Effects of Dietary Supplements of Folic Acid and Vitamin B₁₂ on Metabolism of Dairy Cows in Early Lactation¹

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ABSTRACT

The present experiment was undertaken to determine the effects of dietary supplements of folic acid and vitamin B₁₂ given from 3 wk before to 8 wk after calving on lactational performance and metabolism of 24 multiparous Holstein cows assigned to 6 blocks of 4 cows each according to their previous milk production. Supplementary folic acid at 0 or 2.6 g/d and vitamin B₁₂ at 0 or 0.5 g/d were used in a 2 × 2 factorial arrangement. Supplementary folic acid increased milk production from 38.0 ± 0.9 to 41.4 ± 1.0 kg/d and milk crude protein yield from 1.17 ± 0.02 to 1.25 ± 0.03 kg/d. It also increased plasma Gly, Ser, Thr, and total sulfur AA, decreased Asp, and tended to increase plasma Met. Supplementary B₁₂ decreased milk urea N, plasma Ile, and Leu and tended to decrease Val but increased homocysteine, Cys, and total sulfur AA. Liver concentration of phospholipids was higher in cows fed supplementary B₁₂. Plasma and liver concentrations of folates and B₁₂ were increased by their respective supplements, but the increase in plasma folates and plasma and liver B₁₂ was smaller for cows fed the 2 vitamins together. In cows fed folic acid supplements, supplementary B₁₂ increased plasma glucose and alanine, tended to decrease plasma biotin, and decreased *K_m* of the methylmalonyl-coenzyme A mutase in hepatic tissues following addition of deoxyadenosylcobalamin, whereas it had no effect when cows were not fed folic acid supplements. There was no treatment effect on plasma nonesterified fatty acids as well as specific activity and gene expression of Met synthase and methylmalonyl-coenzyme A mutase in the liver. Ingestion of folic acid supplements

by cows fed no supplementary B₁₂ increased total lipid and triacylglycerols in liver, whereas these supplements had no effect in cows supplemented with B₁₂. The increases in milk and milk protein yields due to folic acid supplements did not seem to be dependent on the vitamin B₁₂ supply. However, when vitamin B₁₂ was given in combination with folic acid, utilization of the 2 vitamins seems to be increased, probably more so in extrahepatic tissues. Metabolic efficiency seems also to be improved as suggested by similar lactational performance and dry matter intake for cows fed supplementary folic acid but increased plasma glucose and decreased hepatic lipids in cows fed folic acid and vitamin B₁₂ together.

Key words: dairy cow, lactation, folic acid, vitamin B₁₂

INTRODUCTION

Separate previous findings (Girard and Matte, 1998, 2005; Girard et al., 2005) showed that, in early lactation, a positive response of milk production and milk component yields to supplementary folic acid was observed in cows with higher plasma concentrations of vitamin B₁₂. However, in cows with low plasma concentrations of vitamin B₁₂, milk production and milk protein yield were either unchanged or depressed following folic acid supplementation.

Folic acid is a B-complex vitamin for which the sole biochemical function in mammals is to mediate the transfer of 1-C units (Choi and Mason, 2000). Folic acid, as 5,10-methylene-tetrahydrofolic acid (THF), and 10-formyl-THF gives 1-C units for pyrimidine and purine synthesis, essential for DNA formation. Moreover, 5,10-methylene-THF can also be irreversibly reduced to 5-methyl-THF, which under the action of the vitamin B₁₂-dependent cytosolic enzyme, Met synthase (EC 2.1.1.13), will transfer its methyl group to homocysteine for regeneration of Met and THF (Bässler, 1997). Methionine can then form S-adenosylmethionine, the primary intracellular methylating agent (Bailey and Gregory, 1999).

Received October 31, 2006.

Accepted March 12, 2007.

¹Contribution number 951 of the Dairy and Swine Research and Development Centre.

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A lack of vitamin B₁₂ would block 5-methyl-THF demethylation and reduce folate utilization at the cell level in spite of the accumulation of 5-methyl-THF in plasma (Scott, 1999). Moreover, in mammals, one other enzyme is vitamin B₁₂-dependent, methylmalonyl-CoA mutase (EC 5.4.99.2). This enzyme plays a part in the conversion of propionate to succinyl-CoA, an essential step for its entry in the Krebs cycle and its use as a gluconeogenic substrate (Kennedy et al., 1990; Taoka et al., 1994). Thus, this latter mitochondrial enzyme is probably highly solicited in high-producing dairy cows, due to the huge needs in energy and glucose to sustain milk production.

An insufficient supply of vitamin B₁₂, especially in early lactation, could possibly interfere with folate metabolism and even with gluconeogenesis. Therefore, the present project was undertaken in an attempt to elucidate the interaction between the vitamin B₁₂ status and the mode of action of supplementary folic acid on lactational performance of multiparous dairy cows in early lactation.

MATERIALS AND METHODS

Cows and Treatments

Twenty-four multiparous gestating cows from the dairy herd at the Agriculture and Agri-Food Canada Research Centre (Sherbrooke, Québec, Canada) with a BCS from 3.0 to 4.0 at drying off were selected for the experiment. Cows were kept in a tie-stall barn under 16 h of light per day (0530 to 2130 h). Care of cows followed the recommended code of practice of Agriculture Canada (1990) and the guidelines of the Canadian Council on Animal Care (1993). At drying off, approximately 60 d before the expected time of calving, all cows were fed a similar diet (Table 1). The experimental period began 3 wk before the expected time of calving and lasted until 8 wk of lactation. All cows were fed a close-up diet from 3 wk before calving until calving and then a lactation diet (Table 1). Forage samples were collected once a week. One part was dried for wet chemistry analysis, and the other part was immediately analyzed by near infrared spectroscopy to adjust, if necessary, the amounts of energy and protein supplements to maintain similar concentrations of energy and degradable and undegradable proteins throughout each diet period. However, during the experiment, no such adjustments were needed.

The cows were assigned to 6 blocks of 4 cows each 3 wk before the expected calving date according to their previous milk production, BCS at the beginning of the drying-off period, and plasma concentration of vitamin B₁₂ at 8 wk before the expected calving date. In each block, the cows were randomly assigned to one of the

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

Item	Dry cows	Close-up cows	Lactation cows
Ingredient (%)			
Grass hay	88.0 ¹	26.2 ¹	7.0 ²
Legume-grass silage ³	—	24.2	27.3
Corn silage ⁴	—	18.0	18.2
Cracked corn	7.8	22.9	32.4
Soybean meal, 49%	2.5	5.5	8.6
Micronized soybean	—	0.31	0.74
Distillers grain (wheat)	—	0.32	0.77
Distillers grain (corn)	—	0.24	0.57
Corn gluten meal	—	0.40	0.95
Canola meal	—	0.32	0.77
Mineral and vitamin premix	1.3 ⁵	1.5 ⁵	2.3 ⁶
Calcium carbonate	0.4	—	0.4
Nutrient composition			
DM (%)	85.5	46.8	44.4
CP (%)	9.4	13.8	16.4
RDP ⁷ (%)	5.9	9.7	11.0
RUP ⁷ (%)	3.6	4.2	5.4
ADF (%)	36.4	24.3	18.5
NDF (%)	61.8	40.8	31.1
NE _L ⁸ (Mcal/d)	14.6	18.2	33.9
MP ⁸ (g/d)	664	1,019	2,211
Met ⁸ (% MP)	1.86	1.97	1.87
Lys ⁸ (% MP)	6.53	6.86	6.44
K (%)	2.07	1.75	1.38
Ca (%)	0.71	0.62	0.84
P (%)	0.46	0.48	0.48
Mg (%)	0.32	0.38	0.30
S (%)	0.24	0.23	0.23
Cl (%)	0.53	0.45	0.47
Co (%)	1.71	1.89	0.84

¹8.3% CP, 1.0% ADF – CP, 3.0% NDF – CP, 3.4% soluble protein, 40.9% ADF, 69.1% NDF, 4.9% lignin.

²13.4% CP, 1.0% ADF – CP, 4.0% NDF – CP, 5.7% soluble protein, 40.9% ADF, 69.1% NDF, 4.9% lignin.

³18.5% CP, 1.6% ADF – CP, 4.8% NDF – CP, 8.4% soluble protein, 34.4% ADF, 54.0% NDF, 3.0% lignin.

⁴8.8% CP, 0.6% ADF – CP, 1.2% NDF – CP, 5.1% soluble protein, 21.3% ADF, 35.1% NDF, 1.9% lignin.

⁵Contains the following per kilogram: 31.3 g of Ca, 114 g of P, 120 g of Mg, 24 g of Na, 105 g of Cl, 16 g of K, 20 g of S, 4,453 mg of Fe, 6,500 mg of Mn, 7,700 mg of Zn, 1,610 mg of Cu, 202 mg of I, 120 mg of Co, 40 mg of Se (25% organic Se), 800,000 IU of vitamin A, 245,000 IU of vitamin D, and 7,500 IU of vitamin E.

⁶Contains the following per kilogram: 67.8 g of Ca, 66.4 g of P, 39 g of Mg, 151 g of Na, 108 g of Cl, 6.7 g of K, 10 g of S, 2,887 mg of Fe, 2,004 mg of Mn, 1,896 mg of Zn, 462.6 mg of Cu, 57.6 mg of I, 34.2 mg of Co, 24 mg of Se (25% organic Se), 398,800 IU of vitamin A, 70,458 IU of vitamin D, and 2,293 IU of vitamin E.

⁷Calculated according to the NRC (2001) model.

⁸Calculated according to the NRC (2001) from averaged DMI of control cows (B₉–B₁₂–) during the drying (11.0 kg/d), close-up (11.4 kg/d), and lactation (21.3 kg/d) periods.

4 following treatments: 1) no folic acid (B₉) or vitamin B₁₂ (B₁₂) supplementation (B₉–B₁₂–), 2) 2.6 g/d of folic acid (pteroylmonoglutamic acid) (B₉+B₁₂–), 3) 0.5 g/d of vitamin B₁₂ (cyanocobalamin; B₉–B₁₂+), and 4) folic acid (2.6 g/d) and vitamin B₁₂ (0.5 g/d) (B₉+B₁₂+). Previous milk production, BCS, and plasma concentration of vi-

tamin B₁₂ at 8 wk before the expected calving date (mean $\pm$ SD) were 28.1 ± 3.4 kg/d, 3.3 ± 0.3 and 312.2 ± 62.8 pg/mL for B₉-B₁₂⁻, 29.0 ± 5.9 kg/d, 3.5 ± 0.3 and 375.5 ± 38.9 pg/mL for B₉+B₁₂⁻, 26.1 ± 5.4 kg/d, 3.4 ± 0.4 and 359.9 ± 68.7 pg/mL for B₉-B₁₂⁺ and 28.3 ± 3.5 kg/d, 3.4 ± 0.4 and 429.9 ± 57.1 pg/mL for B₉+B₁₂⁺. They were similar among groups ($P = 0.20, 0.85$, and 0.08 for milk production, BCS, and plasma vitamin B₁₂, respectively). Plasma concentrations of vitamin B₁₂ and folates, 3 wk before the expected time of calving, were also similar ($P = 0.19$ and 0.53 , respectively) among groups; 249.5 ± 58.3 pg/mL and 8.0 ± 2.2 ng/mL for B₉-B₁₂⁻, 299.0 ± 27.5 pg/mL and 9.7 ± 3.7 ng/mL for B₉+B₁₂⁻, 321.4 ± 58.1 pg/mL and 9.1 ± 2.0 ng/mL for B₉-B₁₂⁺, and 283.2 ± 67.1 pg/mL and 8.1 ± 1.4 ng/mL for B₉+B₁₂⁺.

Cows had free access to water and were fed ad libitum. Hay was served at 0730 h, and the mixed ration was served twice a day at 0800 and 1600 h. Vitamin supplements were top-dressed with the morning meal. Refusals were weighed daily at 0700 h. Body weight and BCS were recorded once weekly. During the dry period, average DMI and BW were 13.7 ± 0.5 kg/d and 712 ± 12 kg, respectively. After calving, the cows were milked twice daily at a 12-h intervals. Feed intake and milk production were not recorded for the first 3 d following calving. Consequently, data for the first week of lactation were not included in statistical analysis.

Feed Analysis

Samples were analyzed for DM, CP, ash, ether extract, (AOAC, 1990), ADF, NDF, and acid detergent lignin (Ankom200 fiber analyzer, Ankom Technology Corp., Fairport, NY). Minerals were analyzed by inductively coupled plasma emission spectrometry.

Milk Sampling Procedure and Analysis

Milk production was recorded at each milking. Milk samples were collected at 2 consecutive milkings, the evening 2 d before blood sampling, and the following morning. Milk composition (DM, fat, CP, and MUN) as well as folates and vitamin B₁₂ contents were determined as described by Girard et al. (2005).

Blood Sampling Procedure and Analysis

Blood samples were collected after milking but before the morning meal by venipuncture using a Vacutainer system (Becton, Dickinson and Co., Franklin Lakes, NJ) with tubes with EDTA at 14.0 ± 1.8 , 28.2 ± 2.1 , 41.7 ± 2.1 , and 55.1 ± 2.1 d after calving. Folates and vitamin B₁₂ in plasma were determined in duplicate by

radioassay with a commercial kit designed for human plasma (Quantaphase Folate II and Quantaphase B₁₂; BioRad Laboratories Ltd., Mississauga, Ontario, Canada) and validated for bovine plasma by recovery and parallelism tests (Girard and Matte, 1988; Girard et al., 1989). Interassay CV were 4.2 and 4.1% for folates and vitamin B₁₂, respectively. Biotin was determined using an ELISA test developed and validated for bovine plasma in our laboratory (Santschi et al., 2005). Plasma glucose and NEFA were determined using commercial kits (Boehringer Ingelheim GmbH, Germany; NEFA-C from Wako Chemical GmbH, Neuss, Germany). Plasma AA composition was determined by HPLC according to the procedure of Sedgwick et al. (1991). Homocysteine, Met, and Cys concentrations were determined in blood plasma by a modification of the method of Malinow et al. (1989) described in Girard et al. (2005).

Liver Sampling Procedure and Analysis

Liver biopsies were performed 14.0 ± 1.8 , 28.2 ± 2.1 , and 55.1 ± 2.1 d after calving under local anesthesia and ultrasound guidance to minimize the hemorrhagic risks. The procedure was approved by the Institutional Committee on Animal Care of the research center. Thirty milliliters of procainic penicillin (20,000 IU/kg of BW) was given twice, at least 1 h before and 6 h after the biopsy. Liver samples were generally taken up at the level of the 10th intercostal space using a liver biopsy needle (20 cm $\times$ 0.6 cm) with a removable core. After introduction in the liver, the core was removed. The needle was then removed while applying a light suction with a syringe to keep the liver sample inside the needle. Liver tissue was immediately blotted to remove blood. Approximately 0.25 g was immediately homogenized, and mitochondria were purified by cell fractionation for the immediate determination of methylmalonyl-CoA mutase activity. The remaining tissue samples were frozen into liquid N and stored at -80°C until use.

Cell Fractionation and Protein Quantification. Methylmalonyl-CoA mutase activity needs to be determined on fresh tissue, whereas Met synthase activity could be measured on frozen tissue. However, both activities had to be assayed on their freshly purified respective cell subfractions (i.e., mitochondria and cytosol, respectively). Homogenization of liver tissue (253.0 ± 0.6 mg, $n = 138$) was performed in K₃PO₄ buffer, pH 7.47 (0.05 M), supplemented with protease inhibitors according to the procedure of Wilkemeyer et al. (1993). Cell fractionation based on differential centrifugations was realized as previously described (Graulet et al., 1998), except for the following adaptations. The mitochondrial pellet, obtained after 30 min of centrifugation

at $10,000 \times g$ and 4°C , was washed and returned in suspension in Na_3PO_4 buffer, pH 7.2 (0.02 M), with protease inhibitors. Due to the small quantity of tissue used in this method, at each step of the fractionation, the tubes and their contents were weighted to precisely determine recovery of the cell material. Average yield of recovery was $95.5 \pm 0.2\%$ ($n = 138$). Protein concentration in the cellular fractions was determined in triplicate according to the method developed by Lowry et al. (1951) adapted to 96-well plates.

Methylmalonyl-CoA Mutase Activity. The method is based 1) on the conversion of methylmalonyl-CoA into succinyl-CoA and 2) on the spectrophotometric detection of the CoA resulting from the hydrolysis of the succinyl-CoA mediated by the succinyl-CoA synthetase. It was developed by Taoka et al. (1994) and adapted in the present experiment for quantification in 96-well plates.

Membranes of the freshly isolated mitochondria (250 μL) were disrupted by sonication (3×15 s at 45 W) on ice just before the incubation. The preparation was diluted in 1.4 mL of Na_3PO_4 buffer, pH 7.2 (0.02 M), and centrifuged for 5 min at $13,000 \times g$ and 4°C to collect the supernatant (average protein concentration of 2.32 ± 0.05 $\mu\text{g}/\mu\text{L}$).

The linearity of succinyl-CoA (Sigma Chemical Corp., St. Louis, MO) quantification in the medium was established from 0 to 333 μM ($r^2 = 0.992$). The activity of the methylmalonyl-CoA mutase was linear ($r^2 = 0.9967$) from 20 to 140 μg of intramitochondrial proteins. Therefore, in the present study, the samples were diluted to maintain the average protein quantity in the assay medium close to 70 μg . The reaction was linear in time at least for the first 45 min (data not shown).

A volume of 180 μL of the basal reaction mixture [final concentrations per well: 100 mM Tris, 50 mM H_3PO_4 , 7.5 mM MgCl_2 , 1 mM guanosine 5'-diphosphate, 0.15 mM 5, 5'-dithiobis (2-nitrobenzoic acid), all from Sigma Chemical Corp.] and 3.3 mU/ μL of succinyl-CoA synthetase (also called succinic thiokinase, EC 6.2.1.4, from Boehringer Mannheim, Mannheim, Germany) was placed in each well. Appropriate volumes of methylmalonyl-CoA and desoxyadenosylcobalamine (both from Sigma Chemical Corp.) were added. The reaction started by addition of 30 μL of diluted intramitochondrial supernatant into wells (final volume: 300 μL per well). The assay of methylmalonyl-CoA mutase activity was realized by using a standard curve into which methylmalonyl-CoA was replaced by increasing amounts of succinyl-CoA ($y = 0.0046x - 0.0066$, $r^2 = 0.9986$, where y = the optic density and x = the succinyl-CoA concentration) ranging from 0 to 300 μM . The plates were placed in darkness, shaken for 1 min, and incubated 40 min at 37°C . Optic density was measured at 412 nm. Each

analysis was conducted in duplicate with a mean CV of 3.38%.

The specific activity of the enzyme was determined in the presence of saturating concentrations of 500 μM methylmalonyl-CoA as substrate and 5 μM desoxyadenosylcobalamine as cofactor. The holoenzyme activity was determined in the absence of exogenous cobalamine in the incubation medium. The activity of the methylmalonyl-CoA mutase was also determined according to increasing methylmalonyl-CoA concentrations (0 to 500 μM) on the one hand and to increasing desoxyadenosylcobalamine concentrations (0 to 2.5 μM) on the other hand. The kinetic parameters of the enzyme (K_M and V_{max}) according to its substrate (methylmalonyl-CoA) and its cofactor (desoxyadenosylcobalamine) were determined by a nonlinear regression on the Michaelis-Menten equation calculated with SAS (1999).

Met Synthase Activity. The method was derived from Garras et al. (1991) and adapted to a cytosol preparation freshly isolated as described above (9.29 ± 0.17 μg of protein/ μL). The reaction medium was composed of 10 mM K_3PO_4 buffer, pH 7.4, containing approximately 250 μg of cytosolic proteins, 900 μM reduced glutathione (Sigma Chemical Corp.), 500 μM methyl-THF (Sigma Chemical Corp.), 300 μM S-adenosylmethionine (Sigma Chemical Corp.), and 300 μM homocysteine (prepared from L-homocysteine thiolactone, Sigma Chemical Corp.), with (total activity) or without (holosynthase activity) 50 μM of methylcobalamine (another biologically active form of the vitamin B₁₂; ICN Biomedicals Inc., Irvine, CA). Each sample was assayed in triplicate. The reaction mixture was placed in 5-mL Vacutainers vials (Becton, Dickinson and Co.), sealed, kept on ice, and protected from light. Oxygen was flushed from each vial with an N flux for 1 min. The reaction started when all vials were placed in a closed water bath, protected from light at 37°C for 3 h. At the end of the anaerobic incubation period, the reaction was stopped by urea treatment, and the amount of Met in the reaction medium was quantified after separation of the intracellular sulfhydryl components by isocratic HPLC analysis (Gold system, Beckman, Mississauga, Ontario, Canada) on a reversed-phase C₁₈ column (5 μm bead size, 4.6×150 mm; MCM, ESA, Concord, Ontario, Canada) with coulometric electrochemical detection (Coulchem II, ESA) according to Melnyk et al. (2000). The quantification of in vitro Met synthesis by each sample was performed taking into account the Met concentration in the purified cytosolic fractions.

The linearity of Met quantification by HPLC analysis with coulometric electrochemical detection in the medium was established from 0 to 150 μM ($r^2 = 0.999$). The enzymatic reaction was linear with the cytosolic protein concentration from 30 to 150 μg ($r^2 = 0.994$)

and with time for at least 5 h ($r^2 = 0.994$) at 37°C in the dark and in anaerobic conditions (data not shown). In these conditions, intra- and interassay variations were estimated to 3.3 and 7.2%, respectively.

RNA Extraction and cDNA Synthesis. Total RNA was extracted from liver tissue according to the modified method of Chomczynski and Sacchi (1987) using Trizol reagent (Invitrogen, Burlington, Ontario, Canada) as recommended by the suppliers. Extracted RNA was quantified by UV spectrometry at 260 nm, and purity was monitored by the 280:260 ratio. The complete integrity of the RNA was verified on 1% agarose gel. Five micrograms of total RNA was treated with 3 U of DNase I (Invitrogen) for the complete elimination of genomic DNA. Total RNA was reverse-transcribed to cDNA using the SuperScript II preamplification system (Invitrogen) and 500 ng of oligo-(dT)12-18 (Amersham Pharmacia Biotech, Baie d'Urfée, Québec, Canada) in 50 μ L of total reaction volume.

Cloning and Sequencing of Bovine Met Synthase. To determine the bovine-specific sequence of Met synthase, primers were designed for the PCR amplification based on homology among human (accession number U71285; Leclerc et al., 1996), rat (accession number AF034214; Yamada et al., 1998), and pig (accession number AF276463). Forward 5'-CAGGAGAGGAT TATGGTGCT-3' and reverse 5'-TTTCCACAGATGGC GACACA-3' primers were used to amplify bovine Met synthase. Amplifications were performed in a total reaction volume of 100 μ L consisting of 200 μ M deoxynucleoside triphosphate, 20 pmol of each primer, 1.0 mM $MgCl_2$, 2.5 U of *Taq* polymerase in 1 $\times$ *Taq* polymerase buffer (Amersham Pharmacia Biotech). The PCR profile consisted of an initial denaturation step at 94°C for 2 min, followed by 35 repetitive cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 68°C. A final extension at 68°C for 5 min was also performed. A 403-bp fragment was gel-extracted using the QIAquick gel extraction kit (Qiagen, Mississauga, Ontario, Canada) and according to the instructions of the manufacturer. Bovine Met synthase was sequenced (3 independent PCR amplifications) using the Big Dye terminator cycle sequencing ready reactions (PE Applied Biosystems, Foster City, CA) and according to the instructions of the manufacturer and run on an ABI Prism 377 DNA sequencer (PE Applied Biosystems). A partial bovine Met synthase mRNA sequence of 402 bp was submitted to GenBank (accession number AY128710).

Methylmalonyl-CoA Mutase and Met Synthase mRNA Levels. Liver tissue cDNA was analyzed for methylmalonyl-CoA mutase, Met synthase, and cyclophilin mRNA levels using real-time PCR amplification. For methylmalonyl-CoA mutase, the forward 5'-GCGACACATCGTGGCTATGA-3' and reverse 5'-G

TCAATAGCAACTCCAGCCATTC-3' primers were designed based on the sequences of bovine methylmalonyl-CoA mutase mRNA, kindly provided by J. Retey (Karlsruhe University, Germany; accession number AJ300476). All primers were designed using the Primer Express software (PE Applied Biosystems). Real-time PCR amplifications were performed in a 25- μ L reaction volume consisting of 300 nM forward and 300 nM reverse primers, 1 μ L of cDNA, and 1 $\times$ SYBR Green Master Mix (PE Applied Biosystems). Cycling conditions were 10 min at 95°C, followed by 40 repetitive cycles of 15 s at 95°C, and 1 min at 60°C. All procedures were performed on an ABI Prism 7700 sequence detector (PE Applied Biosystems).

Bovine Met synthase was amplified using forward 5'-AGCAGCACTTCTATTGCCCAA-3' and reverse 5'-CTCCCGCAGAGCACATGTT-3' primers, designed from the bovine sequence (GenBank accession number AY128710). Real-time PCR amplifications were performed using the same conditions as described above for methylmalonyl-CoA mutase.

Bovine cyclophilin was used as a housekeeping gene. Forward 5'-GGATTTATGTGCCAGGGTGGTGA-3' and reverse 5'-CAAGATGCCAGGACCTGTATG-3' were based on the available bovine cyclophilin sequence (accession number NM178320). Real-time PCR conditions were the same as those described for methylmalonyl-CoA mutase. The PCR amplifications were performed in triplicate. Standard curves were prepared in duplicate for methylmalonyl-CoA mutase, Met synthase, and cyclophilin. A pool of liver biopsy cDNA was used to create the standard curve for quantification of the transcripts, using the relative standard curve method as described by Applied Biosystems (1997). Standard curve arbitrary units were set at 1 for the undiluted cDNA pool, and n-fold dilutions of 0.75, 0.50, 0.25, 0.10, 0.05, and 0.005 were then performed. The relative mRNA abundance of each studied gene and cyclophilin was determined by interpolating the threshold cycle values to their respective standard curves. Specificity of the amplified products was verified on 3% agarose gel and with the melting curve analysis.

Folates. Approximately 40 mg of frozen liver tissue was homogenized in a glass grinder tube with 2 mL of cold buffer (0.457 mM monohydrate citric acid and 8 mM dibasic Na_3PO_4 , pH 5.5) for 30 s. The tubes were always kept on ice. From the homogenate, 200 μ L was placed in a 2-mL tube with 1,800 μ L of 56.8 mM ascorbic acid in ultrapure water, pH 6.0, and vortexed. The tubes were incubated at 75°C for 30 min for hydrolysis, then cooled on ice and centrifuged for 10 min at 16,010 $\times g$ and 4°C. Supernatants were frozen at -20°C until analyzed. Folates were determined in duplicate for each hydrolysis (2 hydrolyses per sample) by radioassay with

a commercial kit designed for human plasma (Quantaphase Folate II, BioRad Laboratories Ltd.). The response was linear from 20 to 60 mg of liver ($r^2 = 0.9926$). The interassay CV was 4.35%.

Vitamin B₁₂. Approximately 30 mg of frozen liver tissue was homogenized in a glass grinder tube with 2 mL of ultrapure water for 30 s. Homogenates were transferred to 15-mL Falcon tubes. Glass tubes and grinders were rinsed with ultrapure water to a final volume of 8 mL. After vortexing, 1 mL of homogenate was transferred to another 15-mL Falcon tube with 1 mL of acetate buffer (0.5 M). One milliliter of a solution of NaCN (20 µg/mL) and papain (50 mg/mL) was added to each tube and incubated for 1 h in a water bath at 60°C for hydrolysis. The tubes were placed on ice for 5 min and then autoclaved for 10 min at 121°C, put on ice for another 5 min, and finally centrifuged at 1,730 × g, 4°C for 15 min. Supernatants were transferred to 16 × 100 weighted tubes, and the weight was adjusted to 6 g with ultrapure water. A volume of 800 µL was transferred in a 1.5-mL tube. The pH was adjusted from 6.0 to 6.5 with 60 µL of 1 N NaOH, and the volume was completed to 1 mL with 140 µL of ultrapure water. The tube was frozen at -20°C until analyzed. Vitamin B₁₂ was determined in duplicate for each hydrolysis (2 hydrolyzes per sample) by radioassay with a commercial kit designed for human plasma (Quantaphase Vitamin B₁₂, BioRad Laboratories Ltd.). The response was linear with fresh liver quantity from 10 to 60 mg ($r^2 = 0.9955$), and the interassay CV was 2.0 %.

Lipids. Total lipids were extracted from 500 mg of frozen liver tissue after verification that the total lipid extract increased linearly from 200 to 800 mg of liver tissue. Triacylglycerols, phospholipids, and total cholesterol were quantified according to Gruffat et al. (1997). Tissue concentrations of vitamins and lipids are reported per gram of DNA (Labarca and Paigen, 1980).

Statistical Analysis

One cow was removed from the experiment following an acute mastitis 2 wk after calving. Supplementary folic acid at 0 (B₉-) or 2.6 g/d (B₉+) and vitamin B₁₂ at 0 (B₁₂-) or 0.5 g/d (B₁₂+) were used in a 2 × 2 factorial arrangement in 6 randomized complete blocks. All variables were analyzed using the MIXED procedure of SAS Institute (1999) according to a randomized complete block design with repeated measures in time except for concentrations and yields of milk components which were analyzed as means for the complete period. The spatial power covariance structure was used, because the time intervals were different. Means were assumed to be different at $P \leq 0.05$ and tended to differ at $0.05 < P > 0.07$. Results are reported as least square means

and standard errors except when a natural logarithm transformation was used to restore normal distribution, in which case, the results are reported as antiln least square means with the interval of confidence at 95%. Gene expression of Met synthase or methylmalonyl-CoA mutase was analyzed as the natural logarithm of the ratio of the quantity of mRNA for the studied enzyme (Met synthase or methylmalonyl-CoA mutase) on the quantity of mRNA for cyclophilin. When the interaction folic acid × vitamin B₁₂ reached a level of significance of $P \leq 0.05$, statistical analysis was conducted by level of vitamin to help interpretation.

RESULTS

Production Data

There was no significant treatment or interaction treatment × time effect ($P > 0.2$; Table 2) on DMI, BW, and BCS during the pre- and postcalving periods. During the first 8 wk of lactation, supplementary folic acid increased milk production by 3.4 kg/d, from 38.0 ± 0.9 to 41.4 ± 1.0 kg/d ($P = 0.01$; Table 2). Milk production of cows fed vitamin B₁₂ alone (B₉-B₁₂+) was lower and reached a plateau earlier than the other treatments at 4 wk of lactation (folic acid × vitamin B₁₂ × time, $P = 0.04$; Figure 1). Supplementary folic acid increased daily secretion of milk CP ($P = 0.05$) by 75 g/d as well as concentrations and amounts of folates secreted in milk ($P \leq 0.003$). Supplementary vitamin B₁₂ decreased milk urea from 13.6 ± 0.4 to 12.1 ± 0.4 mg/dL ($P = 0.03$) and increased concentration and total amount of vitamin B₁₂ secreted in milk ($P = 0.001$; Table 2). Milk fat concentration was increased ($P = 0.02$) by supplementary vitamin B₁₂ in cows fed no folic acid supplements but was not affected ($P = 0.6$) in cows fed supplementary folic acid (folic × vitamin B₁₂, $P = 0.03$; Table 2). Milk CP and lactose concentrations as well as lactose yield were not modified by treatments ($P > 0.15$).

Plasma Variables

Plasma concentrations of folates were increased by folic acid supplements, but the increase was smaller for cows also fed supplementary vitamin B₁₂ (folic acid × vitamin B₁₂, $P = 0.05$; Table 3). Plasma concentrations of folates were relatively stable from 2 to 8 wk of lactation in cows fed supplementary folic acid, whereas they increased in cows fed no supplementary folic acid (time × folic acid, $P = 0.006$).

Plasma concentrations of vitamin B₁₂ were increased by vitamin B₁₂ supplements, but the increase was smaller in cows also fed supplementary folic acid (folic acid × vitamin B₁₂, $P = 0.004$; Table 3). Plasma concentrations of vitamin B₁₂ increased steadily from 2 to 8 wk

Table 2. Effect of dietary supplements of folic acid (B_9) and vitamin B_{12} (B_{12}) given to dairy cows from 3 wk before calving to 8 wk of lactation on DMI, milk production, and composition

Item	Treatment ¹				P-value		
	$B_9-B_{12}-$ (n = 6)	$B_9+B_{12}-$ (n = 5)	$B_9-B_{12}+$ (n = 6)	$B_9+B_{12}+$ (n = 6)	B_9	B_{12}	$B_{12} \times B_9$
Pregalving period (3 wk before calving)							
DMI (kg/d)	12.2 $\pm$ 1.0	13.4 $\pm$ 1.0	12.7 $\pm$ 1.0	13.9 $\pm$ 1.0	0.19	0.64	0.98
BW ² (kg)	735 $\pm$ 25	755 $\pm$ 27	721 $\pm$ 25	740 $\pm$ 25	0.36	0.51	0.96
BCS	3.50 $\pm$ 0.16	3.38 $\pm$ 0.17	3.42 $\pm$ 0.16	3.51 $\pm$ 0.16	0.84	0.91	0.40
Postcalving period							
DMI ² (kg/d)	21.3 $\pm$ 0.7	21.5 $\pm$ 0.8	22.0 $\pm$ 0.7	22.7 $\pm$ 0.7	0.52	0.19	0.72
BW ² (kg)	686 $\pm$ 26	699 $\pm$ 29	683 $\pm$ 26	700 $\pm$ 26	0.46	0.95	0.93
BCS ²	2.90 $\pm$ 0.11	2.81 $\pm$ 0.12	2.81 $\pm$ 0.11	2.93 $\pm$ 0.11	0.85	0.88	0.35
Milk production ³ (kg/d)	39.5 $\pm$ 1.3	41.8 $\pm$ 1.6	36.5 $\pm$ 1.3	40.9 $\pm$ 1.3	0.01	0.17	0.45
Milk composition							
TS (g/kg)	118.3 $\pm$ 1.6	119.3 $\pm$ 1.7	122.7 $\pm$ 1.6	120.8 $\pm$ 1.6	0.67	0.08	0.46
Fat (g/kg)	35.9 $\pm$ 1.4	39.2 $\pm$ 1.6	39.9 $\pm$ 1.4	37.4 $\pm$ 1.4	0.71	0.43	0.03
CP (g/kg)	30.3 $\pm$ 0.6	29.5 $\pm$ 0.7	30.7 $\pm$ 0.6	31.2 $\pm$ 0.6	0.83	0.15	0.38
Lactose ⁴ (g/kg)	45.1 $\pm$ 1.2	43.5 $\pm$ 1.0	44.9 $\pm$ 0.6	44.7 $\pm$ 0.8	0.20	0.35	0.27
MUN (mg/dL)	13.9 $\pm$ 0.5	13.2 $\pm$ 0.7	11.9 $\pm$ 0.8	12.4 $\pm$ 0.7	0.92	0.03	0.31
Folates (ng/mL)	45.6 $\pm$ 6.2	71.7 $\pm$ 6.8	58.4 $\pm$ 6.2	71.2 $\pm$ 6.2	0.01	0.39	0.31
Vitamin B_{12} (pg/mL)	1,731 $\pm$ 351	1,834 $\pm$ 391	5,172 $\pm$ 351	4,406 $\pm$ 351	0.47	0.01	0.31
Component yields							
Solids (kg/d)	4.69 $\pm$ 0.16	5.02 $\pm$ 0.17	4.58 $\pm$ 0.16	4.85 $\pm$ 0.16	0.09	0.40	0.87
Fat (kg/d)	1.42 $\pm$ 0.06	1.65 $\pm$ 0.07	1.49 $\pm$ 0.06	1.50 $\pm$ 0.06	0.09	0.49	0.13
CP (kg/d)	1.20 $\pm$ 0.04	1.24 $\pm$ 0.04	1.14 $\pm$ 0.04	1.25 $\pm$ 0.04	0.05	0.53	0.37
Lactose ⁴ (kg/d)	1.79 $\pm$ 0.07	1.84 $\pm$ 0.08	1.68 $\pm$ 0.11	1.80 $\pm$ 0.04	0.24	0.21	0.40
Folates (mg/d)	1.8 $\pm$ 0.3	3.0 $\pm$ 0.3	2.2 $\pm$ 0.3	2.9 $\pm$ 0.3	0.01	0.68	0.30
Vitamin B_{12} (μ g/d)	70 $\pm$ 12	78 $\pm$ 13	189 $\pm$ 12	177 $\pm$ 12	0.91	0.01	0.40

¹ $B_9-B_{12}-$ = no vitamin supplement; $B_9+B_{12}-$ = 2.6 g of folic acid/d; $B_9-B_{12}+$ = 0.5 g of vitamin B_{12} /d; $B_9+B_{12}+$ = 2.6 g of folic acid/d + 0.5 g of vitamin B_{12} /d.

²Time effect ($P \leq 0.01$).

³Vitamin $B_{12} \times$ folic acid $\times$ time ($P = 0.04$).

⁴Obtained from the following difference: milk TS - (fat + CP + ashes).

of lactation in cows fed supplementary vitamin B_{12} , whereas in cows fed no B_{12} supplements, they decreased from 2 to 6 wk of lactation (time $\times$ vitamin B_{12} , $P = 0.001$).

Figure 1. Effects of dietary supplements of folic acid (B_9) and vitamin B_{12} given to dairy cows from 3 wk before calving until 8 wk of lactation on milk production. $B_9-B_{12}-$ (---○---) = no vitamin supplement; $B_9+B_{12}-$ (—x—) = 2.5 g of folic acid/d; $B_9-B_{12}+$ (---■---) = 0.5 g of vitamin B_{12} /d; $B_9+B_{12}+$ (—■—) = 2.5 g of folic acid/d + 0.5 g of vitamin B_{12} /d (SE, 1.8; time $\times$ folic acid $\times$ vitamin B_{12} , $P = 0.04$).

Plasma concentrations of glucose and biotin changed according to the dietary supplements of vitamins (folic acid $\times$ vitamin B_{12} , $P = 0.01$ and 0.06 , respectively; Table 4) but not with time ($P > 0.2$). Supplementary vitamin B_{12} increased plasma glucose ($P = 0.0009$) and tended to decrease ($P = 0.06$) plasma biotin in cows fed folic acid supplements but had no effect in cows not fed supplementary folic acid ($P > 0.3$).

Plasma concentrations of NEFA were not significantly different among treatments ($P = 0.09$; Table 4) but decreased during the experimental period ($P = 0.001$): 663, 451, 299, and 274 (SE 54) μ M at 2, 4, 6, and 8 wk of lactation, respectively.

Dietary supplements of folic acid increased plasma concentrations of Gly, from 316.8 ± 22.9 to 389.5 ± 24.0 μ M; Ser, from 79.9 ± 4.5 to 93.0 ± 4.8 μ M; Thr, from 86.5 ± 5.9 to 109.0 ± 6.2 μ M; and total sulfur AA (TSAA), from 118.6 ± 4.3 to 132.0 ± 4.5 μ M ($P \leq 0.05$; Table 4). They decreased aspartate ($P = 0.004$), from 9.6 ± 0.6 to 7.0 ± 0.6 μ M. Supplementary folic acid also tended to increase plasma Met, from 14.1 ± 1.1 to 17.1 ± 1.2 μ M ($P = 0.07$), and plasma total AA ($P = 0.06$), from $2,110 \pm 53$ to $2,261 \pm 58$ μ M.

Table 4. Effect of dietary supplements of vitamin B₁₂ and folic acid (B₉) given to multiparous dairy cows from 3 wk before calving until 8 wk of lactation on plasma concentrations of biotin, glucose, NEFA, and AA

Item	Treatment ¹				P-value		
	B ₉ -B ₁₂ - (n = 6)	B ₉ +B ₁₂ - (n = 5)	B ₉ -B ₁₂ + (n = 6)	B ₉ +B ₁₂ + (n = 6)	B ₉	B ₁₂	B ₁₂ × B ₉
Biotin (pg/mL)	1,021 ± 23	1,053 ± 25	1,050 ± 23	1,006 ± 23	0.67	0.78	0.06
Glucose (μM)	3.37 ± 0.06	3.15 ± 0.07	3.35 ± 0.06	3.49 ± 0.06	0.02	0.60	0.01
NEFA ² (μM)	481 ± 56	442 ± 60	420 ± 56	344 ± 56	0.09	0.22	0.69
AA (μM)							
Ala ³	192.6 ± 15.4	199.2 ± 16.9	178.8 ± 15.4	261.6 ± 15.4	0.01	0.13	0.02
Arg ²	68.3 ± 3.7	76.5 ± 4.0	71.2 ± 3.7	76.2 ± 3.7	0.09	0.74	0.67
Asn ²	47.5 ± 2.7	48.1 ± 3.0	42.9 ± 2.7	51.7 ± 2.7	0.09	0.86	0.14
Asp	10.2 ± 0.8	6.5 ± 0.9	9.0 ± 0.8	7.5 ± 0.8	0.01	0.94	0.23
Cit	86.6 ± 7.3	91.2 ± 7.7	87.5 ± 7.3	82.6 ± 0.3	0.97	0.49	0.39
Cys ²	88.7 ± 5.8	105.0 ± 6.3	108.3 ± 5.8	112.6 ± 5.8	0.09	0.03	0.31
Gln	223.4 ± 14.0	224.5 ± 15.1	216.3 ± 14.0	247.1 ± 14.0	0.17	0.51	0.21
Glu ⁴	48.3 ± 2.9	44.7 ± 3.1	46.8 ± 2.9	49.8 ± 2.9	0.93	0.53	0.25
Gly ²	317.6 ± 31.7	385.1 ± 34.7	316.0 ± 31.7	392.9 ± 31.8	0.03	0.93	0.90
Hcy	5.2 ± 0.7	5.3 ± 0.8	6.5 ± 0.7	7.4 ± 0.7	0.46	0.02	0.59
His	56.4 ± 5.7	56.8 ± 6.3	46.6 ± 5.8	55.8 ± 5.7	0.42	0.36	0.46
Ile ²	124.7 ± 8.3	124.4 ± 9.0	110.4 ± 8.3	100.4 ± 8.3	0.49	0.01	0.51
Leu ³	181.1 ± 11.1	180.0 ± 12.1	161.3 ± 11.1	151.1 ± 11.1	0.58	0.02	0.66
Lys ²	64.9 ± 4.3	66.3 ± 4.7	63.6 ± 4.3	69.1 ± 4.3	0.44	0.86	0.64
Met	14.6 ± 1.6	17.7 ± 1.8	13.5 ± 1.6	16.5 ± 1.6	0.07	0.50	0.98
Orn ²	36.2 ± 1.9	32.1 ± 2.1	35.8 ± 1.9	39.3 ± 1.9	0.89	0.08	0.06
Phe ²	46.7 ± 2.5	45.7 ± 2.7	46.9 ± 2.5	47.3 ± 2.5	0.93	0.73	0.79
Ser	79.9 ± 6.4	81.6 ± 7.1	79.8 ± 6.4	104.5 ± 6.4	0.05	0.09	0.09
Tau ²	32.8 ± 2.9	34.2 ± 3.2	27.8 ± 2.9	34.7 ± 2.9	0.11	0.39	0.29
Thr ²	89.6 ± 8.4	105.4 ± 9.2	83.5 ± 8.4	112.7 ± 8.4	0.01	0.94	0.44
Trp	42.5 ± 2.1	39.3 ± 2.2	39.0 ± 2.0	43.1 ± 2.0	0.82	0.93	0.06
Tyr ²	59.9 ± 4.4	57.3 ± 4.8	60.2 ± 4.4	62.3 ± 4.8	0.96	0.54	0.59
Val ²	243.7 ± 10.9	245.7 ± 11.8	229.9 ± 10.8	220.7 ± 10.8	0.73	0.07	0.59
Sulfur AA ²	108.9 ± 6.1	128.0 ± 6.7	128.3 ± 6.1	136.0 ± 6.1	0.04	0.03	0.37
Total AA ²	2,117 ± 76	2,241 ± 81	2,103 ± 74	2,281 ± 83	0.06	0.87	0.73

¹B₉-B₁₂- = no vitamin supplement; B₉+B₁₂- = 2.5 g of folic acid/d; B₉-B₁₂+ = 0.5 g of vitamin B₁₂/d; B₉+B₁₂+ = 2.5 g of folic acid/d + 0.5 g of vitamin B₁₂/d.

²Time ($P \leq 0.04$).

³Folic acid × vitamin B₁₂ × time ($P = 0.04$).

⁴Vitamin B₁₂ × time ($P \leq 0.05$).

cows fed vitamin B₁₂ supplements, but it increased them drastically in cows fed no supplementary B₁₂ (folic acid × vitamin B₁₂, $P = 0.02$). Moreover, in all treatments, hepatic cholesterol decreased over time ($P = 0.0001$; Table 5). Liver concentrations of phospholipids were higher in cows fed supplementary B₁₂, being 8.0 and 7.3 (SE, 0.2) g/g of DNA for cows fed or not fed supplementary vitamin B₁₂, respectively ($P = 0.02$), and they increased with time after calving, 7.1, 7.8, and 8.1 (SE, 0.2) g/g of DNA, at 2, 4, and 8 wk of lactation, respectively ($P = 0.0002$).

Met Synthase Activity and Gene Expression. Neither folic acid nor vitamin B₁₂ supplements affected Met synthase-specific activity in cow liver, 0.34 ± 0.01 nmol/min per milligram of cytosolic proteins ($P > 0.17$), but it decreased ($P = 0.02$) over time, 0.38, 0.36, and 0.29 (SE, 0.02) nmol/min per milligram of cytosolic proteins, at 2, 4, and 8 wk after calving, respectively. Holosynthase activity represented 100% of the Met synthase activity.

Gene expression of the Met synthase, expressed as the ratio of the mRNA quantities of Met synthase on cyclophilin, decreased over the studied period, from 0.72 [confidence interval (CI), 0.55 to 0.94] two weeks after calving to 0.55 (CI, 0.42 to 0.72) and 0.47 (CI, 0.36 to 0.61) at 4 and 8 wk after calving, respectively ($P = 0.04$).

Methylmalonyl-CoA Mutase Activity and Gene Expression. There was no effect of treatment or time ($P > 0.16$) on methylmalonyl-CoA mutase-specific activity, averaging 10.8 ± 0.6 nmol/min per milligram of mitochondrial proteins or on holomutase activity ($P > 0.09$). Averaged over the experimental period, holomutase represented approximately 65% of the total activity of the enzyme. The kinetic constants (K_m and V_{max}) of the enzyme regarding methylmalonyl-CoA (as substrate), 154.8 ± 13.4 μM and 14.58 ± 1.15 nmol/min per milligram of protein, respectively, did not vary with vitamin supplementation or time ($P > 0.2$). The V_{max} of the methylmalonyl-CoA mutase following addition of increasing

Table 5. Effect of dietary supplements of vitamin B₁₂ and folic acid (B₉) given to dairy cows from 3 wk before calving until 8 wk of lactation on concentrations of total lipids, triacylglycerols, and cholesterol in the liver

Item	n	Time after calving (wk)		
		2	4	8
Total lipids ¹ (g/g of DNA)				
B ₉ -B ₁₂ -	6	14.0 ± 1.4	12.3 ± 1.4	9.1 ± 1.4
B ₉ +B ₁₂ -	5	29.7 ± 1.5	20.2 ± 1.5	9.4 ± 1.5
B ₉ -B ₁₂ +	6	15.8 ± 1.4	12.2 ± 1.5	10.0 ± 1.4
B ₉ +B ₁₂ +	6	14.1 ± 1.5	11.8 ± 1.4	10.1 ± 1.4
Triacylglycerols ² (g/g of DNA)				
B ₉ -B ₁₂ -	6	6.1 ± 1.3	3.6 ± 1.3	0.8 ± 1.3
B ₉ +B ₁₂ -	5	21.2 ± 1.4	12.3 ± 1.4	1.3 ± 1.4
B ₉ -B ₁₂ +	6	7.2 ± 1.3	3.4 ± 1.4	1.1 ± 1.3
B ₉ +B ₁₂ +	6	6.0 ± 1.4	3.3 ± 1.3	0.8 ± 1.3
Cholesterol ³ (g/g of DNA)				
B ₉ -B ₁₂ -	6	0.97 ± 0.13	0.63 ± 0.13	0.53 ± 0.13
B ₉ +B ₁₂ -	5	1.75 ± 0.15	0.84 ± 0.15	0.59 ± 0.15
B ₉ -B ₁₂ +	6	1.00 ± 0.13	0.65 ± 0.15	0.58 ± 0.13
B ₉ +B ₁₂ +	6	0.86 ± 0.15	0.60 ± 0.13	0.59 ± 0.13

¹Time × folic acid × vitamin B₁₂ ($P = 0.01$). B₉-B₁₂- = no vitamin supplement; B₉+B₁₂- = 2.5 g of folic acid/d; B₉-B₁₂+/B₉+B₁₂+/ = 0.5 g of vitamin B₁₂/d; B₉+B₁₂+/ = 2.5 g of folic acid/d + 0.5 g of vitamin B₁₂/d.

²Time × folic acid × vitamin B₁₂ ($P = 0.01$).

³Time × folic acid × vitamin B₁₂ ($P = 0.07$).

amounts of deoxyadenosylcobalamin (coenzyme) tended to increase with time, averaging 4.7, 6.1, and 6.4 (SE, 0.5) nmol/min per milligram of protein at 2, 4, and 8 wk of lactation, respectively ($P = 0.07$). The K_m of the methylmalonyl-CoA mutase following addition of deoxyadenosylcobalamin did not vary with time after calving ($P > 0.2$) but did vary according to the vitamin supplements (folic acid × vitamin B₁₂, $P = 0.05$). In cows fed no supplementary vitamin B₁₂, the K_m of the methylmalonyl-CoA mutase following addition of deoxyadenosylcobalamin was not modified by folic acid supplementation ($P = 0.39$) and averaged $0.63 \pm 0.07 \mu M$, whereas in cows fed vitamin B₁₂ supplements, supplementary folic acid decreased it ($P = 0.06$) from 0.80 to 0.56 (SE, 0.09) μM . Gene expression of the methylmalonyl-CoA mutase was not affected by supplementary vitamins or time after calving ($P > 0.2$).

DISCUSSION

Milk production and milk protein yield responses to folic acid supplementation were in accordance with previous experiments (Girard et al., 1995; Girard and Matte, 1998), in which supplementary folic acid increased milk production and milk protein concentration or yield in multiparous cows. However, in some other cases, supplementary folic acid had no effect on milk and milk protein yields in primiparous (Girard et al., 1995; Girard and Matte, 1998) or multiparous cows (Girard et al., 2005). Girard et al. (2005) hypothesized that the lack of lactational response to folic acid supplements could be due

to an insufficient supply of vitamin B₁₂. Indeed, intramuscular injections of vitamin B₁₂ given to primiparous cows fed supplementary folic acid in early lactation increased milk TS and fat yields due to a numeric increase in milk production but did not increase milk protein yield or change milk composition (Girard and Matte, 2005). In the present experiment, supplementary folic acid increased milk production and milk protein yield during the first 8 wk of lactation both in cows fed supplementary vitamin B₁₂ or not, even when plasma vitamin B₁₂ of cows not supplemented with vitamin B₁₂ was low. Thus, it seems that the discrepancy among studies in the response of milk and milk protein yields to folic acid supplements is related to a factor other than vitamin B₁₂ supply.

In mammals, folic acid has the single, important biochemical function of accepting and releasing 1-C units (Choi and Mason, 2000). This role is essential for 1) synthesis of purines and pyrimidines for DNA synthesis and 2) de novo synthesis of methyl groups for formation of the primary methylating agent, S-adenosylmethionine (Bailey and Gregory, 1999). The latter is closely related to vitamin B₁₂ metabolism, because the enzyme, Met synthase, essential for the irreversible methylation of homocysteine in Met and regeneration of the biologically active form of folates, tetrahydrofolate, is vitamin B₁₂-dependent. Therefore, because the response in milk production appears independent of vitamin B₁₂ supply at the level observed in the present experiment, it is likely that the effect of folic acid supplementation on lactational performance was due to the former. The de-

crease of plasma concentrations of aspartate in cows fed supplementary folic acid, which, with glutamine, is one of the major donors of N atoms during purine biosynthesis (Salway, 2004), could indirectly support the hypothesis that DNA formation was favored by the dietary addition of folic acid.

In the present experiment, dietary supplements of folic acid and vitamin B₁₂ increased the vitamin supply to the dairy cow. Indeed, as previously observed, supplementary folic acid increased secretion of folates in milk of dairy cows (Girard et al., 1995, 2005) and folate concentration in liver of young dairy heifers (Dumoulin et al., 1991). The increases in milk and plasma concentrations of vitamin B₁₂ in cows fed supplementary vitamin B₁₂ were similar to those observed previously by Girard and Matte (2005) with intramuscular injections of vitamin B₁₂. However, even though the same amounts of vitamins were fed daily, the increase in plasma concentrations of folates in cows fed only supplementary folic acid was larger than in cows fed folic acid and vitamin B₁₂ together. Similarly, supplementary vitamin B₁₂ increased plasma and liver concentrations of vitamin B₁₂, but again the response was reduced in cows simultaneously fed folic acid supplements. Because the folate- and vitamin B₁₂-dependent enzymes are present in most tissues in cattle (Kennedy et al., 1995; Lambert et al., 2002), these observations could be an indication of an increased utilization of the vitamins by extrahepatic tissues in cows fed the 2 vitamins in conjunction.

In addition to their role in protein synthesis, Gly and Ser are among the major gluconeogenic precursors as well as the primary sources of 1-C units for de novo synthesis of methyl groups (Armentano, 1994). The increase in plasma concentrations of Ser, Gly, Thr, and total AA observed in cows fed supplementary folic acid could possibly represent an increased availability of these AA for tissues, including mammary gland. Plasma concentration of Met tended to be higher in cows fed supplementary folic acid, whereas plasma TSAA were increased. Plasma concentrations of homocysteine, Cys, and TSAA were higher in cows fed vitamin B₁₂ supplements. In the presence of an adequate supply of 1-C units, supplementary folic acid probably improved the supply of methyl groups (as part of methyl-THF) for regeneration of Met. Methionine synthase catalyzes the transfer of the methyl group from methyl-THF to homocysteine. In humans, Met synthase is widely expressed, the highest mRNA levels being observed in pancreas, skeletal muscle, and heart and lower mRNA levels in liver (Chen et al., 1997). In sheep and cattle, Met synthase activity is observed in most tissues, except blood cells and skin (Kennedy et al., 1992; Lambert et al., 2002). In the present experiment, neither gene expression of Met synthase nor its activity per milligram of

cytosolic proteins in liver was affected by the vitamin supplements. Moreover, nearly all the enzyme was present in its holosynthase form as observed in human and pig (Chen et al., 1995). However, in the present experiment, Met synthase concentrations in hepatic cells were not measured; therefore, it is possible that regulation occurred through a posttranscriptional mechanism.

The response of plasma concentrations of TSAA to vitamin supplementation seems to indicate that these vitamins affect the methylation cycle. The 2 major functions of the methylation cycle are to provide methyl groups and to regulate the balance between Met and Cys for protein synthesis (Finkelstein, 1990). Activation of the methylation cycle increases synthesis of S-adenosylmethionine, the major donor of methyl groups. In liver, formation of phosphatidylcholine happens mostly through 3 successive methylations of phosphatidylethanolamine, and the activity of the hepatic phospholipid methyltransferases is increased during lactation in ewes (Xue and Snoswell, 1985). Stangl et al. (1999) reported that Co-deficient steers had very low hepatic concentrations of vitamin B₁₂ and a decreased phosphatidylcholine:phosphatidylethanolamine ratio in liver. Moreover, in mice, recent data put into evidence that the hepatic phospholipid methylation level (through phosphatidylethanolamine N-methyltransferase) plays a significant role in maintaining the cellular balance between S-adenosylmethionine and S-adenosylhomocysteine levels as well as plasma concentrations of homocysteine, this latter one being a final product of the transmethylation pathway (Jacobs et al., 2005). Neither phosphatidylcholine nor phosphatidylethanolamine was measured in the present experiment, but the hepatic concentrations of phospholipids as well as plasma concentrations of homocysteine and Cys increased with vitamin B₁₂ supplementation, supporting this hypothesis. Increased methylation reactions augment intracellular concentrations of S-adenosylhomocysteine (Finkelstein, 1990). Both S-adenosylmethionine and S-adenosylhomocysteine are activators of the enzyme cystathionine synthase, which is responsible for the irreversible synthesis of cystathionine, the precursor for Cys synthesis (Finkelstein, 1990), and α -ketobutyrate, which will enter the Krebs cycle as succinyl-CoA under the action of methylmalonyl-CoA mutase (Salway, 2004). Therefore, an augmentation of the methylation reactions as well as the effect of the products of these reactions on cystathionine synthase are likely explanations for the observed increase in plasma concentrations of Cys.

Supplementary vitamin B₁₂ also decreased milk concentration of urea, which suggests a reduction in AA catabolism. Vitamin B₁₂ supplements also decreased plasma concentrations of branched-chain AA, Ile, and Leu and tended to decrease Val, which could be an indi-

cation of an increased uptake of these AA by extrahepatic tissues, including mammary gland. On the other hand, Ile and Val can be used to produce glucose via gluconeogenesis after their entry into the Krebs cycle through succinyl-CoA by the same metabolic pathway as propionate, which is a biotin- and vitamin B₁₂-dependent process. However, the more direct link between Leu and vitamin B₁₂ is the vitamin-dependent conversion of Leu into β -Leu by the Leu-2, 3 aminomutase (Poston, 1976), although the existence of this metabolic pathway remains to be confirmed in mammals (Stabler et al., 1988).

The increase in milk production and milk protein yield induced by folic acid supplementation without a concomitant increase in DMI is likely to augment the mammary gland demands for nutrients and to promote mobilization of body reserves, especially during the first weeks of lactation. Therefore, one of the most striking observations was the increase of hepatic concentrations of total lipids, triacylglycerols, and cholesterol during the first weeks following calving in cows fed folic acid supplements alone, whereas no such increase was observed in cows fed the 2 vitamins together. Usually, fatty liver development in dairy cows results from intense body fat mobilization in response to increased energy requirements for maintenance and lactation concomitant to underfeeding just after calving (Bobe et al., 2004), as could be expected for cows fed supplementary folic acid in the present experiment. The values of triacylglycerol content, close to 90 and 50 mg/g of fresh tissue in the liver at the second and the fourth weeks of lactation, were similar to those previously reported in early lactating cows and corresponded to a strong to moderate liver steatosis (Gruffat et al., 1997; Bobe et al., 2004). Although plasma concentrations of BHBA were not measured in the present experiment, the absence of treatment effects on plasma concentrations of NEFA, BW, and BCS possibly indicate that lipid mobilization from the adipose tissues was not exacerbated by folic acid supplementation.

In ruminants, methylmalonyl-CoA plays a unique regulating role in gluconeogenesis and β -oxidation of fatty acids (Kennedy et al., 1994). Indeed, in liver of sheep, but not rat or guinea pig, methylmalonyl-CoA inhibits carnitine palmitoyltransferase 1, which converts long-chain fatty acids in their carnitine esters, allowing them to enter the mitochondria where β -oxidation takes place (Brindle et al., 1985). When gluconeogenesis and the propionate-Krebs cycle-glucose pathway are active, the concentration of methylmalonyl-CoA increases and inhibits β -oxidation (Zammit, 1990). However, concentrations of methylmalonyl-CoA, and then methylmalonic acid, are also increased when the entry of propionate in the Krebs cycle is slowed down by a lack of vitamin B₁₂ (Combs, 1998), reducing β -oxidation, even if gluconeogenesis is also reduced.

In the present experiment, cows fed supplementary folic acid, either with or without vitamin B₁₂, had similar milk and milk protein and DMI; consequently, mobilization of body reserves should be similar for these 2 treatments. In cows fed folic acid alone, if a lack of vitamin B₁₂ caused an accumulation of methylmalonyl-CoA, which inhibited β -oxidation of fatty acids in liver, it can result in accumulation of triacylglycerols as observed in the present experiment. In cows fed the combined supplements of folic acid and vitamin B₁₂, supplementary vitamin B₁₂ was likely to reduce the accumulation of methylmalonyl-CoA, allowing β -oxidation of fatty acids to proceed and, as far as the oxaloacetate supply is sufficient, the molecules of acetyl-CoA to enter in the Krebs cycle to provide energy.

It is noteworthy that in cows fed supplementary folic acid, plasma concentrations of glucose and Ala were increased by the simultaneous ingestion of supplementary vitamin B₁₂, whereas plasma biotin was decreased. Alanine is in equilibrium with pyruvate. If β -oxidation of fatty acids is active, large quantities of acetyl-CoA are formed, which stimulate pyruvate carboxylase. This means that pyruvate will be carboxylated to oxaloacetate for metabolism to phosphoenolpyruvate and then to glucose via gluconeogenesis or for the entry of acetyl-CoA in the Krebs cycle (Weil, 1972; Salway, 2004). Pyruvate carboxylase is a biotin-dependent enzyme, and stimulation of this metabolic pathway is likely to increase tissue demand for biotin. On the other hand, propionyl-CoA carboxylase, which transforms propionyl-CoA provided by odd-chain fatty acids, Ile and Val, by homoserine from conversion of Met to Cys and, most importantly, by propionate catabolism to methylmalonyl-CoA, is also a biotin-dependent enzyme. Stimulation of these 2 metabolic pathways likely increased biotin utilization. Therefore, the decrease in plasma biotin, the increase in plasma Ala and glucose, as well as the reduced accumulation of hepatic lipids in cows fed the combined supplement of folic acid and vitamin B₁₂ suggest an improved energy balance as compared with cows fed the folic acid supplement alone.

Hepatic methylmalonyl-CoA mutase activity was not significantly different among treatments (neither total nor holomutase activities or mRNA) in opposition to Peters and Elliot (1984) and Kennedy et al. (1990), who observed a decrease of the holomutase fraction in Co- and vitamin B₁₂-deficient sheep. However, in vitamin B₁₂-deficient cattle, Kennedy et al. (1995) observed an increase in methylmalonic acid plasma concentrations in spite of no changes in total or holoenzyme activity of methylmalonyl-CoA mutase in liver. The mean value of K_m for methylmalonyl-CoA of the bovine enzyme (160 μM) was higher than the values reported for a recombinant human form (60 μM) of the enzyme using the same

spectrometric assay method (Taoka et al., 1994) but lower than values reported for human (300 to 350 μM) and mouse (250 μM) using a radiolabeled methodology (Fenton et al., 1982; Wilkemeyer et al., 1990). Moreover, the average K_m for adenosylcobalamin of methylmalonyl-CoA mutase in the liver of the cows not supplemented with vitamin B₁₂ was 0.58 μM . It was higher than values reported for mouse and human (0.20 and 0.24, respectively; Wilkemeyer et al., 1990), but it relates to the apoenzyme fraction of the mutase, and these fractions were very different between these species (5.4 and 24.7%, respectively) and the bovine (61%). However, it would suggest that the cow enzyme would be more dependent of additional vitamin B₁₂ to be fully active. This K_m value was lower in cows supplemented with vitamin B₁₂ and folic acid together, indicating that the affinity of the methylmalonyl-CoA mutase for vitamin B₁₂ was greater for this treatment and then that the requirement of the enzyme toward its cofactor was decreased, improving the efficiency of this metabolic pathway as discussed previously. Furthermore, this last observation could explain the different responses observed between cows fed supplementary vitamin B₁₂ alone or with folic acid. In the present experiment, although methylmalonyl-CoA mutase is not a folate-dependent enzyme, it seems to be affected by the combined vitamin supplement. Leal et al. (2004) reported that the enzyme Met synthase reductase plays a dual role. It reduced cob(II)alamin to cob(I)alamin for synthesis of adenosylcobalamin (the essential coenzyme for methylmalonyl-CoA mutase) but also for the reductive activation of Met synthase. Methionine synthase reductase seems to be the only known link between the 2 vitamin B₁₂-dependent enzymes, although the mechanism of action is not clearly understood. As stated by Yamanishi et al. (2005): "Intracellular cobalamin concentration is low, and nothing is known about how the cell sequesters and delivers this cofactor to the target enzymes methylmalonyl-CoA mutase and Met synthase."

CONCLUSIONS

Results from the present experiment suggest that the increases in milk and milk protein yields due to folic acid supplements were not dependent of the vitamin B₁₂ supply and were probably closely related to the role of folic acid in the DNA cycle. Lactational performance was not modified by supplementary vitamin B₁₂. Nevertheless, it appears that, when folic acid was given in combination with vitamin B₁₂, utilization of the 2 vitamins seems to be increased, especially in extrahepatic tissues. Moreover, it appears that when folic acid was given in combination with vitamin B₁₂, metabolic efficiency was improved, as suggested by similar lactational perfor-

mance and DMI than cows fed folic acid supplements alone, but increased plasma glucose and decreased hepatic concentrations of lipids.

ACKNOWLEDGMENTS

We thank Dominique Bauchart (Institut National de la Recherche Agronomique, Theix, France), who welcomed B. Graulet in his laboratory and provided expertise and facilities for hepatic lipid analysis. We are also grateful to Eric Martineau, DMV, who performed all the liver biopsies; François Dehours and Richard Lanctôt for animal care; Chrystiane Plante, Véronique Roy, and Danielle Beaudry for technical assistance; and to Steve Méthot for statistical advice. Supplementary vitamin B₁₂ (cyanocobalamin) was kindly provided by Adisseo France SAS (Antony, France) and pteroylmonoglutamic acid by Hoffmann-LaRoche (Cambridge, Ontario, Canada).

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