Effects of supplements of folic acid, vitamin B₁₂, and rumen-protected methionine on whole body metabolism of methionine and glucose in lactating dairy cows¹

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ABSTRACT

The present experiment was undertaken to determine the effects of dietary supplements of rumen-protected methionine and intramuscular injections of folic acid and vitamin B₁₂, given 3 wk before to 16 wk after calving, on glucose and methionine metabolism of lactating dairy cows. Twenty-four multiparous Holstein cows were assigned to 6 blocks of 4 cows each according to their previous milk production. Within each block, 2 cows were fed a diet estimated to supply methionine as 1.83% metabolizable protein, equivalent to 76% of methionine requirement, whereas the 2 other cows were fed the same diet supplemented daily with 18 g of rumen-protected methionine. Within each diet, the cows were administrated either no vitamin supplement or weekly intramuscular injections of 160 mg of folic acid plus 10 mg of vitamin B₁₂. To investigate metabolic changes at 12 wk of lactation, glucose and methionine kinetics were measured by isotope dilution using infusions of D[U-¹³C]glucose, [¹³C]NaHCO₃ and L[1-¹³C, ²H₃] methionine. Milk and plasma concentrations of folic acid and vitamin B₁₂ increased with vitamin injections. Supplementary B-vitamins increased milk production from 34.7 to 38.9 ± 1.0 kg/d and increased milk lactose, protein, and total solids yields. Whole-body glucose flux tended to increase with vitamin supplementation with a similar quantitative magnitude as the milk lactose yield increase. Vitamin supplementation increased methionine utilization for protein synthesis through increased protein turnover when methionine was deficient and through decreased methionine oxidation when rumen-protected methionine was fed. Vitamin supplementation decreased plasma concentrations of homocysteine independently of rumen-protected methionine

feeding, although no effect of vitamin supplementation was measured on methionine remethylation, but this could be due to the limitation of the technique used. Therefore, the effects of these B-vitamins on lactation performance were not mainly explained by methionine economy because of a more efficient methylneogenesis but were rather related to increased glucose availability and changes in methionine metabolism.

Key words: dairy cow, folic acid, vitamin B₁₂, methionine and glucose kinetics

INTRODUCTION

In the latest edition of *Nutrient Requirements of Dairy Cattle* (NRC, 2001), recommendations were made for 2 AA, Lys and Met, based on works of Rulquin et al. (1993) and Schwab (1996), according to which, under intensive dairy systems, these AA may be limiting in certain diets. Under such circumstances, increasing Met supply through feeding rumen-protected Met (**RPM**) has the potential to augment milk protein and fat concentrations and yields in high-producing dairy cows, probably through increased protein synthesis (NRC, 2001). Methionine is not only an AA involved in the structure of proteins per se but it also plays a unique role as the initiating AA for the synthesis of proteins (Brosnan et al., 2007). In addition to these roles in protein synthesis, Met, as a source of preformed labile methyl groups, is a key player in transmethylation reactions and lactation has been demonstrated to increase the demand for methylated compounds (Xue and Snoswell, 1985). The NRC (2001) requirement for Met was based on the relationship between Met supply and milk protein yield, but very little information is available on Met requirements for methyl group transfer in high-yielding cows.

Methyl group transfer occurs through the activation of Met in S-adenosylmethionine (**SAM**), which is the most important methyl group donor to a wide range of acceptors (Finkelstein, 1990). After the transfer of its methyl group, SAM becomes S-adenosylhomocysteine,

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which is hydrolyzed to homocysteine (**Hcy**) and adenosine. The former can be remethylated into Met by the vitamin B₁₂-dependent enzyme methionine synthase, which mediates the transfer of the methyl group from 5-methyltetrahydrofolate, a folate co-factor (Bässler, 1997). Therefore, both folic acid and vitamin B₁₂ are essential for remethylation of Hcy to Met. A major function of this cycle is to ensure that the cells always have an adequate supply of SAM, even when intake of preformed labile methyl groups such as Met, betaine, or choline is low. Homocysteine can also be catabolized via the transsulfuration pathway (cystathionine synthase), which leads to Cys synthesis (Finkelstein, 1990).

Vitamin B₁₂ also relates to energy metabolism as a co-enzyme for the methylmalonyl-CoA mutase, an enzyme crucial to propionate entry into the Krebs cycle and subsequent gluconeogenesis (McDowell, 2000). During lactation in dairy cattle, large amounts of glucose are required for lactose synthesis and as an energy source. As very limited amounts of glucose are absorbed from intestinal digestion of starch, the dairy cow relies heavily on gluconeogenesis for glucose supply (Reynolds, 2006). In high-yielding dairy cows, propionate is the major glucose precursor, followed by glycerol, lactate, and glycolytic AA (Danfaer et al., 1995).

Milk secretion requires substantial supplies of both glucose and AA. Given the roles of folic acid and vitamin B₁₂ in protein and energy metabolism, these 2 B-vitamins should play an important role in the regulation of these metabolic pathways in lactating dairy cows. In addition, because of their direct link with the regeneration of Met from Hcy, their metabolic effect and subsequent response on animal performance might depend on Met supply. Thus, it was hypothesized that supplements of folic acid plus vitamin B₁₂ would affect lactation performance 1) through the methylation cycle, in which case, this effect would be more important when Met supply is limited, and 2) by improving gluconeogenesis because of the role of vitamin B₁₂ in this metabolic pathway. The present study was therefore undertaken to determine the effects of RPM and folic acid plus vitamin B₁₂ supplementation and their potential interaction on whole body kinetics of Met and glucose in lactating dairy cows at 12 wk of lactation.

MATERIALS AND METHODS

Cows and Treatments

For the purpose of the current study, 24 multiparous lactating Holstein cows from the herd at the Agriculture and Agri-Food Canada Research Centre (Sherbrooke, QC, Canada) were selected from a larger study. The cows were kept in a tie-stall barn under 16-h light cycle

per d (0630 to 2230 h) and were milked twice daily at 12-h intervals. Care of cows followed the recommended Code of Practice of Agriculture Canada (1990) and the guidelines of the Canadian Council on Animal Care (1993). The experimental period began 3 wk before calving and lasted until 16 wk after calving. All cows were fed a close-up diet for 3 wk before the expected date of calving until calving and then a basal lactation diet (Table 1). For the larger study, cows had free access to water and were fed ad libitum allowing 10% of refusals. They were fed once daily before calving and twice daily during lactation. Long hay was given at 0730 h and the mixed ration was served at 0800 and 1300 h.

Cows were assigned to 6 blocks of 4 cows each according to milk production of their previous lactation. Within each block, 2 cows were fed the basal diet calculated (NRC, 2001) to provide Met at 1.83% of MP supply (**M**−), equivalent to 76% of the Met requirement (NRC, 2001), whereas the other 2 cows were fed the same diet supplemented daily with RPM (**M**+; 9 and 18 g of Mepron-85/d, Degussa AG, Hanau, Germany, pre- and postcalving, respectively). With a net Met content of 85%, 73% of rumen by-pass protection, and 82% of intestinal digestibility (Berthiaume et al. 2001), the product used in the present experiment was estimated to provide 9.2 g of Met per d after calving and increased the estimated Met supply to 2.23% of MP, equivalent to 93% of the Met requirement (NRC, 2001). Within each level of Met supplementation (**M**+ or **M**−), the cows received either no vitamin supplement (**B**₉−**B**₁₂−) or weekly intramuscular injections of vitamins (**B**₉+**B**₁₂+): 160 mg of folic acid (pteroylmonoglutamic acid, ICN Biochemicals Inc., Cleveland, OH) plus 10 mg of vitamin B₁₂ (cyanocobalamin, 5,000 µg/mL, Vetoquinol, Lavaltrie, QC, Canada). These levels of supplementation were chosen according to previous experiments (Girard et al., 1995; Girard and Matte, 2005). The parenteral route was chosen, instead of dietary supplementation, to rule out any possible effects of vitamins on ruminal microflora.

The larger study was conducted up to 16 wk of lactation, but the measurements currently reported were conducted during the 12th week of lactation. This stage of lactation was selected based on previous results obtained in dairy cows supplemented with folic acid alone (Girard and Matte, 1998). Measurements of whole-body irreversible loss rate (**ILR**) of Met and glucose were conducted during steady state achieved through 2-h feeding in 12 equal meals per day using automated feeders (Ankom, Fairport, NY); the latter started 3 d before the onset of tracer infusions. Rumen-protected Met was also given with the meals in 12 equal servings of 1.5 g. Long hay was given once per day (0730 h).

Table 1. Ingredients and nutrient composition of the diets fed to dairy cows

Item	Diet	
	Close-up	Lactation
Ingredients, %		
Grass hay	33.0 ¹	8.2 ²
Legume-grass silage ³	11.0	23.4
Corn silage ⁴	14.5	23.1
Cracked corn	16.9	28.5
Soybean meal 49%		9.6
Beet pulp	13.3	
Micronized soybean	3.9	2.0
Distillers grains (wheat)	1.3	0.7
Distillers grains (corn)	2.2	1.1
Canola meal	1.3	0.7
Mineral and vitamin premix	1.6 ⁵	1.8 ⁶
Calcium carbonate	1.0	0.8
Nutrient composition		
CP, %	12.8	17.3
RDP, ⁷ %	7.9	10.6
RUP, ⁷ %	4.9	6.7
ADF, %	26.5	19.2
NDF, %	45.2	30.4
NE _L , ⁸ Mcal/d	19.5	34.4
MP, ⁸ g/d	1,055	2,341
Met, ⁸ % of MP	1.85	1.83
Lys, ⁸ % of MP	6.18	6.3
K, %	1.27	1.55
Ca, %	0.69	0.87
P, %	0.52	0.44
Mg, %	0.39	0.29
S, %	0.25	0.26
Cl, %	0.43	0.34

¹7.8 ± 1.1% CP, 0.8 ± 0.1% soluble protein, 34.2 ± 5.8% ADF, 58.9 ± 7.0% NDF, 5.6 ± 0.6% lignin, (n = 3).

²10.9 ± 1.7% CP, 2.4 ± 0.1% soluble protein, 31.9 ± 1.8% ADF, 55.1 ± 4.2% NDF, 4.4 ± 0.5% lignin (n = 5).

³16.8 ± 2.3% CP, 8.0 ± 1.2% soluble protein, 32.8 ± 4.1% ADF, 42.3 ± 5.9% NDF, 7.5 ± 2.5% lignin (n = 11).

⁴7.6 ± 0.4% CP, 3.5 ± 0.6% soluble protein, 21.3 ± 1.5% ADF, 37.0 ± 1.9% NDF, 2.1 ± 0.3% lignin (n = 10).

⁵Contains per kg: 30 g Ca, 120 g P, 120 g Mg, 69 g Na, 106 g Cl, 15 g K, 20 g S, 4,343 mg Fe, 6,566 mg Mn, 6,465 mg Zn, 1,616 mg Cu, 202 mg I, 121 mg Co, 40 mg Se (25% organic selenium), 732,323 IU vitamin A, 247,475 IU vitamin D and 7,576 IU vitamin E.

⁶Contains per kg: 95 g Ca, 55 g P, 55 g Mg, 130 g Na, 150 g Cl, 14 g K, 21 g S, 2,745 mg Fe, 2,065 mg Mn, 3,000 mg Zn, 495 mg Cu, 69 mg I, 33 mg Co, 20 mg Se (25% organic selenium), 501,859 IU vitamin A, 65,000 IU vitamin D and 2,600 IU vitamin E.

⁷Calculated according to the model of National Research Council (2001).

⁸Calculated according to National Research Council (2001) from average DMI of control cows (M–B₉–B₁₂–) during the close-up (12.6 kg/d) and lactation (21.8 kg/d) periods.

During the 2-h feeding period, cows were fed 95% of ad libitum DMI measured during the 2 previous weeks to avoid refusals.

Forage and Milk Analyses. Forage samples were analyzed for DM, CP, ash, ether extract (AOAC, 2000), ADF, NDF, acid-detergent lignin (Ankom200 fiber analyzer, Ankom), and minerals by inductively coupled

plasma emission spectrometry (AOAC, 2000; Agri-Food Laboratories, Guelph, ON, Canada).

Milk production was recorded at each milking. Milk samples were collected at 4 consecutive milkings at 82.7 ± 1.9 d of lactation. Milk composition (fat, protein, lactose, and urea) was determined by near infrared reflectance spectroscopy method (Valacta, Sainte-Anne-de-Bellevue, QC, Canada).

Vitamins in Milk. Milk folates were determined by radioassay (interassay CV = 4.8%) with a commercial kit designed for human plasma (Quantaphase Folate II, Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON, Canada) after preparation as described by Girard and Matte (1998).

Samples for determination of vitamin B₁₂ in milk were prepared as follows: 10 mL of milk, 2 mL of 0.2 N HCl, and 100 µL of 1.0 mM NaCN were placed in a 50-mL tube (Sarstedt Inc., Newton, NC), corked, and vortexed. The tubes were placed in boiling water for 15 min, rapidly cooled on ice, and transferred into 15-mL polycarbonate tubes for centrifugation at 51,520 × g for 20 min at 4°C. The supernatants were filtered (Whatman filter 42, 9-mm diameter, Fisher Scientific, Ottawa, ON, Canada) in weighed tubes of 50 mL, weighed again, and the amount of supernatant was obtained by difference. The pH was adjusted to 6.5 with 3.3 N NaOH. The tubes were then filled to 20 mL with distilled water, vortexed, and stored at –20°C or used immediately. Vitamin B₁₂ was determined in duplicate for each hydrolysis using a radioassay [Quantaphase B₁₂, Bio-Rad Laboratories (Canada) Ltd.]. The interassay CV was 6.3% and recovery of a known amount of cyanocobalamin was 103.0%.

Blood Sampling Procedure

At 83.9 ± 1.7 d after calving, after distribution of the morning meal, blood samples were collected by venipuncture of the coccygeal vein, using a vacutainer system (Becton, Dickinson and Co., Franklin Lakes, NJ) using tubes with EDTA for folates, vitamin B₁₂, BHBA, and NEFA analyses. Tubes with heparin were used for AA, glucose, and urea determinations. Immediately after collection, the tubes were placed on ice and within 1 h after sampling, blood was centrifuged for 20 min at 1,854 × g at 4°C. For AA analysis, 400 µL of plasma was mixed with 400 µL of Met sulfone (0.4 mM, internal standard, Sigma-Aldrich, Oakville, ON, Canada). Plasma was frozen at –20°C until assays.

Blood Plasma Analyses

Folates and Vitamin B₁₂. Folates and vitamin B₁₂ in plasma were determined in duplicate using a radio-

assay [Quantaphase Folate II and Quantaphase B₁₂, Bio-Rad Laboratories (Canada) Ltd.] as described by Girard and Matte (1988). Interassay CV were 4.2 and 4.1% for folates and vitamin B₁₂, respectively.

Urea, NEFA, Glucose, and BHBA. Plasma urea, NEFA, glucose, and BHBA were determined using commercial kits (Diagnostic Chemicals Limited Urea assay, BioPacific Diagnostic Inc., Charlottetown, PE, Canada; NEFA-C, Wako Chemicals GmbH, Neuss, Germany; Roche Diagnostics Glucose GmbH, Mannheim, Germany, and β -hydroxybutyrate Reagent Set, Pointe Scientific Inc., Canton, MI, respectively).

Amino Acids. Total Hcy and total Cys concentrations were determined in plasma by a modification of the HPLC method of Malinow et al. (1989) as described in Girard et al. (2005). Two further changes were made: the mobile phase was a mixture of 50 mM sodium phosphate monobasic, 1.0 mM 1-octanesulfonic acid (Sigma Ultra) and 2% acetonitrile and the solvent was pumped through the analytic column (4.6 $\times$ 150 MCM) at 2 mL/min. Met concentration was measured by isotope dilution using GC-MS (Model CG6890-MS5973, Agilent Technologies, Wilmington, DE) as described previously (Calder et al., 1999).

Plasma concentrations of other AA were determined by HPLC with precolumn derivatization according to the Pico-Tag procedure (Waters, Mississauga, ON, Canada) and using a method adapted from White et al. (1986). Processed plasma, with Met sulfone added as an internal standard, was filtered through a 10,000 molecular weight filter and centrifuged for 30 min at 31,000 $\times g$ at 4°C. A Pico-Tag column was used for separation of physiological AA (3.9 $\times$ 300 mm) maintained at 46°C in a column heater. The mobile phase consisted of 2 eluents labeled A (0.07 M sodium acetate trihydrate buffer containing 350 μ L of 10 mg/mL EDTA, adjusted to pH 6.45 with glacial acetic acid, filtered on 0.45- μ m nylon membrane filters and mixed with 25.65 mL of acetonitrile) and B (methanol, water, and acetonitrile; 15:40:45, vol/vol/vol) used according to a gradient elution program. Amino acid standards 18 H (Pierce, Rockford, IL) were dissolved in water using serial dilutions: 160, 240, 320, and 400 μ M, and injected at the beginning of each week.

Kinetic Measurements

Infusions and Blood Sampling. The tracer infusions began on average on 87 $\pm$ 7 d of lactation. The day before the beginning of the infusion period, 2 catheters were inserted into 2 jugular veins, one to perform infusions and the other to collect blood. Tracers were dissolved in sterile saline and were administered as follows: L[1-¹³C,²H₃]Met (¹³C, 99% and methyl-²H₃,

98%, Cambridge Isotope Laboratories, Andover, MA) on d 1, [¹³C]sodium bicarbonate (Cambridge Isotope Laboratories) on d 2 and D[U¹³-C]glucose (¹³C₆, 99%, Cambridge Isotope Laboratories) on d 3. On each of these days, 4 blood samples were collected before onset of the infusion to determine the natural abundance of the infused metabolite and of CO₂. All daily infusions started with a priming dose of either 0.96, 2.5, or 3.3 mmol followed by a 4-h constant infusion performed at the rate of 0.96, 1.8, or 5.5 mmol/h for Met, bicarbonate, and glucose, respectively.

Starting 2 h after the initiation of the infusion, 5 jugular blood samples were taken every 30 min into heparinized syringes. On each sampling day, immediately after collection, three 1.5-mL portions were placed into evacuated vacutainer tubes (Becton, Dickinson and Co.) containing 1 mL of frozen lactic acid and reacted immediately for measurement of CO₂ enrichment on the same day. On d 1 and 3, the remaining blood (10 mL) was distributed into heparinized vacutainer tubes, kept on ice, and centrifuged within 30 min after sampling at 1,854 $\times g$ for 15 min at 4°C. Plasma samples were stored at -20°C until measurement of isotopic enrichment (**IE**) of Met (d 1) and glucose (d 3).

Enrichment Analyses. The CO₂ released from reaction of whole blood with lactic acid was analyzed for [¹³C] IE for m/z ions 44, 45, and 46 on a triple collector isotopic ratio mass spectrometer (Sira 12, VG Masslab, Manchester, UK).

The IE of plasma Met was determined by GC-MS (Agilent Technologies) on deproteinized plasma samples (1 mL of plasma with 250 μ L of 38% sulfosalicylic acid) derivatized with 50 μ L of MTBSTFA:MF (1:1; N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide; N,N-dimethylformamide; Sigma-Aldrich). Infusion of L[1-¹³C,²H₃]Met was associated with 2 plasma species of labeled Met: the original molecule and L[1-¹³C]Met, formed from remethylation of [1-¹³C]Hcy obtained after transmethylation of L[1-¹³C,²H₃]Met. The abundance of specific ions was determined by selected ion monitoring at the following m/z ions: 292:295 for [1-¹³C,²H₃]Met (m+4; this fragment did not include the labeled C) and 320:321 for [1-¹³C]Met (m+1).

Glucose IE was analyzed by GC-MS (Agilent Technologies) using the pentaacetate derivative (Haymond and Snehag, 2000), with mass selective detector operating in the positive chemical ionization mode. Isotopic enrichments were measured by monitoring ions at m/z 331, 332, 333, 334, and 337 to quantify the IE of m+1, m+2, m+3, and m+6 of glucose isotopomers, respectively; isotopic enrichments on m+4 and m+5 isotopomers were undetectable.

Calculations. The IE of the Met, glucose and CO₂ are expressed as mole percent excess. Whole body Met,

Figure 1. A schematic pathway of L[1-¹³C, ²H₃]Met with its components: transmethylation (TM), remethylation (RM), and transsulfuration (TS); CH₃-THF, methyl-tetrahydrofolate, THF, tetrahydrofolate. If Met is labeled on the methyl group and the carboxyl group, the methyl label will be lost during TM, whereas Hcy RM will produce Met labeled only on the carboxyl group. The carboxyl label will be lost during TS, which corresponds to Met oxidation, and will appear in CO₂ (adapted from Mercier et al., 2006).

glucose, and CO₂ ILR (mmol/h) were calculated as follows: $\text{ILR} = \text{INF}/\text{IE}$, where INF is the rate of infusion of labeled Met, glucose, or bicarbonate infusion and IE is the IE of plasma Met, glucose, or blood CO₂. The infusion rate was not removed from the ILR because in further calculations we could not assume that the dose of labeled Met infused was completely oxidized. Therefore, to have a better appraisal of the oxidation relative to whole body ILR, the rate of infusion was not removed from either term.

Methionine kinetics were calculated according to the model described in Figure 1. Whole-body Met-methyl flux rate (**ILR-M**), calculated with the IE of (m+4) Met, estimated the loss rate of Met as soon as the methyl group was transferred to SAM, producing a labeled [1-¹³C]Hcy. Remethylation of this labeled Hcy generated [1-¹³C]Met, considering null the probability of incorporation of a labeled methyl group to a labeled Hcy. The estimation of whole-body Met-carboxyl flux rate (**ILR-C**), using the sum of IE of m+4 and m+1, yielded the ILR of Met excluding remethylation. The difference between ILR-M and ILR-C represents a minimal estimate of remethylation because if a (m+1)Met re-entered the transmethylation-remethylation cycle, the Met molecule was not further altered and therefore, this fraction was not accounted for in the calculation.

The whole-body fractional rate of oxidation (**FO**, %) of Met was calculated using the following equation:

$$\text{FO} = [(\text{IE}_{\text{CO}_2\text{-Met}} \times \text{ILR-CO}_2\text{-bic}) / \text{INF}] \times 100$$

where $\text{IE}_{\text{CO}_2\text{-Met}}$ was the IE of CO₂ measured during the infusion of the labeled Met, $\text{ILR-CO}_2\text{-bic}$ represented CO₂ production estimated during the bicarbonate infusion and INF was the rate of infusion of labeled Met. Whole-body Met oxidation (Ox, mmol/h) was then calculated as follows:

$$\begin{aligned} \text{Ox} &= \text{FO} \times \text{ILR} = [(\text{IE}_{\text{CO}_2} \times \text{ILR-CO}_2\text{-bic}) / \text{INF}] \\ &\times (\text{INF} / \text{IE}_{\text{m}+4}) = (\text{IE}_{\text{CO}_2} \times \text{ILR-CO}_2\text{-bic}) / \text{IE}_{\text{m}+4}. \end{aligned}$$

Methionine used for whole-body protein synthesis was calculated as the difference between whole-body ILR-C and Met oxidation. Transmethylation was the sum of remethylation and Met oxidation (Figure 1).

Uniformly labeled glucose was infused in this study to assess the feasibility of measuring gluconeogenesis using mass isotopomer distribution analysis (m+1, m+2, m+3, and m+6). However, the IE of glucose species m+1, m+2, and m+3 were below detection specification of the GC-MS for more than half of the cows. Therefore, only whole-body glucose ILR is reported using m+6 isotopomer. The FO of glucose was calculated similarly to Met FO with the exception that the infusion rate was multiplied by 6 to account for the 6 labeled carbons per molecule of glucose.

Statistical Analyses

Supplementations of Met (0 or 18 g of RPM) and B-vitamins (0 or both folic acid and vitamin B₁₂) were tested as the main factors in a 2 × 2 factorial arrangement with 6 randomized complete blocks. Milk production and composition as well as DMI were averaged for the whole 12th week. All variables were analyzed using the MIXED procedure (SAS Institute, 2004) with supplementations of Met and vitamins as the main factors. Orthogonal contrasts were used to determine the effect of Met and vitamin supplementations and their interaction. Results were reported as least square means with standard errors. The significance level was defined at $P \leq 0.05$ and trends toward significance were considered at $0.05 < P \leq 0.15$. When the Met × vitamin interaction reached a P -value ≤ 0.15 , statistical analyses were conducted for each level of Met supplementation separately.

Table 2. Effects of dietary supplements of rumen-protected Met (M) and intramuscular injections of folic acid (B₉) and vitamin B₁₂ (B₁₂) given to dairy cows on production variables at 12 wk of lactation

Item	Treatment ¹				<i>P</i> -value		
	M+		M–				
	B ₉ –B ₁₂ – (n = 6)	B ₉ +B ₁₂ + (n = 6)	B ₉ –B ₁₂ – (n = 6)	B ₉ +B ₁₂ + (n = 5)	Met	Vitamin	Met × Vitamin
BW, kg	613 ± 17	644 ± 17	619 ± 17	619 ± 19	0.55	0.47	0.36
DMI, kg/d	23.7 ± 1.0	23.1 ± 1.0	23.0 ± 1.0	23.5 ± 1.1	0.86	0.98	0.56
Milk production, kg/d	34.1 ± 1.4	39.2 ± 1.4	35.4 ± 1.4	38.6 ± 1.5	0.82	0.01	0.52
Fat, g/kg	34.5 ± 1.5	34.3 ± 1.5	34.5 ± 1.5	33.3 ± 1.6	0.71	0.62	0.71
Protein, g/kg	30.0 ± 0.8	29.4 ± 0.8	29.0 ± 0.8	27.9 ± 0.9	0.14	0.31	0.74
Lactose, g/kg	46.7 ± 0.7	45.7 ± 0.7	46.8 ± 0.7	46.2 ± 0.8	0.69	0.29	0.77
Urea, mg/kg	123.5 ± 7.9	130.7 ± 7.9	126.1 ± 7.9	144.0 ± 8.5	0.25	0.08	0.43
Total solids, g/kg	111.2 ± 2.2	109.4 ± 2.2	110.3 ± 2.2	107.4 ± 2.4	0.53	0.32	0.81
Folates, ng/mL	81.4 ± 10.5	81.4 ± 10.5	69.1 ± 10.5	85.9 ± 11.1	0.59	0.25	0.25
Vitamin B ₁₂ , pg/mL	5,422 ± 758	6,600 ± 758	4,666 ± 758	7,287 ± 828	0.96	0.02	0.34
Fat, kg/d	1.18 ± 0.06	1.34 ± 0.06	1.22 ± 0.06	1.27 ± 0.07	0.81	0.09	0.35
Protein, kg/d	1.02 ± 0.04	1.15 ± 0.04	1.03 ± 0.04	1.07 ± 0.05	0.40	0.05	0.32
Lactose, kg/d	1.59 ± 0.07	1.79 ± 0.07	1.65 ± 0.07	1.78 ± 0.08	0.70	0.04	0.66
Total solids, kg/d	3.79 ± 0.15	4.29 ± 0.15	3.90 ± 0.15	4.14 ± 0.17	0.90	0.03	0.41
Folates, mg/d	2.78 ± 0.44	3.20 ± 0.44	2.40 ± 0.44	3.35 ± 0.47	0.74	0.07	0.45
Vitamin B ₁₂ , µg/d	182.1 ± 26.7	256.7 ± 26.7	164.3 ± 26.7	276.5 ± 29.0	0.97	0.002	0.45

¹M– and M+ = basal diet – or + rumen-protected Met; B₉–B₁₂– = no vitamin supplement; B₉+B₁₂+ = 160 mg folic acid/wk + 10 mg cyanocobalamin/wk.

RESULTS

Production Data

Body weight and DMI averaged 624 ± 17 kg and 23 ± 1 kg/d, respectively, and did not differ between treatments ($P > 0.55$, Table 2).

Milk production, however, increased ($P = 0.01$) by approximately 12% with the administration of folic acid plus vitamin B₁₂, from 34.8 ± 1.0 to 38.9 ± 1.0 kg/d. Milk concentrations of total solids, fat, and lactose did not differ among treatments ($P > 0.29$), whereas milk CP concentrations tended to increase by 1.3 g/kg in M+ cows ($P = 0.14$, Table 2). Milk yields of protein, lactose, and total solids increased ($P < 0.05$) with vitamin supplements and averaged 1.03 ± 0.03 versus 1.11 ± 0.03 kg/d, 1.62 ± 0.05 versus 1.79 ± 0.05 kg/d, and 3.85 ± 0.11 versus 4.22 ± 0.11 kg/d for B₉–B₁₂– versus B₉+B₁₂+, respectively (Table 2). Milk fat yield only tended to increase ($P = 0.09$) with vitamin supplements, 1.20 ± 0.05 versus 1.31 ± 0.05 kg/d for B₉–B₁₂– versus B₉+B₁₂+, respectively. Increased vitamin supply tended to be associated with an increase in milk urea concentrations ($P = 0.08$, 124.8 ± 6.5 versus 137.3 ± 6.6 g/kg for B₉–B₁₂– versus B₉+B₁₂+, respectively; Table 2). Milk concentrations of vitamin B₁₂ were higher in cows injected with B vitamins ($P = 0.02$), whereas concentrations of folates were not affected ($P > 0.25$, Table 2). Nevertheless, supplementary folic acid plus vitamin B₁₂ increased the amounts of these vitamins secreted daily in milk: 173.2 ± 20.9 versus 266.6 ± 21.7 µg/d for vitamin B₁₂ ($P = 0.002$; Table 2) and 2.59 ± 0.37

versus 3.27 ± 0.38 mg/d for folates ($P = 0.07$; Table 2) for B₉–B₁₂– versus B₉+B₁₂+, respectively.

Plasma Variables

Plasma concentrations of vitamin B₁₂ increased by 72% with vitamin injections (236.7 ± 32.0 versus 407.0 ± 35.1 for B₉–B₁₂– versus B₉+B₁₂+, $P = 0.002$; Table 3) but this increase tended to be greater in cows fed no RPM supplement (Met × vitamin interaction, $P = 0.13$). Plasma concentrations of folates also tended to vary according to vitamin and Met supplementation (Met × vitamin interaction, $P = 0.10$; Table 3); indeed, B-vitamin injections tended to increase plasma concentrations of folates in cows fed RPM ($P = 0.06$) whereas no difference was observed in M– cows ($P = 0.99$).

The response of plasma concentrations of urea to vitamin injections tended to differ within each level of Met supplementation (Met × vitamin interaction, $P = 0.07$, Table 3). Injections of folic acid plus vitamin B₁₂ had no effect in cows fed supplementary Met ($P = 0.86$) but increased plasma urea in M– cows ($P = 0.05$). Plasma concentrations of glucose, BHBA and NEFA did not differ among treatments ($P > 0.34$; Table 3).

Cows injected with folic acid plus vitamin B₁₂ supplements had lower ($P = 0.03$) Hcy concentrations than the control cows, 6.50 ± 0.64 versus 4.88 ± 0.69 µM, but higher ($P = 0.04$) plasma concentrations of Arg, 92.9 ± 4.8 versus 105.0 ± 5.0 µM, for B₉–B₁₂– versus B₉+B₁₂+, respectively (Table 3).

Dietary supplement of RPM decreased plasma concentrations of Ile, from 159.4 ± 9.1 to 137.6 ± 8.8 µM

Table 3. Effects of dietary supplements of rumen-protected Met (M) and intramuscular injections of folic acid (B₉) and vitamin B₁₂ (B₁₂) given to dairy cows on plasma variables at 12 wk of lactation

Item	Treatment ¹				<i>P</i> -value		
	M+		M–				
	B ₉ –B ₁₂ – (n = 6)	B ₉ +B ₁₂ + (n = 6)	B ₉ –B ₁₂ – (n = 6)	B ₉ +B ₁₂ + (n = 5)	Met	Vitamin	Met × Vitamin
Folates, ng/mL	12.4 ± 1.5	17.8 ± 1.5	16.9 ± 1.5	16.8 ± 1.6	0.25	0.10	0.10
Vitamin B ₁₂ , pg/mL	269.6 ± 44.2	364.8 ± 44.2	203.8 ± 44.2	443.8 ± 48.4	0.88	0.002	0.13
Glucose, μ <i>M</i>	3.57 ± 0.12	3.54 ± 0.12	3.62 ± 0.12	3.47 ± 1.07	0.94	0.45	0.61
Urea, m <i>M</i>	5.93 ± 0.39	5.99 ± 0.39	5.64 ± 0.39	7.01 ± 0.43	0.29	0.06	0.07
BHBA, m <i>M</i>	0.68 ± 0.11	0.65 ± 0.11	0.56 ± 0.11	0.71 ± 0.12	0.79	0.55	0.39
NEFA, μ <i>M</i>	171.7 ± 55.0	200.4 ± 55.0	160.3 ± 55.0	242.2 ± 60.2	0.79	0.34	0.64
Amino acids, μ <i>M</i>							
Ala	297 ± 24	313 ± 24	291 ± 24	291 ± 25	0.44	0.68	0.65
Arg	93.8 ± 6.0	100.3 ± 6.0	92.0 ± 6.0	109.7 ± 6.5	0.47	0.04	0.30
Asp	4.88 ± 1.25	6.55 ± 1.25	5.46 ± 1.25	6.24 ± 1.37	0.92	0.36	0.73
Cys	119.9 ± 5.0	101.5 ± 5.4	109.5 ± 5.0	114.2 ± 5.4	0.80	0.15	0.02
Glu	53.4 ± 4.1	52.9 ± 4.1	54.8 ± 4.1	48.3 ± 4.5	0.71	0.40	0.48
Gly	314.3 ± 32.9	377.3 ± 32.9	340.8 ± 32.9	293.1 ± 35.4	0.29	0.78	0.06
His	54.5 ± 6.5	52.7 ± 6.5	59.4 ± 6.5	63.2 ± 7.1	0.19	0.87	0.62
Hcy	7.12 ± 0.78	5.08 ± 0.84	5.87 ± 0.78	4.68 ± 0.84	0.23	0.03	0.53
Ile	139.0 ± 11.5	136.1 ± 11.5	147.9 ± 11.5	170.9 ± 12.6	0.07	0.36	0.28
Leu	215.1 ± 20.4	201.5 ± 20.4	233.2 ± 20.4	274.1 ± 22.3	0.03	0.50	0.18
Lys	99.7 ± 7.8	97.8 ± 7.8	98.8 ± 7.8	126.7 ± 8.4	0.07	0.09	0.06
Met	25.1 ± 1.5	24.5 ± 1.5	20.5 ± 1.5	25.3 ± 1.7	0.24	0.21	0.10
Phe	59.9 ± 5.7	49.5 ± 5.7	65.9 ± 5.7	71.4 ± 6.1	0.01	0.58	0.09
Pro	127.9 ± 13.4	133.8 ± 13.4	138.8 ± 13.4	153.7 ± 14.6	0.24	0.43	0.72
Ser	99.5 ± 6.1	116.9 ± 6.1	114.5 ± 6.1	106.4 ± 6.7	0.73	0.47	0.06
Thr	147.6 ± 11.8	151.1 ± 11.8	149.4 ± 11.8	152.2 ± 13.0	0.90	0.80	0.97
Tyr	74.4 ± 6.5	78.0 ± 6.5	84.7 ± 6.5	91.2 ± 7.1	0.10	0.46	0.83
Val	328.0 ± 22.7	301.9 ± 22.7	351.3 ± 22.7	395.2 ± 24.8	0.02	0.70	0.14
BCAA ²	682.1 ± 53.7	639.2 ± 53.7	732.3 ± 53.7	839.9 ± 58.7	0.03	0.55	0.17
EAA ³	1,069 ± 69	1,013 ± 70	1,126 ± 69	1,278 ± 70	0.03	0.48	0.13
NEAA ⁴	1,185 ± 66	1,277 ± 70	1,232 ± 66	1,214 ± 70	0.87	0.49	0.30
TAA ⁵	2,254 ± 122	2,300 ± 132	2,358 ± 122	2,493 ± 132	0.19	0.42	0.68

¹M– and M+ = basal diet – or + rumen-protected Met; B₉–B₁₂– = no vitamin supplement; B₉+B₁₂+ = 160 mg folic acid/wk + 10 mg cyanocobalamin/wk.

²BCAA = branched-chain amino acids = Ile + Leu + Val.

³EAA = essential amino acids = His + Ile + Leu + Val + Lys + Phe + Thr + Met.

⁴NEAA = nonessential amino acids = Ala + Arg + Asp + Glu + Gly + Pro + Ser + Tyr + Cys.

⁵TAA = total amino acids.

($P = 0.07$), Leu, from 253.6 ± 16.1 to 208.3 ± 15.5 μM ($P = 0.03$), Val, from 373.2 ± 17.3 to 314.9 ± 17.3 μM ($P = 0.02$) and consequently, branched-chain amino acids (**BCAA**), from 786.1 ± 41.7 to 660.7 ± 40.3 μM ($P = 0.03$). Supplement of RPM also decreased plasma Tyr, from 87.9 ± 4.8 to 76.2 ± 4.6 μM ($P = 0.10$) and essential AA ($P = 0.03$) from $1,202 \pm 55$ to $1,041 \pm 53$ μM, for M– and M+ cows, respectively (Table 3).

Folic acid plus vitamin B₁₂ administration tended to increase plasma concentrations of Lys and Met in cows fed no supplementary Met ($P < 0.06$), whereas they had no effect ($P \geq 0.73$) when the cows received RPM (Met × vitamin interaction, $P = 0.06$ and 0.10 for Lys and Met, respectively, Table 3). In M+ cows, plasma concentrations of Cys decreased with B-vitamin supply ($P = 0.01$) whereas in M– cows, vitamins had no effect on Cys concentrations ($P = 0.65$; Met × vitamin interaction, $P = 0.02$, Table 3). A similar trend was

observed for plasma Phe (Met × vitamin interaction, $P = 0.09$, Table 3) notwithstanding that plasma Phe was higher for M– than M+ cows, 68.6 ± 5.0 to 54.7 ± 4.9 μM, respectively ($P = 0.01$). The response of Gly and Ser to vitamin injections tended to differ within each level of Met supplementation (Met × vitamin interaction, $P = 0.06$, Table 3); injections of folic acid plus vitamin B₁₂ had no effect in cows not supplemented with Met ($P \geq 0.29$) but they tended to increase concentrations of these 2 AA in cows fed RPM ($P = 0.10$). Plasma concentrations of Ala, Asp, Glu, His, Pro, Thr, total nonessential AA, and total AA were not affected by treatments ($P \geq 0.19$).

Whole-Body Kinetics

Glucose ILR tended to increase with administration of folic acid plus vitamin B₁₂ (802 ± 16 versus 838 ± 17

Table 4. Effects of dietary supplements of rumen-protected Met (M) and intramuscular injections of folic acid (B₉) and vitamin B₁₂ (B₁₂) given to dairy cows on whole body glucose kinetics at 12 wk of lactation

Item	Treatment ¹				<i>P</i> -value		
	M+		M–				
	B ₉ –B ₁₂ – (n = 6)	B ₉ +B ₁₂ + (n = 6)	B ₉ –B ₁₂ – (n = 6)	B ₉ +B ₁₂ + (n = 5)	Met	Vitamin	Met × Vitamin
Glucose kinetics (mmol/h)							
ILR	817 ± 22	843 ± 22	787 ± 22	833 ± 24	0.36	0.11	0.63
Ox ²	190 ± 19	182 ± 19	183 ± 19	197 ± 21	0.81	0.81	0.49
FO (%) ³	23.2 ± 2.0	21.5 ± 2.0	23.1 ± 2.0	23.3 ± 2.1	0.55	0.63	0.54
Milk lactose/glucose ILR	0.47 ± 0.02	0.52 ± 0.02	0.51 ± 0.02	0.52 ± 0.02	0.38	0.22	0.48

¹M– and M+ = basal diet – or + rumen-protected Met; B₉–B₁₂– = no vitamin supplement; B₉+B₁₂+ = 160 mg folic acid/wk + 10 mg cyanocobalamin/wk.

²Ox = oxidation = FO × ILR glucose.

³FO = fractional oxidation.

mmol/h for B₉–B₁₂– versus B₉+B₁₂+ respectively; *P* = 0.11, Table 4). On average, 23% of glucose ILR was oxidized whereas 50% was used for lactose milk production but these variables were not affected by treatments (*P* > 0.49, Table 4).

Results on whole-body Met kinetics are reported in Table 5. Dietary supplements of Met increased ILR-M from 20.0 ± 0.9 to 24.5 ± 0.9 mmol/h (*P* = 0.003). The ILR-C rate was also higher (*P* = 0.003) in M+ than M– cows, averaging 17.9 ± 0.7 versus 21.4 ± 0.7 mmol/h for M– versus M+, respectively. The effects of vitamin supplements on the ILR-M and ILR-C, however tended to differ according to the level of dietary Met (Met × vitamin interaction, *P* = 0.11 and 0.09, respectively). Supplementary folic acid plus vitamin B₁₂ tended to increase the ILR-M and ILR-C by approximately 20% in M– cows (*P* = 0.02) but had no effect on M+ cows (*P* > 0.71).

Folic acid plus vitamin B₁₂ supplements decreased Met entry in the transmethylation pathway (TM in Figure 1) in cows fed RPM (*P* = 0.02), whereas it had no effect (*P* = 0.20) when the cows did not receive Met supplementation (Met × vitamin interaction, *P* = 0.01, Table 5). The entry of Met into the transmethylation pathway relative to ILR-M followed the same pattern except that the interaction was only a tendency (*P* = 0.10). A similar tendency was observed for Met oxidation (Met × vitamin interaction, *P* = 0.06, Table 5), whereas fractional oxidation of Met did not reach significance (*P* > 0.16).

The rate of Met going to Hcy and remethylated (RM in Figure 1) was higher in M+ cows (*P* = 0.04), but the ratio of remethylation on ILR-M was not affected by treatments (*P* > 0.20), averaging 12% of ILR-M. Methionine used for protein synthesis increased from 14.5 ± 0.8 to 17.9 ± 0.8 mmol/h with RPM supplements (*P* = 0.01) and tended to increase (*P* = 0.06), from 15.1 ± 0.86 to 17.3 ± 0.8 mmol/h with vitamin supplements.

DISCUSSION

In the present experiment, intramuscular injections of folic acid plus vitamin B₁₂ successfully increased vitamin supply to dairy cows. Indeed, as previously observed with intramuscular injections (Girard et al., 1995; Girard and Matte, 2005), supplementary vitamin B₁₂, and to a lesser extent, folic acid increased secretion of folates and vitamin B₁₂ in milk and their plasma concentrations.

The increase in milk production and milk component yields following administration of a combined supplement of folic acid and vitamin B₁₂ observed in the present experiment is in agreement with observations from previous experiments. Intramuscular injections of vitamin B₁₂, given to cows fed a basal diet supplemented with folic acid and Met, increased milk component yields compared with cows only supplemented with Met and folic acid (Girard and Matte, 2005). Graulet et al. (2007) also observed that in cows fed a diet providing a low Met supply, dietary supplements of folic acid, given alone or in combination with vitamin B₁₂, increased milk and milk protein yields as compared with unsupplemented cows and in addition, the 2 vitamins given together increased plasma glucose and decreased accumulation of hepatic lipids as compared with folic acid alone.

Glucose Kinetics

In the current study, whole-body glucose ILR was higher than previously reported in dairy cows infused with uniformly labeled glucose (Clark et al., 1977; Hammon et al., 2008) but DMI and milk production of cows in these previous studies were lower than in the current experiment. Nevertheless, the ratio of milk lactose to whole-body glucose ILR averaged 51% in the current study, as reported for this stage of lactation

Table 5. Effects of dietary supplements of rumen-protected Met (M) and intramuscular injections of folic acid (B₉) and vitamin B₁₂ (B₁₂) given to dairy cows on whole body Met kinetics at 12 wk of lactation

Item	Treatment ¹				<i>P</i> -value		
	M+		M–				
	B ₉ –B ₁₂ – (n = 6)	B ₉ +B ₁₂ + (n = 6)	B ₉ –B ₁₂ – (n = 6)	B ₉ +B ₁₂ + (n = 5)	Met	Vitamin	Met × Vitamin
Met kinetics (mmol/h)							
ILR-M ²	24.8 ± 1.2	24.1 ± 1.2	18.3 ± 1.2	21.8 ± 1.3	0.003	0.27	0.11
ILR-C ³	21.6 ± 1.0	21.3 ± 1.0	16.2 ± 1.0	19.5 ± 1.1	0.003	0.15	0.09
RM ⁴	3.2 ± 0.4	2.8 ± 0.4	2.0 ± 0.4	2.3 ± 0.4	0.04	0.84	0.42
Ox ⁵	4.5 ± 0.6	2.6 ± 0.6	3.1 ± 0.6	3.6 ± 0.6	0.74	0.22	0.06
FO (%) ⁶	18.6 ± 2.5	11.1 ± 2.5	16.7 ± 2.5	16.5 ± 2.7	0.50	0.16	0.18
TM ⁷	7.7 ± 0.6	5.4 ± 0.6	5.2 ± 0.6	5.7 ± 0.6	0.02	0.06	0.01
PS ⁸	17.1 ± 1.1	18.7 ± 1.1	13.1 ± 1.1	15.9 ± 1.1	0.01	0.06	0.59
Ox/ILR-M	0.19 ± 0.03	0.11 ± 0.03	0.17 ± 0.03	0.17 ± 0.03	0.50	0.16	0.18
RM/ILR-M	0.13 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.21	0.52	0.81
Ox/TM	0.58 ± 0.06	0.48 ± 0.06	0.61 ± 0.06	0.63 ± 0.07	0.20	0.24	0.23
RM/TM	0.42 ± 0.06	0.56 ± 0.06	0.41 ± 0.06	0.39 ± 0.07	0.20	0.34	0.23
TM/ILR-M	0.31 ± 0.02	0.23 ± 0.02	0.28 ± 0.02	0.27 ± 0.02	0.98	0.05	0.10

¹M– and M+ = basal diet – or + rumen-protected Met; B₉–B₁₂– = no vitamin supplement; B₉+B₁₂+ = 160 mg folic acid/wk + 10 mg cyanocobalamin/wk. One cow in M+B₉+B₁₂+ group has been removed for Ox, FO, transmethylation, and protein synthesis calculation.

²ILR-M = Met-methyl flux; irreversible loss rate of ²D₃ labeled Met. It corresponds to the sum of protein synthesis, oxidation, and remethylation.

³ILR-C = Met-carboxyl flux; irreversible loss rate of ¹³C labeled Met. It corresponds to the sum of protein synthesis and oxidation.

⁴RM = remethylation cycle = ILR-M – ILR-C.

⁵Ox = oxidation = FO × ILR-M.

⁶FO = fractional oxidation.

⁷TM = transmethylation = RM + Ox.

⁸PS = protein synthesis = ILR-M – RM – Ox = ILR-C – Ox.

(Clark et al., 1977; Bruckental et al., 1980; Rigout et al., 2002). Glucose oxidation was not altered by the treatments and averaged 23% of ILR, a value higher than previously reported in dairy cows (17%, Bauman et al., 1988; 14%, Hammon et al., 2008). The oxidation rate, measured through the recovery of glucose carbons in CO₂, is a minimum estimate as it only accounted for the carbons of uniformly labeled glucose that were recovered in CO₂, omitting other carbons of glucose that were incorporated into intermediate products.

Administration of folic acid plus vitamin B₁₂ tended to increase glucose ILR by 160 g/d compared with control cows and was associated with a similar increment in milk lactose secretion. Glucose is the primary precursor for mammary lactose synthesis. Therefore, the amount of glucose available may have an effect on milk production because lactose is the major osmoregulator for mammary uptake of water (Linzell, 1972) and a close relationship between whole-body glucose flux and milk volume has been proposed (Danfaer et al., 1995). The increased milk production of 4 kg/d observed with the folic acid plus vitamin B₁₂ supplements may reflect an increase of the quantity of glucose available. However, it has been recently reported that the increment in whole-body ILR of glucose induced by

infusions of either glucose, a mixture of 5 gluconeogenic nonessential AA in the duodenum, or propionate in the rumen of mid-lactation dairy cows was not paralleled by changes in lactose yields (Lemosquet et al., 2005). Therefore, glucose ILR might be important but is not the sole driver of milk and lactose yields.

Whole-body glucose ILR, as the rate of appearance, represents the sum of glucose available from portal absorption, glycogenolysis, and gluconeogenesis. In the current study, it is unlikely that portal absorption of glucose or glucose precursors differed between treatments because DMI of the same basal diet was similar between treatments. Glucose turnover from the glycogen pool could not be evaluated with this model, but there are no data available from the literature indicating that supplementary folic acid or vitamin B₁₂ would modify glycogen kinetics. Therefore, it is likely that the increase in whole-body ILR of glucose observed in cows supplemented with folic acid plus vitamin B₁₂ was because of enhanced gluconeogenesis. In fed ruminants, 50 to 60% of gluconeogenesis is provided by propionate (Danfaer et al., 1995). Propionate, originating from rumen fermentation of carbohydrates, provides propionyl-CoA, which is carboxylated to methylmalonyl-CoA by propionyl-CoA carboxylase, a biotin-dependent

enzyme. This pathway can also include catabolism of some AA (Ile, Val, Thr, Met). Methylmalonyl-CoA is then isomerized in succinyl-CoA in a reaction including the vitamin B₁₂-dependent enzyme methylmalonyl-CoA mutase (Le Grusse and Watier, 1993). Succinyl-CoA finally enters into the Krebs cycle where it can be used for gluconeogenesis. Bergman (1990) emphasizes that the methylmalonyl-CoA mutase step becomes limiting when vitamin B₁₂ supply is low. Plasma concentrations of methylmalonic acid were decreased by 18% when vitamin B₁₂ was injected intramuscularly to primiparous cows fed a diet supplemented with rumen-protected methionine and folic acid from 4 to 18 wk of lactation (Girard and Matte, 2005). Graulet et al. (2007) observed a decrease of the K_m (Michaelis constant) for adenosylcobalamin of methylmalonyl-CoA mutase in liver of cows supplemented with folic acid plus vitamin B₁₂, indicating an increased affinity of this enzyme for its coenzyme, thus improving the efficiency of this metabolic pathway. In addition, the cows in the present study were selected from a larger study for which gene expression of methylmalonyl-CoA mutase in liver increased by 15% when the cows were injected with folic acid plus vitamin B₁₂ as compared with control cows (our unpublished data). Therefore, supplementary folic acid plus vitamin B₁₂ may improve the efficiency of utilization of glucose precursors for gluconeogenesis, thus allowing positive effects of these B-vitamins on glucose availability and subsequently milk yield.

The increased milk protein yield in cows injected with folic acid plus vitamin B₁₂, also reported by Graulet et al. (2007), could result from the improvement of glucose supply observed with the vitamin treatment discussed above. Such a hypothesis is in agreement with previous studies which showed that an increased supply of energy increases the amounts of proteins secreted in milk (Rulquin and Delaby, 1997; Rulquin et al., 2004; Raggio et al., 2006). The enhanced availability of specific energy sources could explain the milk protein increase by a sparing effect on gluconeogenic AA, the spared AA being available at mammary level for the synthesis of milk protein. This effect may also be due to an increase of mammary blood flow favoring AA uptake as already seen with posttruminally glucose infusion (Rulquin et al., 2004).

Methionine Kinetics

Whole-body Met fluxes in M-B₉-B₁₂- cows are in the range of those previously reported in humans; around 30 and 25 $\mu\text{mol/kg per h}$ for ILR-M and ILR-C, respectively (Storch et al., 1990; Mercier et al., 2006) and similar to those observed in sheep fed a diet in which Met was calculated to be the first-limiting AA (Lobley

et al., 1996). The major outlets for Met are its incorporation into newly synthesized proteins and formation of SAM, through the transmethylation pathway. These outlets are in equilibrium with Met entry rate, the sum of Met absorbed, Met arising from protein degradation, and Met coming from Hcy methylation. Storch et al. (1988) measured the Met kinetics with [methyl-²H₃] and [1-¹³C]Met in humans. They pointed out that, whatever the supply in dietary Met (deficiency or not), the major part of Met (77%) was used for protein synthesis, whereas a smaller proportion (23%) entered in the transmethylation pathway. This partition was also observed in the current study with, on average, 73% of ILR-M directed toward protein synthesis of which approximately 53% was secreted in milk protein.

As previously observed in humans fed a diet supplemented with Met and Cys (Storch et al., 1990; Fukagawa et al., 1998; DiBuono et al., 2003), both ILR-M and ILR-C also increased with Met supplementation in dairy cows, with a concomitant increment in Met utilization for whole body protein synthesis. However, Met supplementation only tended to increase milk protein concentration (+0.13 g/100g). As there was no effect of supplementary Met on milk protein yield and assuming no effect of Met on N retention, it is likely that a general stimulation of protein turnover occurred with RPM.

In M- cows, intramuscular injections of folic acid plus vitamin B₁₂ tended to increase ILR-M and ILR-C by approximately 20%, whereas oxidation of Met tended to decrease with vitamin supplementation in M+ cows. Altogether, this resulted in increased utilization of Met for protein synthesis with vitamin supplementation, independent of the level of Met supply. It still remains unclear, however, if the positive effect of vitamin supplementation on protein synthesis observed in this study was related to an effect on protein synthesis itself, or was a consequence of the positive effect of the vitamin supplements on glucose availability, but is certainly linked with the increased milk protein yield induced by vitamin supplementation.

In M- cows, intramuscular injections of vitamins tended to increase plasma concentrations of Met and Lys. Coupled with the observation that vitamin supplementation increased Met ILR in M- cows, this suggested that vitamin supplementation in M- cows increased protein turnover, which might increase circulating concentrations of AA not extracted by the liver as Lys (Lapierre et al., 2005). On the other hand, Graulet et al. (2007) also observed that supplements of folic acid, alone or combined with vitamin B₁₂, tended to increase plasma concentrations of Met. In this last experiment also, milk protein yield was increased by these vitamin supplements.

In the transmethylation pathway, Met is transformed in SAM, the major donor of methyl group, which after giving its methyl group, will form S-adenosylhomocysteine and finally, provide the carbon skeleton of Hcy. Homocysteine could be either remethylated in Met, using folates (5-methyl-tetrahydrofolate) as one-carbon unit donor and vitamin B₁₂ as coenzyme for the enzyme methionine synthase or, alternatively, catabolized through the transsulfuration pathway. In this last pathway, Hcy undergoes a condensation reaction with serine via cystathionine synthase to form cystathionine, the direct precursor of Cys (Selhub, 1999). Cysteine sulfur is derived from Met, whereas the carbon and nitrogen are derived from Ser.

According to the metabolic pathways described above, weekly intramuscular injections of both folic acid plus vitamin B₁₂ decreased by 33% the plasma concentrations of Hcy. These results are in agreement with previous observations in cows (Girard et al., 2005) and growing-finishing pigs (Giguère et al., 2008) reporting a decrease in plasma concentrations of Hcy with a dietary supplement of folic acid, irrespective of the level of Met added to the diet. These observations would suggest increased remethylation of Hcy with vitamin supplementation. In humans, Met regeneration through Hcy remethylation is decreased by folate deficiency (Cuskelly et al., 2001), whereas a supplement of folic acid given to healthy adults improved the remethylation by 59% and reduced plasma Hcy by 18% (Stam et al., 2005). In farm animals, the fate of Hcy between transsulfuration or remethylation has been studied only by Lobbey et al. (1996) who observed that, in sheep, the provision of the major methylated products (creatine and choline) reduced the need for remethylation. In humans, remethylation is primarily regulated by the need for methyl groups (Brosnan et al., 2007; Mudd et al., 2007). Methyl groups are provided directly by dietary Met, choline or betaine, or from methylneogenesis through the folate pathway (Stead et al., 2006). Therefore, in humans, when the intake of preformed labile methyl groups, as Met, decreases, methylneogenesis prevails. Surprisingly, in the present experiment, the calculated value of remethylation increased with the intake of Met (M+). In humans, Mudd and Poole (1975) demonstrated that even with diets providing an adequate supply of Met, the homocysteinyl moiety cycled more than once between Met and Hcy, the number of cycles increasing when the intake of methyl groups decreased. Recycling of Hcy also increased in the liver of rats when the Met supply decreased (Eloranta et al., 1990). Moreover, these authors observed that the capacity of the skeletal muscle for Met catabolism, through the transsulfuration pathway, is limited and, consequently, there is a continuous flow of Met from

extrahepatic tissues to liver (Eloranta et al., 1990). Within the limit of the methods used, calculated remethylation values are likely to represent only the flux of remethylated Hcy released in the plasma pool and cannot detect if the same molecule has made more than one cycle. If the number of cycles of the homocysteinyl moiety was higher within hepatic cells of cows fed no supplementary RPM or fed supplementary folic acid plus vitamin B₁₂, then the limitation of the method used would not allow for detection of these differences.

Methionine molecules that enter the transmethylation pathway, transformed into Hcy and not remethylated to Met, are going through the transsulfuration pathway and oxidation. In the present experiment, supplementation with vitamins of M+ cows decreased Met oxidation, with simultaneous increment in plasma concentrations of Ser and decrease of plasma concentrations of Cys, all suggesting a real decreased oxidation under this treatment. No explanation can actually be offered to explain this unexpected interaction, the decrease of Met oxidation with vitamin supplementation only with RPM feeding.

CONCLUSIONS

In the current study, folic acid plus vitamin B₁₂ supplementation increased both milk lactose and protein yields. These increases were related to the positive effect of vitamins on glucose ILR and Met ILR, the later being mostly due to an increase of Met directed toward protein synthesis. Milk protein yield was not modified by supplementary Met, despite an increment in Met ILR and the use of Met for protein synthesis, suggesting that Met increased protein turnover. In conclusion, the effects of the supplements of folic acid and vitamin B₁₂ on lactation performance were not mainly explained by Met economy through a more efficient methylneogenesis as hypothesized, but were rather related to increased glucose availability and changes in Met metabolism.

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