of sheep and cattle or differences caused by the use of hepatocytes vs liver slices. Because PPA and CA may have achieved maximal inhibition of propionate metabolism individually (.05 mM), the current data do not provide a satisfactory test of the possible interactive effects of the reduced phenolics.

Of the reduced phenolics tested in the current study, PPA was the most concentrated reduced phenolic observed in blood perfusing the liver of sheep fed roughage diets (Cremin et al., 1995). This observation, combined with the observation that PPA is one of the most inhibitory reduced phenolics at the lowest concentration tested (Table 3), supports the conclusion that PPA is most likely to cause inhibition of propionate metabolism by hepatocytes *in vivo*.

3-Phenylpropionic acid or CA inhibited conversion of propionate to glucose in ovine hepatocytes at the lowest concentration (50 μ M) thus far observed. Glycine linearly alleviated inhibition of conversion of propionate to glucose caused by BA, PPA, or CA, suggesting that within the range of glycine concentrations tested, glycine concentration limits alleviation of inhibition of propionate metabolism caused by these reduced phenolics. This observation, combined with the observation that HA production in the presence of PPA or CA linearly increased with glycine concentration, suggests that the alleviation observed for PPA or CA is the result of conversion of PPA or CA to HA. Bovine serum albumin failed to alleviate inhibition of conversion of propionate to glucose caused by BA, PPA, and CA and only slightly alleviated inhibition caused by 4OHPPA in ovine hepatocytes.

Implications

3-Phenylpropionic acid, a compound derived from ruminal metabolism of forages, inhibited hepatic gluconeogenesis from propionate by 57% at a relatively low concentration (50 μ M). Furthermore, two mechanisms that were anticipated to alleviate this inhibition, conversion of 3-phenylpropionic acid to hippuric acid and adsorption of 3-phenylpropionic acid to bovine serum albumin, had small or no effects on inhibition. These observations support the conclusion that 3-phenylpropionic acid might inhibit hepatic gluconeogenesis from propionate in ruminants *in vivo*, but further research on the effects of physiological concentrations of PPA are needed.

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