Effects of Dietary Supplements of Folic Acid and Rumen-Protected Methionine on Lactational Performance and Folate Metabolism of Dairy Cows*

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ABSTRACT

The present experiment was undertaken to determine the interactions between dietary supplements of folic acid and rumen-protected methionine on lactational performance and on indicators of folate metabolism during one lactation. Fifty-four multiparous Holstein cows were assigned to 9 blocks of 6 cows each according to their previous milk production. Within each block, 3 cows were fed a diet calculated to supply methionine as 1.75% metabolizable protein, equivalent to 70% of methionine requirement, whereas the 3 other cows were fed the same diet supplemented with 18 g of a rumen-protected methionine supplement. Within each diet, the cows received 0, 3, or 6 mg/d of folic acid per kg of body weight. Rumen-protected methionine increased milk total solid concentration but not yield. Supplementary folic acid increased crude protein and casein concentrations in milk of cows fed no supplementary methionine and the effect increased as lactation progressed; it also decreased milk lactose concentration. Folic acid supplements had the opposite effects on milk crude protein, casein, and lactose concentrations in cows fed rumen-protected methionine. Milk and milk component yields and dry matter intake were unchanged. Folic acid supplementation increased serum folates and this response was greater at 8 wk of lactation. It decreased serum cysteine in cows fed rumen-protected methionine, whereas it had no effect in cows fed no supplementary methionine. The highest serum concentrations of cysteine but the lowest of vitamin B₁₂ were observed at 8 wk of lactation. Serum clearance of folic acid following an i.v. injection of folic acid was slower at 8 wk of lactation. During this period, the high concentrations of serum folates and cysteine, the low serum concentrations of vitamin B₁₂ and methionine,

and the slow serum clearance of folates strongly suggest that the vitamin B₁₂ supply was inadequate and interfered with folate use. It could explain the limited lactational response to supplementary folic acid observed in the present experiment.

(**Key words:** dairy cow, lactation, folic acid, rumen-protected methionine)

Abbreviation key: ECM = energy-corrected milk, ILR = irreversible loss rate, MP = metabolizable protein, 5-methyl-THF = 5-methyl-tetrahydrofolate, RPM = rumen-protected methionine, THF = tetrahydrofolate.

INTRODUCTION

Folic acid is a B-complex vitamin and has the single and important biochemical function in mammals to accept and release one-carbon units (Choi and Mason, 2000). This role is essential for the synthesis of purine and pyrimidine, the initiation of protein synthesis, and the formation of the primary methylating agent, S-adenosylmethionine (Bailey and Gregory, 1999). In most species, ingested folic acid is rapidly reduced and methylated across the gastrointestinal wall and the liver to 5-methyl-tetrahydrofolate (**5-methyl-THF**) (Le Grusse and Watier, 1993). This reaction is irreversible and the methyl group of 5-methyl-THF can only be released through transfer to homocysteine to form methionine and tetrahydrofolate (**THF**) (Bässler, 1997). Tetrahydrofolate is the active form of folates and acts as the acceptor of one-carbon units from different reactions (Bässler, 1997).

Lactation increases the demand for methylated compounds (synthesis of milk choline, creatine, creatinine, and carnitine) and for methionine to support milk protein synthesis (Xue and Snoswell, 1985). In ruminants, as net absorption of these methylated compounds is inadequate to meet requirements, they need to be synthesized de novo (Snoswell and Xue, 1987) from gluconeogenic precursors, such as glycine and serine, the primary sources for the required methyl groups (Arm-

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entano, 1994). During early lactation, however, these increased demands occur at the same time as the increased pressure to synthesize glucose (to support the elevated lactose output) and this may create a shortage in precursors for de novo synthesis of methylated compounds. Under such circumstances, other sources of methyl groups need to be used. These include methionine, which can be converted to homocysteine and then metabolized to cysteine if insufficient sources of methyl groups are available to enable remethylation of homocysteine to methionine. Less methionine may be available to support milk protein output due to the activity of this other role of methionine as a methyl group precursor. This concept is supported by the observations where estimated supply of methionine did not meet requirements of the lactating dairy cow (NRC, 2001), and supplementary folic acid increased milk and milk protein yields and milk protein concentrations (Girard et al., 1995, Girard and Matte, 1998). These data suggested that synthesis of folic acid by the rumen microflora did not fulfill the needs of the animal and that when there is a shortage in precursors for de novo synthesis of methylated compounds, supplementary folic acid improved efficiency of transfer of one-carbon units and spared methionine for anabolic outputs. Thus, optimal milk production involves adequate supply of both methyl group precursors and the appropriate cofactors (folates, B₁₂) to support the various metabolic transfers.

Therefore, it was hypothesized that supplementary folic acid affects lactational performance through increased regeneration of methionine from homocysteine. The present experiment was undertaken to determine the interactions between dietary supplements of folic acid in the presence or absence of additional dietary methionine [supplied as a rumen-protected methionine (RPM) supplement] given daily from 1 mo prepartum throughout a 305-d lactation period on milk production and composition as well as on uptake of folates by cow tissues.

MATERIALS AND METHODS

Cows and Treatments

Fifty-four multiparous Holstein cows from the dairy herd at the Agriculture and Agri-Food Canada Research Center (Lennoxville, PQ, Canada) were assigned to 9 blocks of 6 cows each according to their milk production during the previous lactation. Cows were kept in a tie-stall barn equipped with mattress under 16 h/d of light (0530 to 2130 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 of Animal Care (1993). The experiment began approximately 1 mo be-

Table 1. Composition of the total mixed diet (DM basis).

Ingredients	%
Grass silage ¹	20.01
Corn silage ²	20.05
High moisture corn	13.40
Barley	18.90
Wheat	6.02
Soybean hulls	1.95
Soybean meal (48% CP)	3.94
Protected soybean meal (48% CP)	3.94
Urea	0.25
Blood meal	1.27
Extruded soybeans	3.81
Megalac	1.14
Tallow	0.43
Mineral lime	1.39
Biophosphorus	1.03
Salt	0.59
Magnesium oxide	1.15
Sodium bicarbonate	0.69
Mineral and vitamin premix ³	0.06

¹17.2% CP, 5.7% N-NDF, 3.4% N-ADF, 39.2% ADF, 51.3% NDF, 8.1% lignin.

²7.9% CP, 1.7% N-NDF, 0.6% N-ADF, 27.3% ADF, 45.8% NDF, 3.3% lignin.

³Composition: 30 g/kg of Ca, 2 g/kg of Mg, 20 g/kg of S, 101 g/kg of Mn, 152 g/kg of Zn, 30 g/kg of Cu, 3 g/kg of I, 1.1 g/kg of Co, 13,780,000 IU/kg of vitamin A, 4,650,000 IU/kg of vitamin D, and 30,800 IU/kg of vitamin E.

fore the expected time of calving and continued for a 305-d lactation period. The basal TMR is described in Table 1. Silage DM was determined weekly to maintain a constant ratio of ingredients.

Within each block, 3 cows were fed a diet calculated (NRC, 2001) to supply methionine as 1.75% of metabolizable protein (MP), equivalent to 70% of methionine requirement (NRC, 2001), and the other 3 cows were fed the same diet supplemented with 9 g of Smartamine M (Rhône-Poulenc Animal Nutrition, Mississauga, ON, Canada) before calving and 18 g of Smartamine M after calving. With a methionine content of 88%, 91% rumen by-pass, and 97.8% digestibility, the batch of Smartamine M used in the present experiment supplied 14.1 g of methionine per day to bring the estimated methionine supply to 2.2% of MP. Within each diet, the cows received 0, 3, or 6 mg of folic acid (Rovimix 10% pteroylmonoglutamic acid, Hoffmann-LaRoche, Cambridge, ON, Canada) daily per kilogram of BW. Those amounts were adjusted for individual BW at the beginning of the experiment, at calving, and at 8, 18, 28, and 38 wk of lactation. Orts were removed every day at 1000 h, and the dietary supplements of folic acid and RPM were incorporated into 500 g of the grass silage used in the total mixed diet and fed immediately to the cows to insure complete ingestion of the supplements. Cows were fed ad libitum. The TMR was distributed in 8 equal meals per day, every 3 h.

Sampling Procedure

Milk production was recorded at each milking throughout lactation. Milk was sampled at 2 consecutive milkings (on evening of one day and morning of the following day) every 4 wk. The amount of feed offered was recorded daily, and orts were recorded on 5 d per wk. Cows were weighed at the beginning of the experiment, at calving, and at 8, 18, 28, and 38 wk after calving.

Whole blood and blood serum were collected by jugular venipuncture, using a Vacutainer system, at the beginning of the experiment (4 wk before the expected time of calving). At 8, 18, 28, and 38 wk after calving, a 3-d blood sampling procedure was performed. On d 0, each cow was implanted with a jugular catheter. On d 1, one basal blood sample was withdrawn (S_0), at 1000 h, approximately 24 h after the last supplemented meal. Packed cell volume, hemoglobin, serum concentrations of folates, vitamin B₁₂, homocysteine, methionine, and cysteine were determined 4 wk before the expected time of calving, and at 8, 18, 28, and 38 wk of lactation on the basal blood sample (S_0) of d 1.

At 8, 18, 28, and 38 wk of lactation, on d 1, just after the basal blood sample was collected, sterile saline (0.9% NaCl) was injected intravenously through the jugular catheter. On d 2 (at the same time as the previous day), an i.v. bolus of 50 μ g of folic acid per kg of BW, prepared as a solution of 10 mg/mL of pteroylm-noglutamic acid (ICN Biochemicals, Cleveland, OH) was injected through the jugular catheter and the catheter was carefully flushed with 2 mL of a solution of sterile bovine heparin (20 IU/L). On d 1 and 2, blood was collected at 5, 15, 30, 60, 120, 240, and 480 min postinjection and only serum concentrations of folates were determined in these blood samples. During those 2 d, the cows received no folic acid or RPM supplements.

Analyses

Folates and vitamin B₁₂. The procedures for folate determination in serum and milk and vitamin B₁₂ determination in serum using a radioassay (Quantaphase Folate II and Quantaphase B₁₂, Bio-Rad Laboratories (Canada) Ltd, Mississauga, ON, Canada) were as described by Girard and Matte (1988, 1998).

Blood hemoglobin and packed cell volume. Packed cell volume was determined by microcentrifuge, and blood hemoglobin was measured by the method of Drabkin (Manet, 1969).

Milk components. Milk fat was determined using the Rse-Gottlieb method (AOAC, 1990; method 905.02), ash was determined by thermogravimetry (LECO Corp., St. Joseph, MI), total N was determined using a combustion procedure (LECO Corp.), noncasein

N was determined using a modification of method 16.041 (AOAC, 1990; LECO Corp.), casein N was calculated as the difference between total and noncasein N. Lactose was determined by calculation (total DM in milk minus the other milk components chemically determined). Milk urea was determined according to the method of Broderick (1985).

Determination of total homocysteine, cysteine, and methionine in blood serum. Total homocysteine, methionine, and cysteine were determined in blood serum by a modification of the method described by Malinow et al. (1989).

Blood serum was centrifuged at $1854 \times g$ for 10 min and frozen at -20°C less than 1 h after blood collection. Before analysis, 200 μ L of serum, 100 μ L of ultrapure water, 300 μ L of 9.0 M urea (pH 9.0), 50 μ L of n-amylalcohol, and 50 μ L of NaBH₄ (10% wt/vol) in 0.1 N NaOH (NaBH₄ solution prepared fresh daily) were incubated in a borosilicate tube at 50°C for 30 min. The tubes were cooled on ice and 500 μ L of 12% perchloric acid (vol/vol) were added. After 5 min, the content of the tubes was transferred to Eppendorf tubes and centrifuged at $13,000 \times g$ for 7 min. The supernatant was passed through a 0.45- μ m polyvinylidene fluoride filter.

Standard curves for homocysteine, methionine, and cysteine were determined at the beginning and end of each daily run. The concentration of each amino acid was calculated from peak areas. Standards of homocysteine were prepared by dissolving 100 mg of L-homocysteine thiolactone (H-6503; Sigma Chemical Co., St. Louis, MO) in 5 mL of 5.0 M NaOH. After 5 min of incubation at room temperature, 5 mL of 5.0 M HCl was added to achieve pH 4.5. The preparation was partitioned in tubes (1.2 mL) and frozen at -70°C until used. Standard curves of homocysteine and methionine were prepared by adding known amounts of homocysteine and methionine to 200 μ L of pooled serum, and standard curves of cysteine were prepared by adding known amounts of a solution of cysteine to 100 μ L of ultrapure water. Standards were subsequently prepared as previously described for serum samples.

High-pressure liquid chromatography was performed (HPLC system Gold Beckman, Module solvent 126, autosamples 507E, Mississauga, ON, Canada) using an electrochemical detector (Coulchem II (ESA), Concord, ON, Canada) set as follows: guard cell (model 5020; $E = +1400$ mV), analytical cell (model 5010, $E_1 = +400$ mV; $E_2 = +1200$ mV). Sensitivity was set to 10 μ A for detection of homocysteine and methionine and 20 μ A for cysteine. The analytic column was a 4.6×250 micro-sorb-in (R0086200C5 – Rainin, Varian, Mississauga, ON, Canada) protected with a guard column (4.6×4.5 ultrasphere ODS, 243533, Beckman, Mississauga, ON, Canada).

The mobile phase consisted of 10 mM sodium phosphate monobasic, 0.03 mM 1-octanesulfonic acid, and 2.5% acetonitrile. This was prepared by dissolving 2.392 g of NaH_2PO_4 and 0.01296 g of 1-octanesulfonic acid in ultrapure water, adjusting the pH to 2.7 with phosphoric acid, and adding ultrapure water to 2 L. Then 50 mL of acetonitrile was added, mixed, and the solution was filtered through a 0.22- μm nylon membrane filter. During the analysis, helium was bubbled continuously through the buffer. The solvent was pumped through the column at 1.3 mL/min at 3000 psi. An injection volume of 20 μL was used. The system was equipped with an automatic sample injector. Each sample was injected twice, first for detection of homocysteine and methionine and, second for detection of cysteine, with the electrochemical detector set to a lower sensitivity (cysteine is present in high concentrations in cow serum).

Calculations of Serum Folate Kinetics

The serum concentrations following the injection of folic acid were corrected for the background concentrations observed the previous day at the same times after saline injection. A double exponential model (Shipley and Clark, 1972) was fitted to these corrected concentrations:

$$C(t) = A_1 \exp(-g_1 t) + A_2 \exp(-g_2 t)$$

where $C(t)$ denotes the corrected concentration t minutes after injection. This model describes a scenario where some of the injected folates that leave the serum pool are returned within 4 h postinjection. The estimates of A_1 , A_2 , g_1 , and g_2 were obtained from nonlinear regression and then used to calculate the following quantities of interest. The volume of the primary pool (V , l) was calculated as $\text{Dose} / (A_1 + A_2)$, where Dose is the injected folates (μg). The rate constant of the primary pool, k (min^{-1}), is given by $k = A_1 g_1 + A_2 g_2 / (A_1 + A_2)$. The reasonable fits obtained by such an approach indicate that first-order kinetics apply i.e., outflows from the pools can be assumed equal to pool size $\times$ a fixed rate constant.

Clearance rate was calculated as $\text{Dose} / (A_1/g_1 + A_2/g_2)$, irreversible loss rate (**ILR**, $\mu\text{g}/\text{min}$) was calculated as clearance rate $\times C_{\text{initial}}$, where C_{initial} is the concentration ($\mu\text{g}/\text{L}$) before injection of saline on d 0. The flux through the primary pool ($\mu\text{g}/\text{min}$) was obtained from $k \times C_{\text{initial}} \times V$. Flux represents total flows into and out of a pool (i.e., multiple entries and exits of a molecule are quantified), whereas ILR measures unidirectional transfers through the same pool (i.e., the difference between entry and exit).

Statistical Analyses

Four cows, assigned to different treatments, were removed from the experiment at calving or in early lactation due to health problems unrelated to the experimental treatments.

Energy-corrected milk (**ECM**) was calculated according to Tyrrell and Reid (1965). Total milk production, ECM, and feed intake for a 305-d lactation period were analyzed using the MIXED procedure of SAS (1999) according to a randomized complete block design.

Average milk production, ECM, milk composition (concentration and yield of each component) and feed intake, serum concentrations of folates and vitamin B_{12} , packed cell volume, blood hemoglobin, and serum homocysteine, methionine, and cysteine and serum folate kinetics at 8, 18, 28 and 38 wk of lactation as well as BW (5 measurements per cow) were analyzed using the MIXED procedure of SAS (1999) according to a randomized complete block design with repeated measures in time.

The treatment structure was a complete factorial design with 2 levels of methionine and 3 levels of folic acid. Polynomial contrasts were used to further investigate differences among levels of folic acid supplementation. When the interaction folic acid $\times$ methionine reached a level of significance of 95%, contrasts were used within each level of methionine. Results are reported as LSmeans and SE except when a natural logarithm transformation was used to restore normal distribution when the results are reported as anti-natural logarithm LSmeans with the interval of confidence at 95%.

RESULTS

Lactational Performance

At the beginning of the experiment, 4 wk before the expected time of calving, BW was similar between treatments (694 ± 11 kg; $P > 0.3$). There was no effect of dietary treatments on BW during lactation ($P > 0.4$), with BW averaging 637 ± 11 , 624 ± 9 , 653 ± 8 , 679 ± 9 , and 685 ± 10 kg at calving, 8, 18, 28, and 38 wk of lactation, respectively (time effect, $P < 0.001$).

Total milk production ($10,584 \pm 137$ kg), ECM (9943 ± 110 kg), and feed intake (7137 ± 3.1 kg of DM) for the 305-d lactation were unaffected by folic acid supplementation or RPM ($P > 0.2$; Table 2).

There was no effect of folic acid supplements or RPM on milk component yields ($P > 0.14$) and milk fat and ash concentrations ($P > 0.07$; Table 2). Dietary supplements of RPM increased milk total solids concentrations from 120.7 to 124.2 g/kg (SE 1.6; $P = 0.01$). The dietary supply of methionine modified the effects of supplementary folic acid on milk concentrations of lac-

Table 2. Dry matter intake and lactational performance (LSmeans) of dairy cows according to the dietary supplementations in folic acid and rumen-protected methionine (RPM).

Folic acid (mg/kg of BW)	Basal diet			Basal diet + RPM			SE	<i>P</i> values		
	0	3	6	0	3	6		Met	Fol	Met × Fol
No. of cows	9	8	8	9	8	8				
DMI (kg/d)	23.8	23.1	23.2	23.2	24.5	23.7	0.8	0.47	0.90	0.42
Milk production (kg/d)	36.2	33.7	33.9	32.0	34.1	32.9	1.6	0.21	0.90	0.29
Energy-corrected milk (kg/d)	33.5	32.8	32.6	31.6	33.0	33.5	1.5	0.83	0.93	0.60
Milk concentrations (g/kg)										
Total solids	119.5	121.6	121.4	125.3	122.3	127.0	2.3	0.01	0.48	0.33
Fat	32.7	35.1	35.5	36.6	35.3	38.2	1.8	0.08	0.34	0.48
Crude protein ¹	31.9	33.0	32.4	35.0	33.8	34.1	0.8	0.01	0.94	0.31
Casein ¹	26.2	27.5	26.8	29.2	27.9	28.5	0.8	0.01	0.99	0.26
Ash	6.8	6.9	6.7	6.8	6.8	6.7	0.07	0.45	0.07	0.29
Lactose ^{2, 3}	48.3	46.5	46.5	46.9	46.6	48.7	0.7	0.43	0.17	0.04
Milk yields (kg/d)										
Total solids	4477	4329	4289	4166	4408	4364	184	0.73	0.96	0.46
Fat	1213	1232	1251	1188	1254	1301	75	0.80	0.59	0.87
Crude protein	1190	1165	1135	1149	1213	1163	56	0.79	0.76	0.68
Casein	981	967	940	957	1000	977	49	0.69	0.87	0.76
Ash	255	248	239	230	246	229	21	0.19	0.53	0.59
Lactose ²	1820	1693	1667	1585	1652	1664	76	0.14	0.86	0.25
Milk urea (mg of N/dL) ⁴	15.3	14.7	14.9	13.7	14.3	15.0	0.3	0.06	0.40	0.05
Folates concentrations (ng/mL) ⁵	39.3	54.6	43.1	44.1	53.2	43.1	3.7	0.71	0.003	0.66
Total folates (mg/d) ⁵	1.46	2.00	1.63	1.69	1.90	1.43	0.1	0.81	0.009	0.26

¹Folic acid × methionine × time, *P* = 0.04.²Calculated by difference [total solids – (fat + crude protein + ash)].³Quadratic effect of folic acid × methionine.⁴Linear effect of folic acid × methionine.⁵Quadratic effect of folic acid.

tose (quadratic effect of folic acid × methionine, *P* = 0.04; Table 2) and urea (linear effect of folic acid × methionine, *P* = 0.02; Table 2). On average, milk crude protein and casein concentrations were higher in cows fed RPM, (methionine effect, *P* ≤ 0.005). However, the responses of milk crude protein and casein concentrations to supplementations with folic acid and RPM changed during lactation (folic acid × methionine × time, *P* = 0.04; Table 3). With the diet not supplemented with RPM, as lactation progressed, folic acid supplementation increased crude protein and casein concentrations (quadratic effect of folic acid × time, *P* = 0.07), whereas the opposite trend was observed in cows fed RPM (quadratic effect of folic acid × time, *P* = 0.06).

Concentrations and amounts of folates secreted in milk during lactation were increased by folic acid supplements (quadratic effect of folic acid, *P* < 0.009; Table 2). Milk folates decreased as lactation progressed and the decrease is greater in cows fed folic acid supplements (quadratic effect of folic acid × time, *P* ≤ 0.02; not shown).

Blood Variables

Serum concentrations of vitamin B₁₂, blood hemoglobin, and packed cell volume. At the beginning of the experiment, serum vitamin B₁₂ (263.2 ± 12.6 pg/

mL), blood hemoglobin (11.44 ± 0.17 g/dL), and packed cell volume (32.4 ± 0.5%) were similar between treatments (*P* > 0.2).

There was no effect of supplementary folic acid or RPM on serum concentrations of vitamin B₁₂ (*P* > 0.3), blood hemoglobin (*P* > 0.5), or packed cell volume (*P* > 0.3), although those variables changed with stage of lactation (*P* < 0.001; Table 4).

Serum concentrations of folates. At the beginning of the experiment, serum folates (8.52 ± 0.47 ng/mL) were similar between treatments (*P* > 0.2). They increased linearly with the amounts of folic acid ingested but the increase due to folic acid supplementation was larger 8 wk after calving than later in lactation (folic acid × time, *P* = 0.02; Table 4).

Serum concentrations of sulfur amino acids. At the beginning of the experiment, total serum methionine (19.7 ± 1.1 μmol/L), homocysteine (7.1 ± 1.0 μmol/L), and cysteine (231.1 ± 4.7 μmol/L) were similar between treatments (*P* > 0.2).

Total serum methionine and homocysteine were lower in unsupplemented cows, 15.5 (14.2 to 16.9) and 4.0 (3.6 to 4.4) μmol/L than in cows fed RPM, 25.3 (23.3 to 27.7) and 5.0 (4.8 to 5.8) μmol/L (*P* < 0.001). Folic acid supplements did not modify serum methionine (*P* > 0.4) but tended to decrease serum homocysteine (linear effect of folic acid, *P* = 0.06); 5.1 (4.5 to 5.7), 4.4 (3.9

Table 3. Crude protein and casein concentrations (LSmeans) in milk during lactation according to the dietary supplementations in folic acid and rumen-protected methionine (RPM).¹

	No. of cows	Time after calving (wk)				SE
		8	18	28	38	
Milk crude protein concentration ² (g/kg)						
M+F0	9	30.6	33.2	35.7	39.6	0.9
M+F3	8	30.0	33.2	34.4	35.7	1.0
M+F6	8	30.0	33.2	35.7	35.7	1.0
M-F0	9	29.3	30.6	31.3	33.8	0.9
M-F3	8	28.1	31.9	33.2	35.7	1.0
M-F6	8	28.7	30.6	32.5	35.1	1.0
Milk casein concentration ² (g/kg)						
M+F0	9	25.8	27.7	29.8	32.5	0.9
M+F3	8	24.9	27.4	28.2	29.0	0.9
M+F6	8	25.2	27.8	29.7	29.7	0.9
M-F0	9	24.4	25.3	25.9	27.5	0.9
M-F3	8	23.7	26.9	27.8	29.8	0.9
M-F6	8	24.0	25.7	26.8	28.7	0.9

¹M- = basal diet; M+ = basal diet + RPM supplement; F0 = no supplementary folic acid; F3 = 3 mg of folic acid per kg of BW daily; F6 = 6 mg of folic acid per kg of BW daily.

²Folic acid $\times$ methionine $\times$ time, $P = 0.04$.

to 5.0) and 4.4 (3.9 to 5.0) $\mu\text{mol/L}$ for cows fed 0, 3, and 6 mg of folic acid per kg of BW, respectively. The lowest concentrations of methionine (time effect, $P = 0.004$) and homocysteine (time effect, $P = 0.001$) were observed at 8 wk of lactation (Table 4).

On average, serum concentrations of total cysteine were higher in cows fed RPM ($P = 0.002$) but they were altered by the dietary supplements of folic acid (methio-

nine $\times$ quadratic effect of folic acid, $P = 0.008$). They also changed throughout lactation according to methionine supplementation (time effect $\times$ methionine, $P = 0.03$). In cows fed RPM, serum cysteine was lowered by folic acid supplementation; averaging 227.9 (213.8 to 242.0), 190.1 (175.4 to 204.9), and 208.1 (193.2 to 223.1) $\mu\text{mol/L}$ for cows fed 0, 3, or 6 mg of folic acid per kg of BW daily, respectively (quadratic effect of folic acid, $P =$

Table 4. Blood and serum variables and serum folate kinetics following an intravenous bolus of folic acid according to the stage of lactation (LSmeans, 95% confidence interval or SE).

	No. of cows	Time after calving (wk)				SE
		8	18	28	38	
Serum concentrations of folates ¹ (ng/mL)						
0	18	16.0	15.8	16.1	14.2	1.6
3	16	22.8	20.6	18.7	18.4	1.7
6	16	30.1	23.0	20.6	22.4	1.7
Serum vitamin B ₁₂ ² (pg/mL)	50	181.3 (167.3–198.3)	232.8 (214.9–254.7)	257.2 (235.1–278.7)	265.1 (242.3–287.1)	
Hemoglobin (g/dL)	50	10.5	11.1	11.9	11.7	0.2
Packed cell volume ² (%)	50	29.3	32.3	33.2	33.9	0.6
Serum methionine ² ($\mu\text{mol/L}$)	50	16.4 (14.9–18.2)	21.5 (19.5–23.8)	20.9 (18.9–23.1)	20.7 (18.5–23.1)	
Serum homocysteine ² ($\mu\text{mol/L}$)	50	4.18 (3.78–4.57)	4.57 (4.14–5.00)	4.76 (4.31–5.21)	5.05 (4.57–5.53)	
Serum cysteine ³ ($\mu\text{mol/L}$)						
Cows fed rumen-protected methionine	25	237 (221–253)	190 (174–205)	201 (186–217)	207 (191–223)	
Cows fed no rumen-protected methionine	25	202 (187–218)	189 (174–205)	192 (177–208)	188 (173–204)	
Clearance rate ⁴ (L/min)	50	2.37	2.59	2.70	2.62	0.24
Flux ⁵ ($\mu\text{g/min}$)	50	102.0	116.2	90.8	91.9	16.8

¹Folic acid $\times$ time, $P = 0.02$.

²Time effect, $P = 0.001$.

³Methionine $\times$ time, $P = 0.03$.

⁴Time effect, $P = 0.04$.

⁵Time effect, $P = 0.005$.

Table 5. Serum folate kinetics (LSmeans) following an intravenous bolus of folic acid according to the dietary supplementations in folic acid and rumen-protected methionine (RPM).

Folic acid (mg/kg of BW)	Basal diet			Basal diet + RPM			SE
	0	3	6	0	3	6	
No. of cows	9	8	8	9	8	8	
Ka ¹ (/min)	0.077	0.075	0.070	0.068	0.070	0.066	0.015
Kb ^{2,3} (/min)	0.032	0.025	0.035	0.026	0.024	0.022	0.009
Volume observed (L)	38.4	36.5	36.6	37.2	36.7	35.0	2.2
Volume estimated (L)	78.9	82.5	83.3	85.3	90.1	82.2	11.0
Clearance rate (L/min)	2.58	2.55	2.40	2.74	2.64	2.45	0.24
Flux ⁴ (μg/min)	85.8	100.9	118.6	75.1	116.5	110.0	16.8
Irreversible loss rate ⁵ (μg/min)	40.6	48.2	55.5	40.0	54.5	57.3	6.7
Serum folates on d 1 ⁶ (μg/L)	16.0	19.4	24.0	15.0	20.9	23.9	2.2

¹Ka = Fractional rate constant for pool A, calculated as $Ka = A_1g_1 + A_2g_2/A_1 + A_2$.

²Kb = Fractional rate constant for pool B, calculated as $Kb = g_1 + g_2 - Ka$.

³Methionine, $P = 0.08$.

⁴Folic acid, $P = 0.005$.

⁵Folic acid, $P = 0.003$.

⁶Folic acid, $P = 0.001$.

0.005). The highest concentration of cysteine was observed at 8 wk of lactation (time effect, $P \leq 0.04$; Table 4). In cows without supplementary methionine, folic acid supplementation had no effect on serum concentrations of cysteine. Values were 196.7 (182.6 to 210.8), 194.1 (179.4 to 208.9), and 188.3 (173.5 to 203.0) μmol/L, for cows fed 0, 3, or 6 mg of folic acid per kg BW daily, respectively ($P = 0.54$), and they did not vary through lactation (time effect, $P = 0.46$; Table 4).

Serum Folate Kinetics Following an Intravenous Bolus of Folic Acid

From the 2-pool model fit, the estimated size of the initial pool was approximately 2.3-fold the plasma pool and was not affected by treatment ($P > 0.1$). The rate constant (k) of this pool was 0.09/min, again independent of treatment ($P > 0.1$). The clearance rate of folates did vary with stage of lactation ($P = 0.04$; Table 4), however, and was lower during early (2.37 L/min) compared with late (2.66 L/min) lactation. Furthermore, both ILR and flux were increased by dietary folic acid supplementation ($P \leq 0.005$; Table 5). Flux also showed an effect of stage of lactation ($P = 0.005$), with the highest value at 18 wk of lactation (Table 4).

DISCUSSION

Lactational Performance

The absence of response in milk production and DMI to supplementation with RPM is similar to those frequently reported in the literature. In a nonexhaustive review of the literature between 1993 and 2003, only 1 (Vanhatalo et al., 1999) of 22 papers looking at the

effect of RPM fed to dairy cows reported an increase in milk production, and none described an effect on DMI (Aldrich et al., 1993; Guinard and Rulquin, 1995; Jurjanz et al., 1996; Overton et al., 1996; Armentano et al., 1997; Chilliard and Doreau, 1997; Pacheco-Rios et al., 1997, 1999; Rulquin and Delaby, 1997a, b; Robinson et al., 1998; Blum et al., 1999; Pisulewski and Kowalski, 1999; Vanhatalo et al., 1999; Krober et al., 2000; Berthiaume et al., 2001; Younge et al., 2001; Pisulewski et al., 2002; Kowalski et al., 2003; Leonardi et al., 2003; Misciattelli et al., 2003; Noftsker and St-Pierre, 2003). The major effect of RPM supplements is an increase in milk protein concentration and yield (Guinard and Rulquin, 1995; Jurjanz et al., 1996; Armentano et al., 1997; Chilliard and Doreau, 1997; Pacheco-Rios et al., 1997, 1999; Rulquin and Delaby, 1997b a, b; Krober et al., 2000; Kowalski et al., 2003; Leonardi et al., 2003; Misciattelli et al., 2003; Noftsker and St-Pierre, 2003). Overall, in the present experiment, RPM did increase milk crude protein (total nitrogen) and casein concentration but without any changes in milk protein yield. This latter finding maybe due to the numerically lower milk production, 33.0 vs. 34.6 (SE 1.0) kg/d for cows fed or not fed RPM, respectively.

As in previous experiments (Girard et al., 1995; Girard and Matte, 1998), no effect of supplementary folic acid on DMI or BW of cows was observed. Nevertheless, in those experiments, supplementary folic acid increased milk production and milk protein concentrations and yields in multiparous cows but had no such effects on primiparous cows. In the present experiment, however, multiparous cows showed no effect of supplementary folic acid on any of those parameters although as lactation progressed, supplementary folic acid did

increase milk protein concentration in cows fed no supplementary methionine.

Changes in milk concentrations of urea and lactose in response to folic acid supplementation differed according to the level of methionine provided by the diet. An increase in milk concentration of urea reflects an increment in blood concentration. High urea concentrations usually result from an excess of rumen-degradable protein absorbed as ammonia or from an increment in amino acid oxidation, having both as end-product hepatic ureagenesis (Hof et al., 1997). In this study, because all cows were fed the same basal diet, it is unlikely that ammonia absorption differed between treatments. In cows fed no supplementary folic acid, milk concentrations of urea and lactose were higher with the basal diet than with RPM supplementation. Therefore, it can be speculated that in cows receiving no methionine and folic acid supplementations, amino acids were used as gluconeogenic substrates with, consequently, increased nitrogen losses by deamination. When supplementary folic acid was provided, milk urea and lactose concentrations decreased when the basal diet was fed but increased in cows fed RPM. This may indicate a different shift in the use of amino acids as gluconeogenic substrates. How this might happen is unclear but glycine and serine do form an important component of the folate cycle and may be used as sources of one-carbon units in the presence of adequate folates. However, the observed changes in milk urea, although significant, were small, and changes in milk lactose are to be interpreted with caution because these were determined by difference.

As in the present experiment, Girard and Matte (1998) observed a progressive decrease in folate concentrations in milk throughout lactation and a quadratic response of milk folates to folic acid supplementation. However, in the 1998 study, milk folates were increased similarly by the 2 levels of supplementation used (2 and 4 mg/kg of BW per d), whereas in the present experiment, as lactation progressed, the response of milk folates to folic acid supplementation was smaller for cows fed 6 mg (per kg of BW) of folic acid daily than for those fed 3 mg. In women, milk folates are stable, and responses to folic acid supplementation are only seen in malnourished mothers (Tamura et al., 1980; Kirksey, 1986; Keizer et al., 1995; Mackey and Picciano, 1999). Those results confirm that milk secretion of folates in cows, as in humans, is not due to passive transfer but rather is regulated at the level of the mammary gland.

Metabolic Variables

Supplementary folic acid had no effect on packed cell volume or blood hemoglobin, as reported previously in

multiparous cows (Girard et al., 1995; Girard and Matte, 1999).

Serum concentrations of vitamin B₁₂ increased through lactation, a similar pattern to that observed by Girard and Matte (1999), although the concentrations were lower than previously reported for multiparous cows and, instead, were similar to those of primiparous cows (Girard and Matte, 1999). Stangl et al. (2000b) observed plasma concentrations of approximately 108 pg/mL in growing cattle fed a cobalt-deficient diet and 271 pg/mL in those fed a cobalt-adequate diet. In the present experiment, serum vitamin B₁₂ increased from 189 pg/mL at 8 wk of lactation to 275 pg/mL at 38 wk of lactation indicating a possible lack of vitamin B₁₂ in early lactation despite an adequate supply of cobalt.

As previously reported (Girard and Matte, 1999), serum concentrations of folates increased linearly with folic acid supplementation, and reached their highest value in supplemented cows in early lactation. They decreased thereafter to a plateau at 16 to 18 wk of lactation. In the present experiment, blood samples for serum folate determination were taken 24 h after ingestion of the dietary supplements and all serum folates, even in cows fed supplementary folic acid, were in the form of 5-methyl-THF (C. L. Girard, unpublished data). In many species, serum folates are present as the monoglutamate of 5-methyl-THF (Luccock, 2000), but retention and concentration of folates by tissues require conversion of pteroylmonoglutamates to pteroylpolyglutamates (Shane, 1989). 5-Methyl-tetrahydrofolate is a poor substrate for the enzyme responsible for the elongation of the glutamate chain (Shane, 1989) and the methyl group can be removed only through one vitamin B₁₂-dependent reaction, methionine synthase, in the regeneration of methionine from homocysteine (Shane et al., 1977). This demethylation of 5-methyl-THF is rate limiting for the cellular accumulation of folates (Luccock, 2000). Consequently, in the present experiment, the higher serum concentrations of folates in early lactation cows fed supplementary folic acid at a time when serum B₁₂ was low could reflect a decreased ability of the cells to retain and use folates.

This hypothesis is supported by the observation that serum clearance of folic acid during the 8 h following an i.v. bolus of folic acid was slower at 8 wk of lactation. Herbert and Zaluski (1962), as cited by Gregory and Quinlivan (2002), observed that, during vitamin B₁₂ deficiency, serum clearance of total folates (measured by *Lactobacillus casei*) was less rapid than serum clearance of nonmethylated folates (measured by *Streptococcus faecalis*). The authors interpreted this as an accumulation of folates in a nonmetabolically available form, i.e., 5-methyl-THF. In the present experiment, it is possible that a low supply of vitamin B₁₂ interfered

with tissue uptake of folates by inhibiting the demethylation of 5-methyl-THF. This explanation is in accordance with the observations that later in lactation, when serum vitamin B₁₂ increased, serum clearance of folic acid was accelerated while in cows fed supplementary folic acid, serum concentrations of folates were decreased as compared with concentrations earlier in lactation. Overall, the folate kinetics, following a bolus dose, fitted reasonably to a 2-compartment model. Of course, a more complex system may exist biologically but this could only be defined by more detailed experimental approaches using labeled tracer folates and sampling of secondary and tertiary folate pools. The data do indicate, however, that folates both enter and leave the primary pool (plasma), as shown by the difference between flux (total flow) and ILR (net unidirectional flow). At all levels of folic acid supplementation, flux was approximately twice that of ILR (Table 5). At zero supplementation, this means that total flow from the pool was 80 $\mu\text{g}/\text{min}$ with a return of 40 $\mu\text{g}/\text{min}$, leaving an irreversible loss (net unidirectional movement) of 40 $\mu\text{g}/\text{min}$. The model cannot resolve, however, whether the 40 $\mu\text{g}/\text{min}$ return was from the same pool into which the 80 $\mu\text{g}/\text{min}$ flowed or another pool(s).

Ingestion of RPM increased serum concentrations of methionine in a range similar to those reported in the literature (Aldrich et al., 1993; Overton et al., 1996; Pacheco-Rios et al., 1997; Rulquin and Delaby, 1997a, b; Robinson et al., 1998; 1999; Pisulewski and Kowalski, 1999; Vanhatalo et al., 1999; Krober et al., 2000; Pisulewski et al., 2002). Serum concentrations of homocysteine were also higher in cows fed RPM. Overall, however, homocysteine concentrations were lower than observed for growing cattle (7.4 and 7.8 $\mu\text{mol}/\text{L}$; Stangl et al., 2000a, b).

At 8 wk of lactation, MP supply was lower than MP requirements and the decrease in serum concentrations of methionine and homocysteine could reflect the demand for protein synthesis. This should have been concomitant with a decrease in serum concentrations of cysteine but it was not the case in the present experiment in which serum concentrations of cysteine peaked in early lactation in cows fed RPM. Later in lactation, increased dietary intake and lower milk protein output should have resulted in increased catabolism of dietary methionine to cysteine. Nonetheless, as discussed below, other factors must also operate, as plasma cysteine concentrations were lower in late lactation than during early lactation, and were similar in the presence and absence of RPM.

The methionine cycle is of major importance for cellular metabolism. Among its numerous roles, it regulates the balance between methionine and cysteine for protein synthesis and provides the pathway by which

methyl groups are transferred from 5-methyl-THF to a broad variety of substrates (Finkelstein, 1990). According to the model proposed by Reed et al. (2004), based on data obtained in humans and rats, a lack of vitamin B₁₂ (and the subsequent interference with folate use) would decrease methionine concentration in liver due to a reduction of the activity of the methionine synthase and then, homocysteine remethylation. In the present experiment, the lowest serum concentrations of methionine were observed at 8 wk of lactation at a time where serum concentrations of vitamin B₁₂ reached their lowest values. Those observations could give a supplementary indication, although an indirect one, that the vitamin B₁₂ status of the cows was low enough at this physiological stage to interfere with the methionine cycle.

Homocysteine has 2 metabolic fates. First, it is transformed to cystathionine (by the enzyme cystathionine synthase) and then cysteine, which is then used for protein, glutathione, and taurine synthesis, or degraded to sulfate. Second, homocysteine can accept a methyl group from 5-methyl-THF or betaine and be reconverted to methionine (Martinov et al., 2000). In humans and rats, high dietary supply of methionine increases the concentration of S-adenosylmethionine, which activates cystathionine synthase and, thus, cysteine formation (Selhub, 1999). Conversely, decreased methionine supply reduces S-adenosylmethionine concentration, with consequent lower activity of cystathionine synthase and less cysteine formation (Reed et al., 2004). These concepts could account for the higher plasma cysteine concentration for the cows fed RPM.

In cows fed supplementary methionine, supplementary folic acid reduced serum concentrations of both homocysteine and cysteine and this may be explained by increased conversion of homocysteine to methionine, as similar responses have been observed with humans given additional folates (Bunout et al., 2000; Mayer et al., 2002). The regeneration of methionine may have been inhibited during early lactation, however, because of low availability of vitamin B₁₂. This would increase the size of the homocysteine pools and stimulate production of cysteine, particularly in cows fed RPM.

CONCLUSIONS

Dietary supplements of folic acid and RPM modified milk crude protein and casein concentrations but not yield in mid- and late-lactation. In early lactation, 8 wk after calving, serum concentrations of folates and cysteine were increased whereas vitamin B₁₂ and methionine were decreased. These data, coupled with the slower serum clearance of folates following an i.v. bolus of folic acid suggest that low supply in vitamin B₁₂

during early lactation could interfere with folate use. Other studies are necessary to determine if supplementary vitamin B₁₂ given in early lactation, impacts on lactational performance of dairy cows fed supplementary folic acid and RPM.

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REFERENCES

- Agriculture Canada. 1990. Recommended Code of Practice for Care and Handling of Dairy Cattle, Publ. No. 1853/E, Agriculture Canada, Ottawa, ON.
- Aldrich, J. M., L. D. Muller, and G. A. Varga. 1993. Effect of somatotropin administration and duodenal infusion of methionine and lysine on lactational performance and nutrient flow to the small intestine. *Br. J. Nutr.* 69:49–58.
- Association of Official Analytical Chemists. 1990. Official Methods of Analysis. 15th ed. AOAC, Arlington, VA.
- Armentano, L. E. 1994. Impact of metabolism by extragastrointestinal tissues on secretory rate of milk proteins. *J. Dairy Sci.* 77:2809–2820.
- Armentano, L. E., S. J. Bertics, and G. A. Ducharme. 1997. Response of lactating cows to methionine or methionine plus lysine added to high protein diets based on alfalfa and heated soybeans. *J. Dairy Sci.* 80:1194–1199.
- Bailey, L. B., and J. F. Gregory, III. 1999. Folate metabolism and requirements. *J. Nutr.* 129:779–782.
- Bässler, K. H. 1997. Enzymatic effects of folic acid and vitamin B₁₂. *Int. J. Vit. Nutr. Res.* 67:385–388.
- Berthiaume, R., P. Dubreuil, M. Stevenson, B. W. McBride, and H. Lapiere. 2001. Intestinal disappearance and mesenteric and portal appearance of amino acids in dairy cows fed ruminally protected methionine. *J. Dairy Sci.* 84:194–203.
- Blum, J. W., R. M. Bruckmaier, and F. Jans. 1999. Rumen-protected methionine fed to dairy cows: Bioavailability and effects on plasma amino acid pattern and plasma metabolite and insulin concentrations. *J. Dairy Sci.* 82:1991–1998.
- Broderick, G. A. 1985. Alfalfa silage or hay versus corn silage as the sole forage for lactating dairy cows. *J. Dairy Sci.* 68:3262–3271.
- Bunout, D., A. Garrido, M. Suazo, R. Kauffman, P. Venegas, P. de la Maza, M. Petermann, and S. Hirsch. 2000. Effects of supplementation with folic acid and antioxidant vitamins on homocysteine levels and LDL oxidation in coronary patients. *Nutrition* 16:107–110.
- Canadian Council on Animal Care. 1993. Guide to the Care and Use of Experimental Animals, 2nd ed. Vol. 1. E. D. Rolfert, B. M. Cross, and A. A. McWilliam, ed. Canadian Council on Animal Care, Ottawa, ON.
- Chilliard, Y., and M. Doreau. 1997. Influence of supplementary fish oil and rumen-protected methionine on milk yield and composition in dairy cows. *J. Dairy Res.* 64:173–179.
- Choi, S.-W., and J. B. Mason. 2000. Folate and carcinogenesis: An integrated scheme. *J. Nutr.* 130:129–132.
- Finkelstein, J. D. 1990. Methionine metabolism in mammals. *J. Nutr. Biochem.* 1:228–237.
- Girard, C. L., and J. J. Matte. 1988. Blood serum concentrations of folates and vitamin B₁₂ during growth period of white veal calves. *Can. J. Anim. Sci.* 68:455–460.
- Girard, C. L., and J. J. Matte. 1998. Dietary supplements of folic acid during lactation: Effects on the performance of dairy cows. *J. Dairy Sci.* 81:1412–1419.
- Girard, C. L., and J. J. Matte. 1999. Changes in serum concentrations of folates, pyridoxal, pyridoxal-5-phosphate and vitamin B₁₂ during lactation of dairy cows fed dietary supplements of folic acid. *Can. J. Anim. Sci.* 79:107–113.
- Girard, C. L., J. J. Matte, and G. F. Tremblay. 1995. Gestation and lactation of dairy cows: A role for folic acid? *J. Dairy Sci.* 78:404–411.
- Gregory, J. F., III, and E. P. Quinlivan. 2002. In vivo kinetics of folate metabolism. *Annu. Rev. Nutr.* 22:199–220.
- Guinard, J., and H. Rulquin. 1995. Effects of graded amounts of duodenal infusions of methionine on the mammary uptake of major milk precursors in dairy cows. *J. Dairy Sci.* 78:2196–2207.
- Hof, G., M. D. Vervoorn, P. J. Lenaers, and S. Tamminga. 1997. Milk urea as a tool to monitor the protein nutrition of dairy cows. *J. Dairy Sci.* 80:3333–3340.
- Jurjanz, S., O. Colin-Schoellen, and F. Laurent. 1996. Effect of starch nature of the energy concentrate and of a methionine supply on the rearing performance of dairy cows. *Ann. Zoot.* 45:467–476.
- Keizer, S. E., R. S. Gibson, and D. L. O'Connor. 1995. Postpartum folic acid supplementation of adolescents: Impact on maternal folate and zinc status and milk composition. *Am. J. Clin. Nutr.* 62:377–384.
- Kirksey, A. 1986. Effects of vitamin supplementation on vitamin levels in human milk: Vitamin B-6, vitamin C and folacin. Pages 339–348 in *Human Nutrition 2. Maternal and environmental factors*. M. Hamosh and A. S. Goldman, ed. Plenum Press. New York, NY.
- Kowalski, Z. M., P. M. Pisulewski, and M. Gorgolu. 2003. Effects of protected methionine and variable energy supply on lactational responses in dairy cows fed grass silage-based diets. *J. Anim. Feed Sci.* 12:451–464.
- Krober, T. F., M. Kreuzer, M. Senn, W. Langhans, and F. Sutter. 2000. Lactational and metabolic effects in cows of lysine and methionine added to a ration deficient according to the INRA method. *Arch. Tierernähr.* 53:375–394.
- Le Grusse, J., and B. Watier. 1993. Les vitamines. Données biochimiques, nutritionnelles et cliniques. Centre d'étude et d'information sur les vitamines, Produits Roche. Neuilly-sur-Seine, France.
- Leonardi, C., M. Stevenson, and L. E. Armentano. 2003. Effect of two levels of crude protein and methionine supplementation on performance of dairy cows. *J. Dairy Sci.* 86:4033–4042.
- Lucock, M. 2000. Folic acid: Nutritional biochemistry, molecular biology, and role in disease processes. *Mol. Genet. Metab.* 71:121–138.
- Mackey, A. D., and M. F. Picciano. 1999. Maternal folate status during extended lactation and the effect of supplemental folic acid. *Am. J. Clin. Nutr.* 69:285–292.
- Malinow, M. R., S. S. Kang, L. M. Taylor, P. W. K. Wong, B. Coull, T. Inahara, D. Mukerjee, G. Sexton, and B. Upson. 1989. Prevalence of hyperhomocyst(e)inemia in patients with peripheral arterial occlusive disease. *Circulation* 79:1180–1188.
- Manet, L. 1969. Techniques usuelles de biologie clinique. Page 39 in *Hématologie*. Éditions médicales Flammarion. Paris, France.
- Martinov, M. V., V. M. Vitvitsky, E. V. Mosharov, R. Banerjee, and F. I. Ataullakhnov. 2000. A substrate switch: A new mode of regulation in the methionine metabolic pathway. *J. Theor. Biol.* 204:521–532.
- Mayer, O., J. Filipovsky, M. Hromadka, V. Svobodova, J. Racek, P. Stehlik, L. Trefil, and M. Zarybnicka. 2002. Treatment of hyperhomocysteinemia with folic acid: Effects on homocysteine levels, coagulation status, and oxidative stress markers. *J. Cardiovasc. Pharmacol.* 39:851–857.

- Misciattelli, L., V. F. Kristensen, M. Vestergaard, M. R. Weisbjerg, K. Seirsen, and T. Hvelplund. 2003. Milk production, nutrient utilization and endocrine responses to increased postruminal lysine and methionine supply in dairy cows. *J. Dairy Sci.* 86:275–286.
- National Research Council. 2001. *Nutrient Requirements of Dairy Cattle*. 7th rev. ed. National Academy Press, Washington, DC.
- Noftsker, S., and N. R. St-Pierre. 2003. Supplementation of methionine and selection of highly digestible rumen undegradable protein to improve nitrogen efficiency for milk production. *J. Dairy Sci.* 86:958–969.
- Overton, T. R., D. W. LaCount, T. M. Cicela, and J. H. Clark. 1996. Evaluation of a ruminally protected methionine product for lactating dairy cows. *J. Dairy Sci.* 79:631–638.
- Pacheco-Rios, D., W. C. McNabb, J. P. Hill, T. N. Barry, and D. D. S. Mackenzie. 1997. The effects of methionine supplementation upon milk composition and production of dairy cows in late lactation. *Proc. Nutr. Soc. New Zealand.* 22:184–191.
- Pacheco-Rios, D., W. C. McNabb, J. P. Hill, T. N. Barry, and D. D. S. Mackenzie. 1999. The effects of methionine supplementation upon milk composition and production of forage-fed dairy cows. *Can. J. Anim. Sci.* 79:235–241.
- Pisulewski, P. M., and Z. M. Kowalski. 1999. The effect of protected lysine and methionine on milk yield and its composition in lactating dairy cows fed grass silage-based rations. *J. Anim. Feed Sci.* 8:341–353.
- Pisulewski, P. M., Z. M. Kowalski, and M. Gorgulu. 2002. Lactational responses to ruminally-protected methionine in cows fed a low-protein grass silage based-diet. *J. Anim. Feed Sci.* 11:189–203.
- Reed, M. C., H. F. Nijhout, R. Sparks, and C. M. Ulrich. 2004. A mathematical model of the methionine cycle. *J. Theor. Biol.* 226:33–43.
- Robinson, P. H., W. Chalupa, C. J. Sniffen, W. E. Julien, H. Sato, K. Watanebe, T. Fujida, and H. Suzuki. 1998. Ruminally protected lysine and methionine for lactating dairy cows fed a ration designed to meet requirements for microbial and postruminal protein. *J. Dairy Sci.* 81:1364–1373.
- Rulquin, H., and L. Delaby. 1997a. Effects of the energy balance of dairy cows on lactational responses to rumen-protected methionine. *J. Dairy Sci.* 80:2513–2522.
- Rulquin, H., and L. Delaby. 1997b. Lactational responses of grazing dairy cows to rumen-protected methionine. *Ann. Zoot.* 46:409–415.
- SAS Institute. 1999. *SAS User's Guide: Statistics*, Version 6 ed. SAS Institute, Inc., Cary, NC.
- Selhub, J. 1999. Homocysteine metabolism. *Annu. Rev. Nutr.* 19:217–246.
- Shane, B. 1989. Polyglutamate synthesis and role in the regulation of one-carbon metabolism. *Vit. Horm.* 45:263–335.
- Shane, B., J. E. Watson, and E. L. R. Stockstad. 1977. Uptake and metabolism of [3H]folate by normal and by vitamin B-12 and methionine-deficient rats. *Biochim. Biophys. Acta* 497:241–252.
- Shipley, R. A., and R. E. Clark. 1972. *Tracer methods for in vivo kinetics, theory and applications*, Academic Press, London, UK.
- Snoswell, A. M., and G.-P. Xue. 1987. Methyl group metabolism in sheep. *Comp. Biochem. Physiol.* 88B:383–394.
- Stangl, G. I., F. J. Schwarz, B. Jahn, and M. Kirchgeßner. 2000a. Cobalt-deficiency-induced hyperhomocysteinaemia and oxidative status of cattle. *Br. J. Nutr.* 83:3–6.
- Stangl, G. I., F. J. Schwarz, H. Müller, and M. Kirchgeßner. 2000b. Evaluation of the cobalt requirement of beef cattle based on vitamin B12, folate, homocysteine and methylmalonic acid. *Br. J. Nutr.* 84:645–653.
- Tamura, T., Y. Yoshimura, and T. Arakawa. 1980. Human milk folate and folate status in lactating mothers and their infants. *Am. J. Clin. Nutr.* 33:193–197.
- Tyrrell, H. F., and J. T. Reid. 1965. Prediction of the energy value of cow's milk. *J. Dairy Sci.* 48:1215–1223.
- Vanhatalo, A., P. Huhtanen, V. Toivonen, and T. Varvikko. 1999. Response of dairy cows fed grass silage diets to abomasal infusions of histidine alone or in combinations with methionine and lysine. *J. Dairy Sci.* 82:2674–2685.
- Xue, G.-P., and A. M. Snoswell. 1985. Regulation of methyl group metabolism in lactating ewes. *Biochem. Int.* 11:381–385.
- Younge, B. A., J. J. Murphy, M. Rath, and B. K. Sloan. 2001. Effect of dietary absorbable methionine and lysine concentrations on milk production and composition of dairy cows offered grass-silage based diets. *Irish. J. Agric. Food. Res.* 40:1–11.