Effect of Biotin on Activity and Gene Expression of Biotin-Dependent Carboxylases in the Liver of Dairy Cows

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ABSTRACT

Biotin is a cofactor of the gluconeogenic enzymes pyruvate carboxylase (PC) and propionyl-coenzyme A carboxylase (PCC). We hypothesized that biotin supplementation increases the activity and gene expression of PC and PCC and the gene expression of phosphoenolpyruvate carboxykinase (PEPCK) in the liver of lactating dairy cows. Eight multiparous Holstein cows (40 ± 2 kg/d of milk yield and 162 ± 35 d in milk) were randomly assigned to 1 of 2 diet sequences in a crossover design with two 22-d periods. Treatments consisted of a basal diet (60% concentrate) containing 0 or 0.96 mg/kg of supplemental biotin. On d 21 of each period, liver tissue was collected by percutaneous liver biopsy. Activities of PC and PCC were determined by measuring the fixation of [¹⁴C]O₂ in liver homogenates. Abundance of mRNA for PCC, PC, and PEPCK was determined by quantitative reverse-transcription PCR. Biotin supplementation did not affect milk production or composition. Biotin supplementation increased the activity of PC but had no effect on PCC activity. Biotin supplementation did not affect the gene expression of PC, PCC, and PEPCK. The increased activity of PC without changes in mRNA abundance may have been caused by increased activation of the apoenzymes by holocarboxylase synthetase. In conclusion, biotin supplementation affected the activity of PC in the liver of lactating dairy cows, but whether biotin supplementation increases glucose production in the liver remains to be determined.

Key words: biotin, dairy cow, pyruvate carboxylase, propionyl-coenzyme A carboxylase

INTRODUCTION

Several studies (Zimmerly and Weiss, 2001; Majee et al., 2003; Ferreira et al., 2007), but not all (Rosendo et al., 2004), have demonstrated that biotin supplementation increases milk production by lactating dairy

cows. Despite these observations, the mechanism by which biotin supplementation increases milk production is still unknown. Glucose supply to the mammary gland is a major determinant of milk yield in lactating dairy cows (Reynolds et al., 1994). Therefore, the increase in milk production by lactating dairy cows whose diets were supplemented with biotin might be related to an increase in gluconeogenesis, presumably in the liver.

Biotin is a cofactor of the gluconeogenic enzymes pyruvate carboxylase (PC, EC 6.4.1.1) and propionyl-coenzyme A (CoA) carboxylase (PCC, EC 6.4.1.3). Pyruvate carboxylase catalyzes the carboxylation of pyruvate to form oxaloacetate, whereas PCC catalyzes the carboxylation of propionyl-CoA to form methylmalonyl-CoA, a precursor of oxaloacetate. Rodriguez-Melendez et al. (2001) reported lower PCC and PC activities in the liver of biotin-deficient rats than in the liver of biotin-sufficient rats. Mock and Mock (2002) also reported reduced PCC activity in the liver of biotin-deficient rats. Our main hypothesis was that biotin supplementation would increase the activity and expression of genes for the biotin-dependent carboxylases PCC and PC in the liver of lactating dairy cows.

Increased activities of PC and PCC might result in increased production of oxaloacetate. Phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32) catalyzes the decarboxylation of oxaloacetate to produce phosphoenolpyruvate, the precursor for glucose synthesis. Phosphoenolpyruvate was synthesized in liver-extract incubations containing PC and PEPCK, but little phosphoenolpyruvate was synthesized in incubations with only PC, resulting in accumulation of oxaloacetate (Keech and Utter, 1963). Baird and Young (1975) observed that the increase in activity of PCC in the liver of dairy cows fed a diet containing 100% concentrate was accompanied by an increase in the activity of PEPCK. Because of its key role in gluconeogenesis, we expected an increased activity of PEPCK if biotin supplementation increased the production of oxaloacetate. Because the activity of PEPCK is correlated with PEPCK mRNA abundance in dairy cows (Agca et al., 2002), our secondary hypothesis was that biotin supplementation would increase the gene expression of PEPCK in the liver of lactating dairy cows. The objectives of this study were

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to determine the effect of biotin supplementation on the activities of PCC and PC and on the expression of genes for PCC, PC, and PEPCK in the liver of lactating dairy cows.

MATERIALS AND METHODS

Materials and Reagents

NaH[¹⁴C]CO₃ (5.95 mmol/L, 8.4 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Propionyl-CoA (P5397), disodium ATP (A3377), sodium pyruvate (P2256), acetyl-CoA (A2056), reduced NAD (N8129), and malate dehydrogenase (M7032) were purchased from Sigma (St. Louis, MO). The Bradford Assay Kit (23236) was purchased from Pierce (Rockford, IL). Trizol reagent and reverse-transcription enzyme (Super Script II) were purchased from Invitrogen Corporation (Carlsbad, CA). Ribonuclease inhibitor (RNasin, N2111) was purchased from Promega Corporation (Madison, WI). The SYBR Green PCR master mix was purchased from Qiagen, Inc. (Valencia, CA). Random and specific primers were purchased from Operon Biotechnologies, Inc. (Huntsville, AL).

Animals and Diets

All procedures involving animals were approved by the Agricultural Animal Care and Use Committee of The Ohio State University. The experiment was designed as a crossover design with two 22-d periods. Eight multiparous Holstein cows were fed a common diet (Table 1) for a 21-d preliminary period. At the end of the preliminary period, cows were 162 ± 35 DIM and produced 40 ± 2 kg/d of milk. At the end of the experiment, 6 of the 8 cows were pregnant and the average days of gestation for cows that were pregnant was 82. All cows were randomly assigned to 1 of 2 sequences of treatments (control to biotin, and biotin to control). Treatments consisted of experimental diets (Table 1) with 0 (control) or 0.96 mg (biotin) of supplemental biotin per kilogram of DM. The concentration of supplemental biotin was selected to supply 20 mg/d based on an estimated DMI of 22 kg/d. The diet was formulated to meet nutrient requirements (NRC, 2001) for a 650-kg lactating dairy cow producing 40 kg of milk/d. High proportions of grain in the substrate decreased microbial biotin synthesis *in vitro* (Abel et al., 2001); therefore, we formulated the basal diet to contain a high proportion of concentrate (60% DM basis). Supplemental biotin (Rovimix Biotin; DSM Nutritional Products Inc., Parsippany, NJ) was supplied in the pelleted concentrate mix. Cows were housed in individual tie stalls and fed once daily (0400 h). Diets were offered

Table 1. Ingredients and nutrient composition (% DM basis) of the basal diet

Item	Preliminary	Experimental ¹
Ingredient		
Corn silage	20.7	12.1
Alfalfa silage	31.1	30.4
Corn grain, dry and ground	17.4	20.7
Corn gluten meal	8.0	9.6
Soybean hulls	13.7	16.3
Soybean meal, 44% CP	6.4	7.7
Fat, animal and vegetable blend	0.8	1.0
Urea	0.1	0.1
Sodium bicarbonate	0.6	0.8
Minerals and vitamins	1.2	1.4
Chemical analysis		
CP	20.6	20.6
NDF	33.4	33.5
Starch	19.9	19.4

¹The experimental diet with supplemental biotin contained 0.0048% of biotin premix (20 mg/g of Rovimix H-2; DSM Nutritional Products Inc., Parsippany, NJ), which replaced a similar amount of corn gluten meal. The diet contained 0.38% dicalcium phosphate, 0.58% limestone, 0.01% magnesium oxide, 0.20% trace mineral salt, 0.15% selenium (200 mg/kg), 0.0023% zinc sulfate, 0.0012% copper sulfate, 3,000 IU/kg of vitamin A, 870 IU/kg of vitamin D, and 21 IU/kg of vitamin E.

ad libitum (~5% feed refusals, as-fed basis) as mixed rations. The amount of feed offered and refused was measured daily. Diets were adjusted weekly for changes in ingredient DM concentration. Cows were milked twice daily (0200 and 1300 h) and milk weights were recorded electronically at each milking. The average of the daily milk yields and DMI from d 14 to 20 was used for statistical analysis. Cows were weighed on d 21 of each period at 0800 h.

Sample Collection and Analysis

Samples of feed and feed refused were collected once weekly. Aliquots of feed and feed refusal samples were dried in a forced-air oven for 24 h at 100°C. A second aliquot of feed was stored at -20°C until analysis. Forage samples were lyophilized and all feed samples were ground to pass through a 1-mm screen of a Wiley mill (Arthur H. Thomas, Philadelphia, PA). Ground samples were analyzed for DM (24 h at 100°C), NDF (Ankom²⁰⁰ Fiber Analyzer; Ankom Technology, Fairport, NY) with sodium sulfite and α-amylase (Ankom Technology), CP (AOAC, 1990), and starch (Weiss and Wyatt, 2000).

Milk samples (a.m. and p.m. milkings) were collected on d 10 and 17 for determination of milk fat, protein, and lactose concentrations with a B2000 Infrared Analyzer (Bentley Instruments, Chaska, MN) by DHI Cooperative, Inc. (Columbus, OH). An additional milk sample (p.m. milking), and blood and urine samples were collected on d 20. Concentrations of avidin-binding substances (ABS) in the milk, plasma, and urine were de-

terminated (Ferreira et al., 2007). Crude liver homogenates (described below) were diluted (1:20) before a single-step competitive enzyme-binding assay (Ridascreen Biotin kit; R-Biopharm GmbH, Darmstadt, Germany) to measure ABS. Urine was also analyzed for creatinine (Di Giorgio, 1974). Samples of rumen fluid were collected on d 20 approximately 2 h postfeeding via a stomach tube. Rumen fluid was processed and analyzed for VFA (Zimmerly and Weiss, 2001).

Liver Biopsies

On d 21 of each period, liver tissue was collected by percutaneous liver biopsy. Liver biopsies were performed while cows were restrained in a chute. After clipping, locating liver parenchyma and large blood vessels by ultrasonography (5 MHz linear probe, Aloka 500V; Aloka, Wallingford, CT), and appropriately scrubbing the area, 10 mL of 2% lidocaine hydrochloride was injected at the 11th and 12th intercostal space. After making a 2-cm incision, a sterile biopsy instrument (5-mm i.d.) was directed toward the opposite elbow and inserted through the muscle and peritoneal tissue into the liver. After retracting the biopsy instrument, the incision was closed with suture staples. Two aliquots of liver tissue were immediately rinsed with sterile 0.9% NaCl solution, placed in a sterile plastic vial, snap-frozen in liquid N, and stored at -80°C .

Activity of Biotin-Dependent Carboxylases

Thawed liver tissue (0.86 ± 0.22 g, wet weight) was homogenized and subjected to sonication as described by Zempleni et al. (1997). Aliquots of the resulting supernatant (i.e., liver homogenate) were snap-frozen in liquid N and stored at -80°C until ABS, enzyme activity, and protein assays were conducted. Protein concentrations in liver homogenates were determined in triplicate according to the method of Bradford, using BSA as a protein standard. The coefficient of variation among triplicates was 5.3%.

Activity of PCC was determined by measuring the fixation of $^{14}\text{CO}_2$ according to Zempleni et al. (1997), with modifications. Briefly, 5 μL of liver homogenate was vortex-mixed with 100 μL of prewarmed (30°C) incubation buffer. The incubation buffer (Suormala et al., 1985) contained 20 mmol/L of $\text{NaH}^{14}\text{CO}_3$ (specific radioactivity 125 $\mu\text{Ci}/\text{mmol}$), 100 mmol/L of Tris (pH = 8.0), 0.75 mmol/L of dithiothreitol, 6.0 mmol/L of MgCl_2 , 100 mmol/L of KCl, 3.14 mmol/L of disodium ATP, 1% Triton X-100 (vol/vol), and 1.0 mmol/L of propionyl-CoA. The mixture was incubated in a water bath at 30°C for 8 min. The reaction was terminated by adding 20 μL of 1 mol/L of perchloric acid (vortex-mixed). A

100- μL aliquot was transferred into 5-mL scintillation vials, dried in a dry bath at 55°C for approximately 2 h to remove unfixed CO_2 (i.e., until a purplish residue was observed), and mixed with 4 mL of scintillation liquid (Ecolite+; MP Biomedicals, Irvine, CA). Fixed $^{14}\text{CO}_2$ was measured using a liquid scintillation analyzer (BS 6500; Beckman Coulter, Inc., Fullerton, CA). Final values were obtained after a 96-h equilibration of the dried residue with the scintillation liquid.

Activity of PC was determined by measuring the fixation of $^{14}\text{CO}_2$ (Zempleni et al., 1997), with modifications. Briefly, 5 μL of liver homogenate was vortex-mixed with 100 μL of prewarmed (30°C) incubation buffer. The incubation buffer (Warren and Tipton, 1974) contained 15 mmol/L of $\text{NaH}^{14}\text{CO}_3$ (specific radioactivity 680 $\mu\text{Ci}/\text{mmol}$), 100 mmol/L of Tris (pH = 8.0), 5 mmol/L of MgSO_4 , 0.1 mmol/L of acetyl-CoA, 0.1 mmol/L of reduced NAD, malate dehydrogenase (5 units/mL), 1 mmol/L of disodium ATP, and 5 mmol/L of sodium pyruvate. The mixture was incubated in a water bath at 30°C for 5 min. The reaction was terminated, and fixed $^{14}\text{CO}_2$ was measured as described above. Blank reactions were performed in all assays by omitting liver homogenate from the incubations. All samples were assayed in triplicate. The coefficients of variation among triplicates were 9.6 and 10.0% for PCC and PC, respectively. All specific activities are expressed in nanomoles of total CO_2 fixed per milligram of protein in 1 min at 30°C .

RNA Extraction

Total RNA was isolated from liver tissue (0.35 ± 0.11 g, wet weight) using 4 mL of Trizol reagent following the manufacturer's instructions. Precipitated RNA was redissolved in 400 μL of RNase-free water. Protein contamination of the isolated RNA was determined by the ratio of optical absorbance at 260 to 280 nm, which averaged 1.66 ± 0.05 .

Quantification of mRNA

The reverse-transcription reaction was performed using Super Script II reverse transcriptase following the manufacturer's instructions, but using RNasin as the ribonuclease inhibitor. To control for DNA contamination in the extracted RNA, reverse-transcription reactions were also performed without the addition of reverse transcriptase. No products were obtained.

Primers for PC, PCC, and cytosolic PEPCK were designed after obtaining bovine-specific mRNA sequences from the National Center of Biotechnology Information (Table 2). Primers were diluted to a working concentration of 15 $\mu\text{mol}/\text{L}$. Quantitative real-time PCR was per-

Table 2. Primer design for quantitative real-time PCR

Gene ¹	Accession no.	Primer sequence	Position
PC	NM_177946	F 5'-CATGCGCTTCCTGTACGAGT-3'	1,846
		R 5'-CGCAGAACTTGAAGACCACA-3'	1,991
PCC	CB223151	F 5'-GAGGACACAAGCAGCATTCT-3'	250
		R 5'-GCAGGTCTCCTTCTCCAACT-3'	454
PEPCK	NM_174737	F 5'-TCCTTGGGAAGAAGTGCT-3'	388
		R 5'-CGGGAGAGTAGGGTTTCAT-3'	572
GAPDH	NM_001034034	F 5'-AAGATTGTCAGCAATGCC-3'	499
		R 5'-ACAGACACGTTGGGAG-3'	791

¹PC = pyruvate carboxylase; PCC = propionyl-coenzyme A carboxylase; PEPCK = phosphoenolpyruvate carboxykinase.

formed in 96-well plates by incubating 2 μ L of reverse-transcription product with 0.5 μ L of forward primer, 0.5 μ L of reverse primer, 10 μ L of SYBR Green PCR master mix, and 7 μ L of RNase-free water. Quantitative real-time PCR was performed in a continuous fluorescence detector (DNA Engine Opticon 2 System; Bio-Rad Laboratories, Inc., Waltham, MA) using the following temperatures and times per cycle (40 cycles): denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 60 s. The first cycle was preceded by denaturation at 95°C for 15 min. Nonspecific amplification was not evidenced by the melting curve analysis and agarose-gel electrophoresis. Primer pairs were also validated after sequencing the amplified product. For each gene, all samples ($n = 16$) were amplified in triplicate and in one plate (i.e., within-run replication). Expression of the genes for PC, PCC, and PEPCK were normalized to that of GAPDH (Table 2).

Statistical Analysis

All variables were analyzed using the MIXED procedure of SAS (SAS Institute, 2004). The model included cow (random, 7 df), period (random, 1 df), and residual error (random, 6 df).

RESULTS

Biotin supplementation did not affect DMI, milk yield, or milk composition (Table 3). Consistent with

previous studies (Zimmerly and Weiss, 2001; Ferreira et al., 2007), biotin supplementation did not affect molar proportions of VFA in the rumen (59.7, 22.2, 13.8, 0.8, 2.4, and 1.1 mol/100 mol of VFA for acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate, respectively; data not shown). Biotin supplementation increased ($P < 0.01$) the concentration of ABS in plasma, milk, and urine but not in liver (Table 4). Biotin supplementation increased ($P < 0.08$) the activity of PC but did not affect ($P > 0.20$) the activity of PCC in liver tissue (Figure 1). Biotin supplementation did not affect ($P > 0.30$) expression of the genes for PC, PCC, and PEPCK (Figure 2).

DISCUSSION

Biotin is a required cofactor for 4 carboxylase enzymes, 2 of which (PC and PCC) are directly involved in glucose synthesis. In a dairy cow, the peripheral glucose supply comes almost exclusively from gluconeogenesis (Reynolds et al., 1994). Because the glucose supply to the mammary gland is a major determinant of milk yield, increased activity of PC and PCC could explain the increased milk yield often observed when biotin is supplemented. The pathway for glucose synthesis from lactate, glycerol, and alanine involves PC, whereas PCC is needed to synthesize glucose from propionate. In this study, biotin supplementation increased ($P < 0.08$) the activity of hepatic PC but had no effect on PCC. In rats that were marginally or clinically deficient in biotin, activities of both hepatic PC (Rodriguez-Melendez et al., 2001) and hepatic PCC (Rodriguez-Melendez et al., 2001; Mock and Mock, 2002) were depressed. Zemleni and Mock (1999) suggested that in nonruminants, PCC activity may be more responsive than PC activity to changes in biotin status because of the importance of PC in central metabolic pathways. However, in ruminants, propionate is the major precursor of glucose. In dairy cattle near peak production (83 DIM), about 66% of the net glucose released from the liver came from propionate, whereas only 10% came

Table 3. Production measures of cows consuming a diet containing either 0 (control) or 0.96 mg/kg of DM of supplemental biotin (biotin)

Item	Control	Biotin	SEM	P-value
BW, kg	746	752	14	0.13
DMI, kg/d	27.9	28.3	1.3	0.23
Milk yield, kg/d	39.1	39.3	2.3	0.82
Milk fat, %	3.59	3.66	0.34	0.52
Milk true protein, %	3.30	3.32	0.08	0.73
Milk lactose, %	4.80	4.83	0.06	0.50

Table 4. Concentrations of avidin-binding substances (ABS) in plasma, milk, urine, and liver of cows consuming a diet with either 0 (Control) or 0.96 mg/kg DM of supplemental biotin (Biotin)

Item	Control	Biotin	SEM	<i>P</i> -value
Plasma ABS, nmol/L	3.48	4.95	0.34	0.01
Milk ABS, nmol/L	109.7	329.5	32.6	0.01
Urine ABS, nmol/mmol of creatinine	84.1	179.5	19.2	0.01
Liver ABS, pmol/mg of protein	1.05	1.06	0.11	0.91

from lactate, glycerol, and alanine combined (Reynolds et al., 2003). Because of the importance of propionate for glucose synthesis, PCC may have a higher priority for biotin than PC in ruminants, thus explaining the different treatment effects on the activities of PC and PCC. The activities of PC and PCC (Figure 1) were similar to those previously reported for dairy cows (Murondoti et al., 2004), but were substantially greater than those reported in the liver of rats (Zempleni et al., 1997).

Because biotin supplementation did not affect expression of the gene for PC (Figure 2), the increased activity of PC may be attributed to an increased activation of apocarboxylase into holocarboxylase (Rodriguez-Melendez et al., 2001; Solorzano-Vargas et al., 2002) by action of the enzyme holocarboxylase synthetase (HCS) or to limited availability of biotin to biotinylate apocarboxylases (Sealey et al., 2005). Rodriguez-Melendez et al. (2001) observed a lower activity of PC in the liver of biotin-deficient rats, whereas the mRNA abundance for PC in the liver was similar between biotin-deficient and biotin-sufficient rats. Rodriguez-Melendez et al. (2001) and Solorzano-Vargas et al. (2002) reported that biotin deficiency decreased the mRNA abundance of HCS in rats and humans. In this study we attempted to measure the abundance of HCS mRNA as a marker

of HCS activity, but no message amplification was obtained using 3 different primer pairs. Sealey et al. (2005) reported lower abundances of holocarboxylases in biotin-deficient than in biotin-sufficient mice (similar observations for dams and fetuses), whereas the mRNA abundances did not differ. They suggested that biotin deficiency results in a lack of available biotin to biotinylate apocarboxylases.

Contrary to our second hypothesis, biotin supplementation did not affect the expression of PEPCK in the liver of lactating dairy cows. We based our hypothesis on the assumption that an increased activity of PEPCK would be required for conversion of the additional oxaloacetate (i.e., the presumed result of increased activities of PCC and PC). Accordingly, Baird and Young (1975) indicated that the activity of PEPCK in the liver of dairy cows responded to dietary changes (50 vs. 100% of concentrate in the diet), likely in response to an increased load of propionate to the liver. Biotin supplementation may not have increased the substrate load enough to increase the activity of PEPCK. Also, the mRNA abundance of PEPCK may not be an adequate indicator of PEPCK activity. Agca et al. (2002) reported a correlation between mRNA abundance of cytosolic PEPCK and PEPCK activity in adult dairy cows; how-

Figure 1. Enzyme activity (nmol of CO₂/mg of protein per min) of pyruvate carboxylase (PC) and propionyl-coenzyme A carboxylase (PCC) in the liver of lactating dairy cows. Cows were fed a diet containing either 0 (open bars) or 0.96 mg/kg of DM (solid bars) of supplemental biotin. Values are means $\pm$ SEM. Diet effect: PC ($P < 0.08$); PCC ($P < 0.21$). **Figure 2.** Gene expression (arbitrary units) of pyruvate carboxylase (PC), propionyl-coenzyme A carboxylase (PCC), and phosphoenolpyruvate carboxykinase (PEPCK) in the liver of lactating dairy cows. Cows were fed a diet containing either 0 (open bars) or 0.96 mg/kg of DM (solid bars) of supplemental biotin. Abundance of mRNA was normalized using GAPDH. Values are means $\pm$ SEM. Diet effects for all genes ($P > 0.30$).

ever, Hammon et al. (2005) reported no such correlation in milk-fed calves.

Biotin supplementation increased the concentration of ABS in plasma and milk (Table 4), similar to previous data (Zimmerly and Weiss, 2001; Rosendo et al., 2004; Ferreira et al., 2007). Biotin supplementation also doubled the concentration (per mmol of creatinine) of ABS in urine. In a companion study (Ferreira et al., 2007) using different cows but similar diets, plasma and milk concentrations were approximately 1.7 and 3.5 times higher for cows fed supplemental biotin compared with control cows. In the current study, plasma and milk concentrations in cows receiving the biotin supplement were 1.4 and 3 times higher than those of control cows. Cows in this study weighed approximately 100 kg more than those in the previous study; thus, increased basal metabolism (i.e., maintenance) and increased volume of distribution may account for the differences between the 2 experiments. The concentration of free ABS in liver tissue was not affected by biotin supplementation (Table 4). Liver homogenates were not subjected to acid or enzymatic hydrolysis; therefore, biotin that was bound to enzymes would not have been measured. Lewis et al. (2001) reported that rats fed a control diet (nonsupplemented) or a biotin-supplemented diet had similar concentrations of free biotin in the liver. Biotin is transported into human hepatocytes by a sodium-dependent transporter that has a Michaelis constant in the micromolar range (Said et al., 1994). Based on this transport system, biotin uptake by the liver should likely be related to the biotin concentration in plasma. Reidling and Said (2006) observed that biotin uptake by intestinal cells is upregulated during biotin deficiency, suggesting that a mechanism exists to maintain biotin homeostasis. The presence of a similar homeostatic mechanism in the liver could also explain the similar concentration of ABS in liver tissue.

Although biotin supplementation increased the activity of PC, it had no effect on any production measures (Table 3). This could mean that the increase in PC activity for cows fed biotin was not great enough to elicit a milk yield response or that something other than PC activity (i.e., glucose synthesis) was limiting milk production. However, this experiment was designed primarily to examine dietary effects on liver enzymes, and the number of observations is small for production data. In studies using more animals, biotin supplementation has often increased milk production (Zimmerly and Weiss, 2001; Majee et al., 2003; Ferreira et al., 2007). In addition, the liver biopsies at the end of the first period may have influenced intake and milk production during the second period. The effect of period could not be evaluated statistically because it was considered random. However, average milk yield was 4.2

kg/d lower and average DMI was 2.2 kg/d lower in period 2 than in period 1.

Residual or carryover effects may affect interpretations when a sequence of treatments is applied to the same subject, such as in the case of a crossover design. Whether carryover of biotin existed in this experiment is not certain, but seems unlikely. Frigg et al. (1993) measured the clearance of an oral dose of biotin (40 mg) in dairy heifers and observed that the biotin concentration in plasma returned to baseline concentrations 72 h after dosing. Accordingly, during period 2, the concentrations of ABS in the plasma, milk, and urine of cows consuming the control diet were similar to those of cows consuming the control diet during period 1. Weinberg and Utter (1979) reported that the half-life for PC in the rat liver was 4.6 d. Assuming that this value is similar for carboxylases in the liver of dairy cows, a residual effect on the enzyme activity also would be unlikely.

In conclusion, biotin supplementation did not affect the activity of PCC and expression of the genes for PCC, PC, and PEPCK in the liver of lactating dairy cows but increased the activity of PC. The differential response to biotin supplementation suggests that compared with PC, PCC may have a higher priority for biotin in cattle. Although the activity of PC was increased by biotin supplementation, no response was observed in milk yields. This could be a result of the experimental design or it could be that the change in PC activity was not large enough or that something other than glucose synthesis limited milk yields. However, data from this experiment suggest that when biotin is supplemented, the increase in milk production observed in some studies may be related to an increase in biotin-dependent carboxylases. Whether biotin supplementation increases glucose production in the liver still needs to be evaluated.

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