Hepatic metabolism of propionate relative to meals for cows in the postpartum period

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ABSTRACT

The objective of this research was to identify potential short-term metabolic bottlenecks of propionate metabolism in the liver of dairy cows in the postpartum (PP) period and how such bottlenecks are affected by feeding status. Propionate, produced primarily from the fermentation of starch, decreases dry matter intake for cows in the postpartum period, likely by stimulating oxidation of acetyl-CoA in the liver. In this study, 8 dairy cows [2 blocks of 4 cows each, 6.63 ± 1.19 (mean $\pm$ SD) days PP; body condition score of 2.84 ± 0.39] were administered a pulse dose of either 1.5 mol/500 mL of propionic acid (PA) or 500 mL of water (control; CON) to the rumen either 1 h before or 2 h after feeding in a 4×4 Latin square design with a 2×2 factorial arrangement of treatments. Liver tissue was sampled at -1 , 10 and 20 min relative to dosing, and blood was sampled at -30 , -20 , -10 , -1 , 5, 10, 15, 20, 25, 30, and 60 min relative to dosing. We hypothesized that rapid propionate absorption results in bottlenecks as enzymes become saturated and cofactors require regeneration. The PA treatment increased plasma propionate and insulin concentrations rapidly, with peaks reached by 5 min regardless of feeding status and cleared from the plasma within 30 min of dosing. The PA treatment decreased plasma nonesterified fatty acid concentration over 30 min compared with CON before but not after feeding. The PA treatment decreased plasma β -hydroxybutyrate concentration and increased plasma lactate concentration compared with CON both before and after feeding. The PA treatment also increased hepatic pyruvate and lactate concentrations compared with CON. The PA treatment tended to increase hepatic isocitrate and fumarate concentrations but did not affect hepatic malate and oxaloacetate concentrations, suggesting that elevated mitochondrial NADH/NAD⁺ may have slowed the isocitrate dehydrogenase and fumarase reactions. The PA treatment

also increased succinate concentration compared with CON, suggesting that a bottleneck may be present at succinate dehydrogenase. The PA treatment tended to increase citrate concentration despite having no effects on acetyl-CoA or oxaloacetate concentrations. These results are in agreement with our hypothesis that rapid absorption of propionate from the rumen and extraction by the liver results in metabolic bottlenecks in the liver that may affect feeding behavior and dry matter intake in dairy cows in the PP period.

Key words: hepatic oxidation, liver, propionate, dairy cow

INTRODUCTION

Dairy cows in the immediate postpartum (PP) period are in a negative energy balance as energy output in milk increases rapidly and appetite is depressed. This exacerbates the pre-existing lipolytic state, increasing the risk for metabolic disorders that can lead to decreased milk yield, feed conversion efficiency, and ultimately, fertility. According to the hepatic oxidation theory (HOT; Allen et al., 2009), oxidation of fuels in the liver can stimulate satiety by transmitting signals via hepatic vagal afferents to feeding centers in the brain. During meals, tricarboxylic acid (TCA) cycle intermediates increase, stimulating the oxidation of acetyl coenzyme A (CoA) in the TCA cycle. Oxidation of fuels and production of ATP over minutes can vary greatly, likely affecting feeding behavior (Allen et al., 2009).

Hypophagic effects of propionate have been documented extensively among ruminants (Allen, 2000); however, these anaplerotic effects are dependent upon the availability of acetyl-CoA to be oxidized (Allen, 2014). A rapid ruminal infusion rate of propionic acid in dairy cows in the PP period delayed satiety compared with a slower rate of infusion, possibly resulting from metabolic bottlenecks as enzymes become saturated and cofactors require regeneration (Maldini and Allen, 2018). Additionally, a pilot study performed in our laboratory (Kennedy and Allen, 2017) identified potential short-term hepatic metabolic bottlenecks—points

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at which metabolism may be impeded—of propionate metabolism, including malate dehydrogenase (EC 1.1.1.37), methylmalonyl-CoA mutase (EC 5.4.99.2), and succinyl-CoA synthetase (EC 6.2.1.4).

Although much research has been conducted to determine the metabolic effects of propionate on liver metabolism, most of these studies used cells from bovine calves or were conducted over a period of hours, or both (Aiello et al., 1989; Donkin and Armentano, 1995; Zhang et al., 2015). No research has examined the short-term effects of propionate over a time scale of minutes, with particular focus on its relation to the stimulation of oxidation in the TCA cycle and how it may relate to feeding behavior in mature dairy cows. Ruminal absorption and liver metabolism of propionate before and after meals has not been quantified in a short-term time scale and may vary greatly, affecting feeding behavior in accordance with the HOT (Allen et al., 2009). The objective of this research was to identify potential short-term metabolic bottlenecks of propionate metabolism in the liver in dairy cows in the PP period and how they are affected by feeding status. We hypothesized that rapid propionate absorption results in metabolic bottlenecks and that these bottlenecks are greater after feeding due to partial saturation of the propionate metabolism pathway from diet-derived propionate.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at Michigan State University approved all experimental procedures for this experiment.

Animals, Housing, and Diets

Eight Holstein cows (4 primiparous and 4 multiparous) were used in this experiment. Cows were housed in individual tiestalls for the duration of the experiment and milked twice daily in the milking parlor. Cows were fed a TMR once daily at 110% of expected intake at 0800 h, and received a common experimental diet from parturition throughout the experiment. The experimental diet (Table 1) was formulated to contain 28% NDF, 23% starch, and 17.4% CP on a DM basis and consisted of corn silage, alfalfa silage, alfalfa hay, dry ground corn, soybean meal, soybean hulls, and a premix of protein supplement, minerals, and vitamins.

Experimental Design and Treatments

The experimental design was a replicated 4 × 4 Latin square with a 2 × 2 factorial arrangement of treatments balanced for carryover effects. Animals were blocked

based on parity. The primiparous animals were 6.25 ± 1.50 (mean ± SD) d PP at the start of the experiment and had a BCS of 2.63 ± 0.11 and BW of 596 ± 21.7 kg. The multiparous animals were 7.00 ± 0.82 d PP at the start of the experiment, with BCS of 3.06 ± 0.36 and BW of 731 ± 39.6 kg. Treatments consisted of a pulse dose of either 1.5 mol/500 mL of propionic acid (PA; food grade, 99.5%; Kemin Industries Inc., Des Moines, IA) or 500 mL of water (control, CON) into the rumen via orogastric intubation either 1 h before or 2 h after feeding (dose relative to feeding, DRF). Each block was randomly allocated to a treatment sequence in which each animal received each treatment once, and only one treatment was administered per d (period). Period is defined as the day that a treatment was administered (n = 4) and consisted of all sampling conducted within that day. One primiparous cow became ill before treatment or sampling on the first day of treatment and was dropped from the study. She was replaced with another primiparous cow for the remainder of the study; however, no samples were taken from the replacement cow during the first period. A rest day was provided between periods to minimize risk of carryover effects.

Data and Sample Collection

Animals were transferred to a surgery room at the Michigan State University Dairy Cattle Teaching and Research Center (East Lansing) for the dosing of treatment, as well as liver and blood collections. Feed offered was adjusted daily for individual animals. Samples (0.5 kg) of all dietary ingredients and the TMR were col-

Table 1. Ingredients and nutrient composition of experimental diet (% of dietary DM, except for DM)

Item	%
Ingredient	
Corn silage	29.9
Ground corn	19.5
Soybean meal	16.4
Alfalfa silage	15.3
Alfalfa hay	13.6
Vitamin and mineral mix ¹	4.2
Soybean hulls	1.1
Nutrient composition	
DM	53.5
OM	90.0
Starch	22.8
NDF	27.6
Forage NDF	24.0
CP	17.4

¹Vitamin and mineral mix contained 11.4% NaCl, 12.8% Ca, 0.99% Mg, 0.99% P, 10.9% Na, 14 mg/kg of Co, 250 mg/kg of Cu, 9.9 mg/kg of I, 745 mg/kg of Fe, 994 mg/kg of Mn, 7.5 mg/kg of Se, 1,093 mg/kg of Zn, 30.6 kIU/kg of vitamin A, 5.1 kIU/kg of vitamin D, and 152 IU/kg of vitamin E.

lected daily throughout the experiment and stored in plastic bags at -20°C until processed.

Animals were fitted with jugular catheters 2 d before the start of the experiment. Catheters were maintained by flushing the lines twice daily with approximately 5 mL of a sterile 3.5% sodium citrate solution. Blood was collected from the jugular catheter at -30 , -20 , -10 , and -1 min before dosing to determine baseline values and at 5, 10, 15, 20, 25, 30, and 60 min after dosing. Blood samples were collected into 3 tubes containing K_2EDTA and 1 tube containing potassium oxalate with NaF as a glycolytic inhibitor (Vacutainer tubes; VWR International, Radnor, PA), and placed on ice until centrifugation. Tubes were centrifuged at $1,700 \times g$ for 15 min at 4°C immediately after sample collection. Plasma was harvested and frozen at -20°C until analysis.

Liver samples (approximately 300 to 400 mg) were biopsied at -1 , 10, and 20 min relative to dosing. Liver biopsies were performed as modified from Bradford and Allen (2005). Briefly, the liver was accessed on the right side of the cow at the 10th intercostal space between the 11th and 12th ribs, located approximately 5 cm above the line between the olecranon and the tuber coxae. The area for the biopsy was clipped and cleaned using gauze soaked in 7.5% povidone-iodine, followed by gauze soaked in 70% ethanol. The biopsy site was locally anesthetized with up to 40 mL of 2% lidocaine hydrochloride as deemed necessary to maximize cow comfort during the procedure. Lidocaine was injected directly to the biopsy site and intramuscularly on the surrounding area. To ensure that the liver was in the expected location, a sterile biopsy needle (14-gauge, Wilburn Medical USA, Kernersville, NC) was used to take an initial biopsy sample. Upon confirmation of liver tissue, liver was biopsied using a stainless steel biopsy needle of 0.4 cm outer diameter. Samples were flash frozen in liquid nitrogen, stored on dry ice for transportation, and then stored at -80°C until further analysis.

Analysis of Samples

Feed Ingredients. Feed ingredient samples were composited and dried in a 55°C forced-air oven for 72 h, ground with a Wiley mill (1-mm screen; Thomas Scientific, Swedesboro, NJ), and analyzed for DM, ash, NDF, starch, and CP concentrations according to Gualdrón-Duarte and Allen (2017). All nutrients are expressed on a DM basis.

Plasma. Commercial kits were used to determine concentrations of nonesterified fatty acids (NEFA; NEFA HR kit; Wako Chemicals USA, Richmond, VA) and BHB (kit no. 2240; Stanbio Laboratory, Boerne, TX). Glucose was determined using a glucose oxidase

method (Sigma Aldrich, St. Louis, MO). The above metabolites were analyzed colorimetrically with a microplate reader (SpectraMax Plus 384; Molecular Devices, San Jose, CA) to determine the plasma concentrations of metabolites. Insulin concentration was determined using a bovine insulin ELISA kit (kit no. 10-1201-01; Mercodia, Uppsala, Sweden). Plasma propionate concentrations were analyzed by HPLC as described by Gualdrón-Duarte and Allen (2017). Plasma acetate and lactate were analyzed by HPLC, and samples were prepared in the same manner as plasma propionate, as described by Gualdrón-Duarte and Allen (2017). Baseline plasma concentrations for each factor were determined using samples collected before dosing.

Liver Sample Preparation. Liver samples were analyzed for citrate, isocitrate, succinate, fumarate, malate, pyruvate, lactate, BHB, glutamate, aspartate, oxaloacetate (OAA), α -ketoglutarate, glucose, and γ -aminobutyric acid (GABA) by GC-MS and for CoA, acetyl-CoA, propionyl-CoA, succinyl-CoA, and methylmalonyl-CoA by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Approximately 40 mg of frozen tissue was weighed into clean, flat-bottom 2-mL centrifuge tubes stored on dry ice. Tissue weights were recorded, and results were expressed per g of wet weight. We added 500 μL of 1% formic acid in 30% ACN:70% H_2O stored on ice to each tube and immediately homogenized using a Polytron PT 1200E homogenizer (Kinematica, Luzern, Switzerland) for 30 s. Samples were centrifuged at $5,000 \times g$ for 10 min at 4°C . The supernatant was transferred to clean centrifuge tubes for analysis and stored at -80°C . All samples for GC-MS and LC-MS/MS were evaporated to dryness using a Savant SC110 SpeedVac Concentrator (Savant Instruments Inc., Holbrook, NY).

Gas Chromatography–Mass Spectrometry. Samples evaporated to dryness were derivatized by methoximation and trimethylsilylation as modified by Kind et al. (2009), except that *N*-tert-butyltrimethylsilyl-*N*-methyltrifluoroacetamide with 1% *tert*-butyldimethylchlorosilane (MTBSTFA + 1% *t*-BDMCS; Sigma Aldrich) or *N*,*O*-bis(trimethylsilyl)trifluoroacetamide with 1% trichloromethylsilane (BSTFA with 1% TMCS; Sigma Aldrich) was used instead of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide, as noted in Table 2. Briefly, 30 μL of *O*-methylhydroxylamine reagent solution (40 mg/1 mL of pyridine; Sigma Aldrich) was added to each sample and vortexed for 30 s. Samples were then shaken for 3 h at 60°C in a Forma Orbital Shaker Model 420 (Thermo Fisher Scientific, Waltham, MA). An additional 90 μL of either MTBSTFA or BSTFA was added to each sample, vortexed for 10 s, and then shaken for an additional 30 min at 37°C . Samples were then centrifuged for 10 min at $5,000 \times g$

Table 2. Analyte parameters measured on GC-MS; each analyte was monitored using 2 ions (A and B)

Analyte	Retention time (min)	<i>m/z</i> monitored (A)	<i>m/z</i> monitored (B)
Pyruvate ¹	5.54	174.1 ²	216.1
Lactate ¹	6.85	233.1	261.1 ²
3-Hydroxybutyrate ¹	7.53	159.1	275.1 ²
Succinate ¹	8.76	289.1 ²	331.1
Fumarate ¹	9.03	287.1 ²	329.1
Phosphate ¹	10.03	383.2 ²	425.2
Malate ¹	11.60	287.2	419.3 ²
Aspartate ¹	11.98	418.3 ²	460.3
Glutamate ¹	12.97	432.3 ²	474.3
Citrate ¹	15.57	459.3	591.4 ²
Isocitrate ¹	15.65	459.3	591.4 ²
Oxaloacetate ³	6.67	98.0	290.1 ²
GABA ^{3,4}	7.12	174.1 ²	304.1
α -Ketoglutarate ³	7.39	198.1 ²	304.1
Glucose ³	9.8	205.1	319.2 ²

¹Derivatized with *N*-tert-butyl-dimethyl-silyl-*N*-methyltrifluoroacetamide with 1% tert-butyl-dimethylchlorosilane.

²Mass used for analysis.

³Derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide with 1% trichloromethylsilane.

⁴GABA = γ -aminobutyric acid.

at 22°C. The supernatant was transferred into a 250- μ L glass insert within a 2-mL amber autosampler vial (Thermo Fisher Scientific). Vials were stored in a desiccator in the dark until analysis by GC-MS within 48 h of derivatization. Analysis of peaks was conducted with MassLynx Mass Spectrometry Software (version 4.1; Waters Corporation, Milford, MA). Peaks were identified and quantified by comparison with standards.

Analysis was performed on an Agilent Technologies 6890N Network GC system coupled to an Agilent Technologies 5975B inert XL MSD mass spectrometer. The GC features a CombiPAL autosampler, helium carrier gas, and an Agilent VF-5ms 30-m $\times$ 0.25-mm $\times$ 0.25- μ m capillary column with an integrated 10-m EZ-Guard guard column (Agilent Technologies, Santa Clara, CA; P/N CP9013). Method-specific parameters include an inlet temperature of 280°C, a 10:1 injection split ratio, 70 eV electron ionization, and selected ion monitoring MS analysis of the target compounds (Table 2). The oven program was as follows: initial temperature of 40°C and held for 1 min, programmed at 40°C/min to 110°C and held for 1 min, then programmed at 40°C/min to 170°C, then programmed at 10°C/min to 300°C and held for 2.75 min, for a total run time of 21.25 min per sample.

Liquid Chromatography–Tandem Mass Spectrometry. Samples evaporated to dryness were re-suspended in 400 mM ammonium formate with 10 mM tris(2-carboxyethyl)phosphine HCl (Sigma Aldrich). Samples were centrifuged twice at 5,000 $\times g$ for 10 min each at 4°C and the supernatant aliquoted to new centrifuge tubes between bouts of centrifugation. The supernatant was then transferred into a 250- μ L glass

insert within a 2-mL amber autosampler vial (Thermo Fisher Scientific) for analysis on LC-MS/MS. Peaks were identified and quantified by comparison with standards. Analysis of peaks was conducted with MassLynx Mass Spectrometry Software (version 4.1, Waters Corporation).

Analysis of samples was performed on an Acquity ultra-performance LC system coupled to a Quattro Premier XE triple quadrupole MS using an Acquity UPLC BEH C18 1.7 μ m 2.1 $\times$ 100-mm column (Waters Corporation). This is a modified version of the method by Gotoh et al. (2015). The LC operation used the mobile phases: 400 mM ammonium formate (A) and acetonitrile (B). The column oven temperature was set to 50°C. The flow rate used on the program was set at 0.4 mL/min, with the initial parameters set at 98% mobile phase A and 2% mobile phase B until 4 min. From 4 min to 6 min the gradient shifted to 50% mobile phase A and 50% mobile phase B and held for 1 min. At 7 min until 9 min, the gradient shifted back to 98% mobile phase A and 2% mobile phase B, for a total run time of 9 min per sample. The electrospray ionization source parameters included a source temperature of 120°C, a gas flow of 600 L/h, a gas temperature of 350°C, and an electron spray ionization voltage of 2.5 kV. The MS analysis was performed using multiple reaction monitoring for the target compounds; detailed information is available in Table 3.

Statistical Analysis

Liver and plasma metabolite and hormone data were analyzed using the MIXED procedure of SAS software

Table 3. Multiple reaction monitoring settings for coenzyme A (CoA) compounds on LC-MS/MS

Analyte	Neutral mass (Da)	Precursor m/z	Product m/z	Cone voltage (V)	Collision energy (V)
CoA	767.5	768.1	261.1	45	23
Acetyl-CoA	809.6	810.1	303.1	45	30
Propionyl-CoA	823.6	824.1	317.1	30	40
Methylmalonyl-CoA	867.6	868.1	317.1	45	30
Succinyl-CoA	867.6	868.1	361.1	45	30

(version 9.4, 2013; SAS Institute Inc., Cary, NC) as a 4×4 Latin square with a 2×2 factorial design with double repeated measures (2 factors within the model are classified as repeating). Before statistical analysis, Box-Cox transformation analyses were applied to each response variable, to determine necessary transformations on the data using JMP Pro (version 13.2, 2016; SAS Institute Inc.). All liver and plasma metabolites and hormones were analyzed using the following model:

$$Y_{ijklmn} = \mu + B_i + C_j(B_i) + P_k(B_i) + T_l + S_m + F_n \\ + T_l S_m + T_l F_n + T_l B_i + F_n S_m + S_m P_k(B_i) \\ + T_l F_n S_m + e_{ijklmn},$$

where Y_{ijklmn} = the response variable; μ = overall mean; B_i = fixed effect of block i ; $C_j(B_i)$ = random effect of cow j nested within block i ; $P_k(B_i)$ = fixed effect of period k nested within block i ; T_l = fixed effect of treatment l ; S_m = fixed effect of sampling time m ; F_n = fixed effect of dose relative to feeding n ; $T_l S_m$ = interaction of treatment and sampling time; $T_l F_n$ = interaction of treatment and dose relative to feeding; $T_l B_i$ = interaction of treatment and block; $F_n S_m$ = interaction of dose relative to feeding and sampling time; $S_m P_k(B_i)$ = interaction of sampling time and period nested within block; $T_l F_n S_m$ = interaction of treatment, dose relative to feeding, and sampling time; and e_{ijklmn} = residual. Period was treated as the macro repeated measure and sampling time as the micro repeated measure. The covariance structure was compound symmetry for all metabolites and hormones except hepatic BHB, which was first-order autoregressive due to a lower Bayesian information criterion (BIC). Denominator degrees of freedom were estimated by using the Kenward-Roger option in the MODEL statement. The covariance structure Toeplitz was also examined for plasma samples; however, compound symmetry yielded a lower BIC for all plasma metabolites and hormones. Pairwise tests were determined using the diff option in PROC MIXED for those variables that had significant interactions or tendencies for significant interactions.

Treatment effects were declared at $P \leq 0.05$, and tendencies for treatment effects were declared at $P \leq 0.10$. Interactions were declared significant at $P \leq$

0.10, and tendencies for interactions were declared at $P \leq 0.15$. Data transformed are noted in the tables and figures, along with the transformation used for statistical analysis. All transformed data has been back-transformed for interpretation purposes. Percent differences were calculated by back-transforming the least squares means from the statistical models, subtracting the baseline from the time point of interest, dividing that number by the baseline, and multiplying it by 100.

RESULTS

Plasma Metabolites and Insulin

Treatment effects on plasma metabolites and hormones are summarized in Table 4. Treatment interacted with sampling time to affect all plasma metabolites and hormones measured (Figure 1; $P < 0.01$). The PA treatment rapidly increased plasma propionate concentration, peaking at 5 min both before feeding (BF) and after feeding (AF) compared with CON, and returned to baseline within 20 and 25 min, respectively (Figure 1A; interaction, $P = 0.05$). Plasma propionate concentration increased at a rate of 0.03 and 0.07 mM/min BF and AF, respectively, and cleared from circulation at a rate of 0.01 and 0.02 mM/min BF and AF, respectively. Additionally, the plasma propionate concentration peak was greater at 5 min AF (0.53 mM, 225% increase from baseline) than BF (0.32 mM, 109% increase from baseline) for PA (pairwise test, $P = 0.05$). The PA treatment increased mean plasma propionate concentration compared with CON (0.20 vs. 0.15 mM; $P = 0.04$). The PA treatment increased plasma glucose concentration BF, but not AF, compared with CON (Figure 1B; $P < 0.01$), with the peak concentration at 10 min after dosing. The PA treatment also increased plasma insulin concentration more BF than AF compared with CON ($P = 0.09$), with concentrations peaking at 5 min and returning to baseline by 30 min (Figure 1C; $P = 0.02$). Plasma NEFA concentration was higher BF (1,121 μ Eq) than AF (514 μ Eq/L; $P < 0.01$) and PA decreased plasma NEFA concentration to a greater extent over time BF than AF (Figure 1D; $P = 0.01$). The PA treatment decreased plasma BHB concentration over 30 min, which remained lower at

60 min and was lower overall for PA compared with CON (Figure 1E; $P < 0.01$). The PA treatment reduced plasma acetate concentration compared with CON over time (Figure 1F; $P < 0.01$), which was higher AF (1.34 mM) than BF (0.67 mM; $P < 0.01$). The PA treatment also interacted with time to increase plasma lactate concentration compared with CON (Figure 1G; $P < 0.01$) and increased mean plasma lactate concentration compared with CON (0.50 vs. 0.38 mM; $P < 0.01$), which tended to be higher AF than BF (0.47 vs. 0.40 mM; $P = 0.06$).

Liver Metabolites

Hepatic metabolite concentrations are reported in Table 5 and Figures 2 and 3. The PA treatment tended to increase hepatic propionyl-CoA concentration over time (Figure 2A; $P = 0.13$), with greater concentration at 10 min compared with CON (11.1 vs. 2.1 nmol/g; pairwise, $P = 0.02$) and declining to near baseline by 20 min, resulting in greater mean concentration (7.80 vs. 2.82 nmol/g; $P = 0.04$). Propionyl-CoA concentration was greater AF than BF (7.18 vs. 3.22 nmol/g; $P = 0.05$). Methylmalonyl-CoA concentration tended to be greater AF than BF (2.25 vs. 1.43 nmol/g; $P = 0.08$) but was not affected by PA treatment.

Treatment interacted with dose relative to feeding and sampling time to affect succinyl-CoA concentration (Figure 2B; interaction, $P = 0.10$). Relative to baseline concentration, PA did not affect succinyl-CoA concentration either BF or AF at 10 or 20 min (pairwise test, $P > 0.10$); however, succinyl-CoA tended to be greater for PA compared with CON AF at 20 min (26.5 vs. 8.75 nmol/g; pairwise test, $P = 0.06$). The concentration of CoA was not affected by treatment either BF or AF.

The PA treatment increased succinate concentration compared with CON (227 vs. 163 nmol/g; $P = 0.01$) and interacted with sampling time (Figure 2C; $P = 0.10$). Succinate concentration was greater at 10 min for PA compared with CON (286 vs. 146 nmol/g; pairwise test, $P < 0.01$), and compared with baseline, PA increased succinate concentration 46% at 10 min (pairwise test, $P = 0.02$). The PA treatment tended to increase mean fumarate concentration (55.0 vs. 46.9 nmol/g; $P = 0.07$), which was greater AF than BF (55.5 vs. 46.5 nmol/g; $P = 0.05$).

Malate and OAA concentrations were not affected by treatment either BF or AF. The PA treatment increased mean citrate concentration compared with CON ($P = 0.01$), but the effect was detected BF (96.2 vs. 46.2 nmol/g) but not AF (102 vs. 81.5 nmol/g; $P = 0.10$). Treatments tended to interact with sampling time ($P = 0.12$); PA increased citrate concentration 41% at 10 min compared with baseline (Figure 2D; pairwise test,

Table 4. Effects of a pulse dose of either 1.5 mol of propionic acid (PA) or water (control, CON) to the rumen either 1 h before or 2 h after feeding on plasma metabolites and hormones in lactating dairy cows over the first 60 min

Metabolite	Before			After			P-value ¹			
	CON	PA		CON	PA		TRT	DRF	TRT × DRF	TRT × ST
Propionate ² (mM)	0.18 ^{ab}	0.18 ^{ab}		0.13 ^a	0.23 ^b		0.04	0.72	0.03	<0.01
Glucose (mg/dL)	44.7 ^a	51.5 ^b		47.3 ^a	45.4 ^a		0.06	0.16	<0.01	<0.01
Insulin ² (μg/L)	0.05 ^a	0.25 ^{bc}		0.14 ^b	0.31 ^c		<0.01	0.01	0.09	<0.01
NEFA ^{3,4} (μEq/L)	1,360	904		560	470		0.02	<0.01	0.16	<0.01
BHB ² (mg/dL)	16.8	10.6		16.3	14.4		0.06	0.35	0.25	<0.01
Acetate ² (mM)	0.67 ^a	0.67 ^a		1.51 ^b	1.19 ^c		0.16	<0.01	0.12	<0.01
Lactate ⁵ (mM)	0.34	0.47		0.42	0.54		<0.01	0.06	0.47	<0.01

^{a-c}Means within a row with different superscripts differ ($P \leq 0.05$).
¹TRT = treatment; DRF = dose relative to feeding (before or after); ST = sampling time.
²Data log-transformed for statistical analysis and back-transformed for interpretation purposes.
³NEFA = nonesterified fatty acid.
⁴Data transformed using square root for statistical analysis and back-transformed for interpretation purposes.
⁵Data transformed using the inverse of the square root for statistical analysis and back-transformed for interpretation purposes.

Figure 1. Effects of a pulse dose of either 1.5 mol of propionic acid (PA) or water (CON) to the rumen on (A) plasma propionate, (B) glucose, (C) insulin, (D) nonesterified fatty acid (NEFA), (E) BHB, (F) acetate, and (G) lactate in dairy cows in the postpartum period over the first 60 min either 1 h before or 2 h after feeding. Associated *P*-values can be found in Table 4. For plasma propionate, insulin, BHB, and acetate, data were log-transformed for statistical analysis and back-transformed for interpretation purposes. For NEFA, data were transformed using square root for statistical analysis and back-transformed for interpretation purposes. For lactate, data were transformed using the inverse for statistical analysis and back-transformed for interpretation purposes.

Table 5. Effects of a pulse dose of either 1.5 mol of propionic acid (PA) or water (control, CON) to the rumen on hepatic metabolites and select couplets of lactating dairy cows, either 1 h before feeding or 2 h after feeding

Metabolite ¹	Before			After			P-value ²					
	CON	PA		CON	PA		TRT	DRF	TRT × DRF	TRT × ST	DRF × ST	TRT × DRF × ST
CoA ³	15.1	12.9		14.6	16.6		0.97	0.55	0.51	0.21	0.25	0.87
Propionyl-CoA ⁴	2.04	4.67		3.73	11.7		0.04	0.05	0.26	0.13	0.64	0.19
M-CoA ^{3,4}	1.62	1.24		2.34	2.15		0.51	0.08	0.71	0.71	0.58	0.18
Succinyl-CoA	16.6	15.9		16.3	25.8		0.30	0.23	0.17	0.69	0.39	0.10
Succinate ⁴	156	202		170	253		0.01	0.14	0.48	0.10	0.70	0.55
Fumarate ⁵	44.9	48.1		49.1	62.8		0.07	0.05	0.31	0.29	0.77	0.54
Malate ⁴	166	198		197	291		0.12	0.13	0.53	0.22	0.76	0.40
OAA ^{3,5}	8.96	7.67		8.14	8.82		0.57	0.75	0.17	0.56	0.72	0.48
Citrate ⁵	46.2 ^a	96.2 ^b		81.5 ^b	102 ^b		0.01	0.04	0.10	0.12	0.04	0.60
Isocitrate ⁴	7.88	9.11		8.48	10.6		0.06	0.23	0.55	0.66	0.87	0.23
α-KG ^{3,4}	77.9	79.2		86.5	86.7		0.92	0.31	0.94	0.33	0.46	0.16
Glutamate ⁴	1,521	2,051		1,573	2,017		0.08	0.96	0.87	0.26	0.63	0.74
Glu/α-KG ⁴	22.6	28.0		18.9	23.4		0.13	0.18	0.95	0.56	0.18	0.37
GABA ^{3,4}	267	296		259	294		0.31	0.87	0.90	0.04	0.53	0.98
Acetyl-CoA ⁴	7.18	7.12		3.77	6.04		0.24	0.07	0.44	0.74	0.18	0.28
BHB ⁵	3,128 ^a	1,418 ^b		1,602 ^b	1,567 ^b		0.08	0.19	0.04	0.08	0.94	0.33
Aspartate ⁶	166	223		214	306		0.02	0.03	0.94	0.02	0.07	0.53
Pyruvate ⁴	25.8 ^a	36.2 ^b		34.5 ^b	55.0 ^c		<0.01	<0.01	0.14	<0.01	0.56	0.27
Lactate ⁹	449	531		557	708		0.20	0.14	0.72	0.07	0.42	0.14
Lac/Pyr ^{3,4}	22.8	16.1		22.5	15.4		<0.01	0.75	0.76	0.25	0.06	<0.01
Glucose ⁵	980 ^a	1,306 ^b		1,182 ^{ab}	1,179 ^{ab}		0.15	0.63	0.10	0.13	0.02	0.04

^{a-c}Means within a row with different superscripts differ ($P \leq 0.05$).

¹Metabolite values reported in nmol/g.

²TRT = treatment; DRF = dose relative to feeding (before or after); ST = sampling time.

³M-CoA = methylmalonyl-CoA; OAA = oxaloacetate; α-KG = α-ketoglutarate; GABA = γ-aminobutyric acid; and Lac/Pyr = lactate/pyruvate.

⁴Data transformed using square root for statistical analysis and back-transformed for interpretation purposes.

⁵Data log-transformed for statistical analysis and back-transformed for interpretation purposes.

⁶Data transformed using the inverse of the square root for statistical analysis and back-transformed for interpretation purposes.

Figure 2. Effects of a pulse dose of either 1.5 mol of propionic acid (PA) or water (CON) to the rumen on hepatic concentrations of (A) propionyl-CoA, (B) succinyl-CoA, (C) succinate, (D) citrate, (E) BHB, (F) aspartate, and (G) γ -aminobutyric acid (GABA), in dairy cows in the postpartum period over the first 20 min either 1 h before or 2 h after feeding. Associated *P*-values can be found in Table 5. For propionyl-CoA, succinate, and GABA, data were transformed using square root for statistical analysis and back-transformed for interpretation purposes. For citrate and BHB, data were log-transformed for statistical analysis and back-transformed for interpretation purposes. For aspartate, data were transformed using the inverse of the square root for statistical analysis and back-transformed for interpretation purposes.

$P = 0.02$), and PA increased citrate concentration at 10 and 20 min compared with CON (pairwise tests, $P = 0.01$ and $P < 0.01$, respectively). Additionally, mean citrate concentration was higher AF than BF (91.0 vs. 66.7 nmol/g; $P = 0.04$). The PA treatment tended to increase mean isocitrate concentration compared with CON (9.84 vs. 8.18 nmol/g; $P = 0.06$) but did not affect α -ketoglutarate concentration relative to feeding time. The PA treatment tended to increase mean hepatic glutamate concentration compared with CON (2,034 vs. 1,546 nmol/g; $P = 0.08$) but did not affect the ratio of glutamate to α -ketoglutarate concentration relative to feeding time.

Hepatic acetyl-CoA concentration was not affected by PA treatment but tended to be lower AF compared with BF (4.84 vs. 7.15 nmol/g; $P = 0.07$). The PA treatment decreased hepatic BHB concentration compared with CON BF (1,418 vs. 3,128 nmol/g) but not

AF (1,567 vs. 1,602 nmol/g; $P = 0.04$), and differences between treatments increased over time (Figure 2E; $P = 0.08$).

The PA treatment increased hepatic aspartate concentration compared with CON (259 vs. 188 nmol/g; $P = 0.02$). Treatments interacted with sampling time to affect aspartate concentration (Figure 2F; interaction, $P = 0.02$), which was greater AF than BF (254 vs. 191 nmol/g; $P = 0.03$); PA increased aspartate concentration 58% (pairwise test, $P < 0.01$) whereas CON decreased it 22% (pairwise test, $P = 0.04$) at 10 min compared with baseline, and the concentration began to return to baseline by 20 min. Treatments interacted with sampling time to affect GABA concentration (Figure 2G; $P = 0.04$), with PA decreasing GABA concentration 37% at 10 min compared with baseline (pairwise test, $P = 0.02$) and CON tending to increase GABA concentration 39% at 10 min compared with

Figure 3. Effects of a pulse dose of 1.5 mol of propionic acid (PA) or water (CON) to the rumen on hepatic concentration of (A) pyruvate, (B) lactate, (C) ratio of lactate to pyruvate, and (D) glucose in dairy cows in the postpartum period over the first 20 min either before or after feeding. Associated P -values can be found in Table 5. For pyruvate and lactate:pyruvate ratio, data were transformed using square root for statistical analysis and back-transformed for interpretation purposes. For lactate and glucose, data were log-transformed for statistical analysis and back-transformed for interpretation purposes.

baseline (pairwise test, $P = 0.10$). Mean GABA concentration was greater for PA than for CON at baseline (381 vs. 241 nmol/g; pairwise test, $P = 0.02$).

The PA treatment increased hepatic pyruvate concentration compared with CON ($P < 0.01$), and the increase tended to be greater AF (55.0 vs. 34.5 nmol/g) than BF (36.2 vs. 25.8 nmol/g; $P = 0.14$). Treatments interacted with sampling time (Figure 3A; $P < 0.01$), and PA increased pyruvate concentration compared with CON at 10 min (62.8 vs. 26.1 nmol/g; pairwise test, $P < 0.01$) and 20 min (42.7 vs. 27.0 nmol/g; pairwise test, $P = 0.01$). Mean pyruvate concentration was increased AF compared with BF (44.1 vs. 30.8 nmol/g; $P < 0.01$). Treatment significantly interacted with block ($P = 0.05$) in which PA increased pyruvate concentration compared with CON in multiparous cows (31.0 vs. 14.2 nmol/g; $P < 0.01$) and tended to increase pyruvate concentration compared with CON in primiparous cows (61.8 vs. 51.7 nmol/g; $P = 0.08$). The PA treatment increased hepatic lactate concentration at 10 min compared with CON (843 vs. 396 nmol/g; pairwise test, $P = 0.04$) and differed or tended to differ compared with CON AF but not BF (Figure 3B; interaction, $P = 0.14$). The PA treatment decreased the ratio of lactate to pyruvate (15.8 vs. 22.7; $P < 0.01$), but treatment interacted with dose relative to feeding and sampling time (Figure 3C; $P < 0.01$). The PA treatment did not affect the ratio of lactate to pyruvate compared with baseline BF but tended to increase it at 10 min (pairwise test, $P = 0.06$) and increased it at 20 min AF (pairwise test, $P = 0.04$), whereas CON increased the ratio at 10 and 20 min BF (pairwise tests, $P < 0.05$) and decreased it over the sampling period AF (pairwise tests, $P \leq 0.01$) compared with baseline. Treatment significantly interacted with block ($P = 0.01$) in which PA decreased the ratio of lactate to pyruvate compared with CON in multiparous cows (19.7 vs. 33.9 nmol/g; $P < 0.01$) and did not differ compared with CON in primiparous cows (12.2 vs. 13.7 nmol/g; $P = 0.42$).

Treatments interacted with feeding status and sampling time to affect hepatic glucose concentration (Figure 3D; $P = 0.04$). The PA treatment did not change glucose concentration over the sampling time BF but increased glucose between 10 and 20 min AF (pairwise test, $P = 0.01$), whereas CON decreased glucose concentration BF at 20 min (pairwise test, $P = 0.04$) and increased it AF throughout the sampling period compared with baseline (pairwise tests, $P < 0.05$).

DISCUSSION

Propionate absorption from the rumen occurred more rapidly than expected, with effects from the pulse dose immediately measurable at the 5-min time point. These

effects were short-term and cleared within 30 min of administration of treatments. Furthermore, many of the hepatic reactions returned to baseline values by 20 min, which supports that diet and individual meals have acute metabolic effects that need to be considered and further explored. Observed peaks and increases in measured plasma and hepatic metabolites from a pulse dose of PA suggest possible areas of interest in which metabolism of propionate may be hindered. Because the measured hepatic metabolites follow a well-established pathway of metabolism, we are able to use the observed concentrations to identify enzymes within the pathway that may be delaying propionate metabolism, which we refer to as metabolic bottlenecks of propionate metabolism. Identification of possible metabolic bottlenecks allows us to focus future research on these specific enzymes to help us improve management strategies (nutrition, genetics, etc.) to optimize nutrient extraction and utilization during the PP period.

Therefore, a peak of plasma propionate suggests that plasma propionate is bypassing the liver despite its very efficient extraction by the liver (Baird et al., 1980; Reynolds et al., 2003). However, metabolism of propionate by other tissues is minimal (Brockman, 2005), and as such, the liver is expected to extract the vast majority of propionate in plasma, albeit requiring additional passes through the circulation. Propionyl-CoA synthetase (EC 6.2.1.17), the enzyme responsible for the activation of plasma propionate to propionyl-CoA, likely delayed the extraction of propionate from plasma. This is evidenced by the tendency toward an increase in propionyl-CoA concentration but lack of change in methylmalonyl-CoA concentration. The available CoA pool may be too large to detect minute changes caused by propionyl-CoA synthetase, which would explain why we were unable to detect treatment differences in free CoA; as such, the availability of free CoA does not appear to be a limiting factor. However, propionyl-CoA carboxylase (EC 6.4.1.3), the enzyme responsible for converting propionyl-CoA to methylmalonyl-CoA, may also be a potential metabolic bottleneck that might explain the tendency for an increase in propionyl-CoA concentration but lack of change in methylmalonyl-CoA concentration.

An increase in succinate concentration suggests a possible metabolic bottleneck at succinate dehydrogenase (EC 1.3.5.1), the enzyme responsible for converting succinate to fumarate. Sun et al. (2005) hypothesized that succinate dehydrogenase may provide a feedback mechanism that maintains a balance between energy utilization and storage, given its position as part of both the TCA cycle and the electron transport chain. Therefore, a potential bottleneck at this enzyme could be a major source of interest for control and regulation

of feed intake in accordance with the HOT (Allen et al., 2009). Also, succinate dehydrogenase is strongly inhibited by OAA (Das, 1937) and malate (Dervartanian and Veeger, 1965; Ackrell et al., 1974). However, malate and OAA are found in both the mitochondria and cytosol of cells, and malate is exported from the mitochondria to the cytosol for gluconeogenesis. Siess et al. (1977) reported that cytosol contains more than 75% of total malate concentrations found in rat liver and an even greater proportion in the presence of glucagon. We did not separate mitochondrial and cytosolic metabolites so, although neither malate nor OAA concentrations were elevated in liver tissue, they might have been elevated in the mitochondria and been diluted by the cytosolic compartment. NADH is a regulator of the TCA cycle, affecting many enzymes that use NAD^+ as a reducing factor, such as isocitrate dehydrogenase (EC 1.1.1.41; Williamson and Cooper, 1980) and mitochondrial malate dehydrogenase, the enzyme responsible for conversions between malate and OAA. Therefore, a tendency toward an increase in isocitrate concentration suggests reduced isocitrate dehydrogenase activity and increased NADH/NAD^+ . A mitochondrial increase of NADH/NAD^+ may cause malate to be shunted toward gluconeogenesis, which aligns with Zhang et al. (2015), who reported a tendency for a greater flux of OAA to pyruvate and glucose in cows receiving propionate infusions. Additionally, mitochondrial malate may convert to fumarate or decrease the rate of fumarate conversion to malate when accumulated. Therefore, a tendency for fumarate concentration to increase with PA further supports that an accumulation of mitochondrial malate may have occurred.

The spike in plasma insulin concentration was likely a result of an increase in plasma propionate concentration (Horino et al., 1968; Bines and Hart, 1984) because it coincides with the peak in plasma propionate concentration, whereas plasma glucose concentration did not peak until later. Furthermore, the increase in plasma insulin concentration likely caused the decrease in plasma NEFA concentration observed BF. Whereas a reduction in plasma NEFA concentration would be expected to decrease hepatic concentration of acetyl-CoA (Piantoni et al., 2015), acetyl-CoA was unaffected by treatment. This was likely because PA decreased hepatic BHB concentration. Citrate concentration, however, increased 108% with PA compared with control BF and tended to increase over time by PA compared with CON both BF and AF. Generally, the availability of acetyl-CoA and OAA as substrates for citrate synthase (EC 2.3.3.1), the enzyme responsible for the formation of citrate, is low, which can affect citrate formation (Williamson and Cooper, 1980). In addition, the conversion between citrate and isocitrate

is a reversible reaction, and as such, the tendency for an increase in isocitrate concentration could contribute to the accumulation of citrate. However, no spikes of isocitrate concentration were observed, and the accumulated isocitrate concentration would most likely be derived from citrate. Therefore, the increase in citrate concentration is likely from the conversion of acetyl-CoA and OAA to citrate through citrate synthase.

Circulating plasma NEFA concentration was lower AF compared with BF, as expected, and did not decrease with PA AF, as observed BF, despite an increase in plasma insulin concentration AF. Furthermore, the overall decrease in plasma NEFA concentration AF compared with BF is likely responsible for the tendency for hepatic acetyl-CoA concentration to decrease AF compared with BF, which is in agreement with previous research (Piantoni et al., 2015). Plasma and hepatic BHB concentration decreased both BF and AF; however, this decrease is not as evident AF as BF, consistent with the decreased plasma NEFA concentration AF.

The increase in pyruvate and lactate concentrations suggests one or a combination of the following: (1) excess carbons and reducing equivalents are contributing to gluconeogenesis from transfer of mitochondrial malate to the cytosol; (2) another metabolic bottleneck associated with pyruvate may be present; (3) the system is high in cytosolic NADH/NAD^+ ; or (4) the system is likely trying to regenerate NAD^+ from $\text{NADH} + \text{H}^+$. High NADH levels, along with other inhibitors, can reduce the conversion of pyruvate to acetyl-CoA and may shunt carbons toward gluconeogenesis. Along with an excess of carbon supplied by propionyl-CoA metabolism in the mitochondria, the accumulation of pyruvate may also signify a metabolic bottleneck. However, multiple enzymes are associated with pyruvate metabolism and determining which enzyme or enzymes may be contributing to the increase in pyruvate is not possible from our experimental results. The lactate/pyruvate couplet, which has been used to estimate the ratio of cytosolic NAD^+/NADH (Williamson et al., 1967), decreased with PA compared with CON in multiparous cows, supporting the assumption of a high ratio of cytosolic NADH/NAD^+ . Although the ratio increases BF with PA compared with the baseline over the sampling period, these increases are not as great as with CON, suggesting a larger pool of cytosolic NADH with PA treatment, likely resulting from exportation of mitochondrial malate to the cytosol. Furthermore, the increase in lactate concentration noted with PA treatment is likely directly related to the increase in pyruvate concentration because pyruvate can be converted to lactate, a reaction that regenerates NAD^+ in the process. The increase in plasma lactate concentration may be the result of the increased hepatic lactate con-

centration being released into circulation or a decrease in hepatic uptake of lactate (Baird et al., 1980).

Glutamate, a major excitatory neurotransmitter, has been reported to excite abdominal vagal afferents in rats (Tsurugizawa et al., 2009). Conversely, glutamate can convert to GABA, an inhibitory neurotransmitter that may also play a role in signaling satiety through the vagus hepatic nerve. The hepatic concentration of GABA in humans is 252 nmol/g, and an experiment with rats observed almost a doubling of hepatic GABA concentration within 4 h following a 70% partial hepatectomy (Minuk, 1992). However, GABA concentration decreased with PA treatment, whereas glutamate concentration had a tendency to increase, suggesting that glutamate was not converting to GABA. Firing rate is decreased when energy status increases, inhibiting feeding (Friedman, 1997), and previous studies have reported hypophagic effects of propionate (Allen, 2000; Gualdrón-Duarte and Allen, 2017; Maldini and Allen, 2018). Therefore, we would expect to see the opposite patterns of glutamate and GABA concentrations if these neurotransmitters were responsible for regulating feeding behavior through the HOT. Aspartate has been reported to be an excitatory neurotransmitter as well (Dingledine and McBain, 1999); however, aspartate concentration also increased, suggesting that it is not a likely neurotransmitter candidate for the HOT.

For those metabolites affected by dose relative to feeding main effects (BF vs. AF), the concentrations of respective hepatic metabolites were generally higher AF than BF, as hypothesized, suggesting that some metabolic bottlenecks may be more apparent AF, such as propionyl-CoA synthetase. Recently consumed feed in the rumen would cause greater propionate production, particularly if feeds consumed were high in starch. As such, enzymes and cofactors associated with propionate metabolism may be reaching their saturation and depletion limits, respectively, as a result of these pre-existing higher levels of metabolites before dosing.

Frequently, DMI is evaluated and reported daily. However, daily DMI intake is a function of: (1) the number of meals eaten per day, (2) the amount of feed eaten per meal, (3) the length of each meal, and (4) the length of time between meals (Allen, 2014). Previous research has shown that propionate decreases meal frequency and meal size (Maldini and Allen, 2018) and that hepatic vagotomy diminishes the hypophagic effects of propionate in sheep (Anil and Forbes, 1988). As such, understanding the short-term metabolism of propionate within a meal may help with describing daily DMI and feeding behavior. By understanding mechanistically what is occurring within the meal, future studies and research can be directed toward maximizing daily DMI through manipulation of the aforementioned

parts. This is especially important during the first few weeks postpartum, when appetite is suppressed.

We propose that elevated NADH/NAD⁺ during the postpartum period likely created bottlenecks for certain reactions within the TCA cycle, slowing TCA activity and ATP production despite anaplerosis from propionate, and shunting carbons and reducing equivalents to the cytosol for gluconeogenesis. As such, these metabolic bottlenecks likely delay satiety within a meal, increasing meal size but extending intermeal interval. This study shows that metabolism is occurring very quickly. We hope it stimulates further research on metabolism focused on a short timescale.

CONCLUSIONS

This study demonstrated the following: (1) absorption of propionate from the rumen into the bloodstream, and subsequent extraction by the liver, is very rapid; (2) the metabolic effects of propionate are rapid, occurring within the time frame of a meal; and (3) metabolic bottlenecks for propionate metabolism occur that might affect feeding behavior. Specifically, we suggest potential metabolic bottlenecks of propionate metabolism at propionyl-CoA synthetase and succinate dehydrogenase. Additionally, as expected, feeding status can affect metabolite concentrations and response to propionate with metabolite concentrations generally higher after than before feeding. Understanding the metabolic mechanisms that contribute to feeding behavior builds the foundation for developing methods to alter energy intake and partitioning in lactating dairy cows, ultimately increasing health and milk production. Future research is required to understand how different fuels affect liver metabolism, oxidation, and DMI within the time frame of a meal for dairy cows in the PP period.

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