Fate of Supplementary B-Vitamins in the Gastrointestinal Tract of Dairy Cows*

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ABSTRACT

Four lactating Holstein cows equipped with ruminal, duodenal, and ileal cannulas were used in 2 studies to evaluate the disappearance of supplementary B-vitamins before and from the small intestine. The cows were fed a total mixed ration with chromic oxide in 12 daily meals. Each study consisted of a control (no vitamin supplementation) and a treatment period (with vitamin supplementation). Amounts of vitamins (mg/d) supplemented in studies 1 and 2, respectively, were: thiamin: 300 and 10; riboflavin: 1600 and 2.0; niacin: 12,000 and 600; vitamin B₆: 800 and 34; biotin: 20 and 0.02; folic acid: 2600 and 111; vitamin B₁₂: 500 and 0.4. In study 1, vitamins were added to the feed 5 d before and during the 4-d collection period. In study 2, vitamins were infused postruminally 1 d before and during the 4-d collection period. Substantial disappearance before the duodenal cannula was noted in study 1 (67.8% thiamin, 99.3% riboflavin, 98.5% nicotinamide, 41.0% pyridoxine, 45.2% biotin, 97.0% folic acid, and 62.9% vitamin B₁₂). Except for nicotinamide and folate, there was almost no disappearance of postruminally infused vitamins before the duodenal cannula (study 2), suggesting extensive ruminal destruction or use. Apparent intestinal absorption values differed greatly among vitamins, but the proportion of vitamins disappearing from the small intestine was not negatively influenced by supplementation. Except for riboflavin and niacin, absolute amounts disappearing from the small intestine were greater during the treatment than the control periods, suggesting that B-vitamin supply in dairy cows is increased by supplementation, although losses in the rumen are extensive.

(**Key words:** dairy cow, B vitamins, ruminal destruction, intestinal absorption)

Abbreviation key: CON = control period, NA = nicotinic acid, NAM = nicotinamide, P5P = pyridoxal-5-phosphate, PAL = pyridoxal, PAM = pyridoxamine, PYR = pyridoxine, TRT = treatment period.

INTRODUCTION

Most of the research studying the requirements of dairy cattle for B-vitamins was conducted almost half a century ago. It was concluded then that the mature ruminant animal does not require an exogenous supply of B-vitamins, because its rumen microflora should synthesize enough of these compounds to avoid deficiency. Since then, dairy cows have greatly increased their average milk and milk component yields. It is likely that their B-vitamin requirements increased accordingly and that ruminal synthesis alone may not be sufficient to meet the new needs, as supported by studies reporting beneficial effects of dietary supplementation of thiamin (Shaver and Bal, 2000), niacin (Fronk and Schultz, 1979; Riddell et al., 1981), biotin (Zimmerly and Weiss, 2001), and folic acid (Girard and Matte, 1998). Nevertheless, the exact fate of these vitamins in the digestive tract of ruminants is still unknown. Data are often extrapolated from nonruminant animals, but the precision of such estimates has not been verified.

Zinn et al. (1987) presented 2 studies looking at ruminal destruction and intestinal absorption of most of the B-vitamins in growing steers. According to their results, extensive loss of supplementary vitamins occurs before the small intestine, which complicates supplementation. Intestinal absorption rates reported by Zinn et al. (1987) were similar to those of Miller et al. (1986) estimated with heavier steers and were close to values observed in nonruminant animals. However, the literature still lacks information about the availability of dietary supplements of B-vitamins for dairy cows.

The present experiment was undertaken to evaluate the disappearance of supplementary B-vitamins before and from the small intestine of lactating dairy cows. As extensive ruminal destruction or use was expected,

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the effect of postruminal infusions of vitamins on their availability was also investigated. In addition, estimates of daily amounts of vitamins being synthesized in the rumen of dairy cows were calculated.

MATERIALS AND METHODS

Animals and Diets

Four multiparous lactating Holstein cows [DIM: between 29 and 178; milk production during the trial: 27.7 ± 1.4 kg/d (mean $\pm$ SE)] equipped with cannulas in the rumen, the proximal duodenum (~30 cm from the pylorus), and the distal ileum (~60 cm from the ileocecal valve) were used. The experimental procedures of this study were approved by the Institutional Animal Care Committee of the Dairy and Swine Research and Development Center, Lennoxville, QC, and followed the guidelines of the Canadian Council on Animal Care (1993). The cows were fed a TMR containing chromic oxide as digesta passage marker 12 times daily (Table 1). Chromic oxide was included in the protein supplement pellets. Amounts of feeds offered were limited to 95% of the ad libitum intake measured the week before the beginning of the experiment, to avoid refusals and maintain a similar feed intake between the periods [average DMI during studies 1 and 2: 19.8 ± 0.5 kg/d (mean $\pm$ SE)]. The cows were milked twice daily at 0900 and 2100 h. Feed and water intakes, feed refusals (when present), and milk production were recorded daily. Feed samples were taken weekly and frozen at -20°C for later analyses of chemical composition and vitamin concentrations.

Treatments and Collection of Samples

The experiment was conducted as 2 separate studies. Each study consisted of a control period (**CON**; no vitamin supplementation) and a treatment period (**TRT**; with vitamin supplementation). The vitamins provided in the TRT periods were: thiamin (thiamin hydrochloride; Sigma, St. Louis, MO), riboflavin (Sigma), niacin (nicotinamide; Sigma), vitamin B₆ (pyridoxine hydrochloride; Sigma), biotin (Sigma), folic acid (pteroylmoglutamic acid; ICN Biomedicals, Cleveland, OH), and vitamin B₁₂ (cyanocobalamin; Hoffmann-LaRoche, Cambridge, Ontario, Canada). Daily amounts of vitamins supplemented in each study are reported in Table 2. The amounts of thiamin, niacin, biotin, folic acid, and vitamin B₁₂ provided in study 1 were based on the literature (Frigg et al., 1993b; Shaver and Bal, 2000; Girard et al., 2001), whereas quantities of riboflavin and vitamin B₆ (in the form of pyridoxine) were calculated on a BW basis from Zinn et al. (1987) for a cow weighing 650 kg. In study 2, levels of vitamins infused

Table 1. Ingredients, chemical composition, and vitamin concentrations of the basal diet.

Item		
DM	40.0	
Ingredients (% DM basis)		
Grass-legume silage	43.6	
Corn silage	14.7	
High moisture corn	33.6	
Soybean meal	0.8	
Protein supplement	4.9	
Corn gluten meal		1.1
Extruded soybean		1.0
Wheat distillers		1.0
Canola meal		1.0
Corn distillers		0.7
Chromic oxide		0.08
Mineral and vitamin premix ¹	2.3	
Chemical composition (% DM basis)		
CP	17.7	
Soluble protein	7.1	
ADICP ²	7.4	
NDICP ³	14.4	
ADF	20.0	
NDF	28.2	
Nonstructural carbohydrates	44.3	
Fat	2.6	
Ash	7.2	
B-Vitamin concentrations (ug/g DM)		
Thiamin	2.09	
Riboflavin	5.18	
Niacin ⁴	23.80	
Nicotinic acid (NA)		23.80
Nicotinamide (NAM)		0.00
Vitamin B ₆ ⁵	8.50	
Pyridoxamine (PAM)		0.64
Pyridoxal (PAL)		1.56
Pyridoxine (PYR)		6.30
Biotin	7.04	
Folates	0.35	
Vitamin B ₁₂	0.010	

¹Composition per kg: 95.3 g Ca, 54.7 g P, 34.9 g Mg, 132.6 g Na, 74.3 g Cl, 14.4 g K, 20.8 g S, 2.7 g Fe, 2.1 g Mn, 3.0 g Zn, 499 mg Cu, 69 mg I, 33 mg Co, 17 mg Se, 369 500 IU vitamin A, 65 130 IU vitamin D, and 1.8 g of DL-tocopherol acetate.

²Acid detergent insoluble CP.

³Neutral detergent insoluble CP.

⁴Sum of the molar concentrations of NA and NAM; expressed as NA-equivalents.

⁵Sum of the molar concentrations of PAM, PAL, and PYR; expressed as PYR-equivalents.

were based on preliminary trials conducted on one dry cow equipped with ruminal, duodenal, and ileal cannulas.

Duodenal and ileal samples (approximately 350 mL) were collected twice daily over 4 consecutive days in both the CON and the TRT periods (d 1: 0800 and 1400 h; d 2: 1000 and 1600 h; d 3: 1100 and 1700 h; d 4: 0900 and 1500 h). Samples were considered missing if they could not be collected within 45 min of the target time. Three ileal samples were missing: two in study 1, and one in study 2. A rest period of 8 d was allowed between the 2 studies to reduce carryover effects.

Table 2. Amounts of B-vitamins supplemented in the diet (study 1) or infused postruminally (study 2) (mg/d).

Vitamin	Dietary supplement (study 1)	Postruminal infusion ¹ (study 2)
Thiamin	300	10 ± 0.3
Riboflavin	1600	2.0 ± 0.04
Nicotinamide	12,000	1173 ± 7
Pyridoxine (vitamin B ₆)	800	34 ± 2
Biotin	20	0.02 ± 0.0005
Folic acid	2600	111 ± 5
Vitamin B ₁₂	500	0.4 ± 0.01

¹The amounts infused are calculated as the infusion rates multiplied by vitamin concentrations in the infused solutions. These variables were measured twice daily during the 5 d of infusion.

In study 1, the vitamins were given as dietary supplements. Vitamins were mixed with ground corn and added to the feed at each meal. Supplementation took place 5 d before and during the 4 d of collection in the TRT period. Biotin values from one cow were removed due to an error during preparation of the vitamin supplement.

In study 2, the vitamins were infused postruminally as described by Griinari et al. (1997). For this purpose, the B-vitamins were dissolved in a sufficient amount of water to infuse at a rate of approximately 4 L/d [3.79 ± 0.03 L/d (mean ± SE)]. The rate of infusion was determined by difference in the weight of the vitamin solution remaining to be infused, which was taken twice daily. Infusions lasted 5 d, that is, 1 d before and during the 4-d collection period of the TRT period.

Laboratory Analyses

Digesta samples were frozen at -20°C until being freeze-dried and ground through a 1-mm screen. The samples were then composited by day, cow, and collection site. Analyses of B-vitamin and chromic oxide concentrations were performed on the pooled digesta samples and in the feed.

The HPLC system used for the analysis of thiamin, riboflavin, and vitamin B₆ consisted of the following components: a solvent delivery (model 110B; Beckman, Fullerton, CA), an autosampler with a 10-μL injection loop (model 9300; Varian, Walnut Creek, CA), and a fluorimetric detection system (model LC240; Perkin Elmer, Woodbridge, Ontario, Canada).

Thiamin. Thiamin concentrations were analyzed in duplicate by HPLC according to a method adapted from Botticher and Botticher (1986). A sample of 0.1 g was mixed with 5 mL of sulfuric acid (0.1 M) in a 15-mL polypropylene conical tube and was autoclaved for 15 min at 121°C. After cooling, 0.5 mL of sodium acetate (4.5 M) and 0.25 mL of Clara-diestase (amylase EC

3.2.1 from *Aspergillus oryzae*; 50 mg/mL) were added. The samples were incubated for 18 h in a water bath at 45°C and then centrifuged at 1784 × g for 10 min at 4°C. Immediately after centrifugation, 1 mL of the supernatant was mixed with 225 μL of oxidative solution (175 μL of NaOH 50% and 50 μL of potassium ferricyanide 5% per sample). After 5 min of incubation, approximately 0.25 g of NaCl was added to each tube. Following mixing, 2.0 mL of 2-butanol was added, the samples were agitated for 1 min and were then injected into the HPLC. Routine HPLC analysis was carried out on a Nucleosil-NH₂ column (250 mm × 4.6 mm, 5 μ; Varian) preceded by a guard column. The mobile phase was composed of 250 mL of KH₂PO₄ and 750 mL of acetonitrile at a flow rate of 1.5 mL/min, with the fluorimetric detection system adjusted to 425 and 370 nm for emission and excitation, respectively. Recovery rates averaged 95.8 and 106.9% for the duodenal and ileal samples, respectively. Rates of conversion of thiamin monophosphate and thiamin triphosphate into thiamin were both 100%. Interassay coefficients of variation averaged 12.2%. An intraassay coefficient of variation of less than 10% was accepted between duplicates.

Riboflavin. Measurements of riboflavin concentrations were done in duplicate according to a method adapted from Giguère et al. (2002). All forms of the vitamin were transformed into riboflavin for analysis. Briefly, 0.2 g of feed or digesta and 20 mL of HCl (0.1 M) were combined in 12 × 75-mm borosilicate tubes and centrifuged for 5 min at 1784 × g. Five hundred microliters of supernatant was combined with 200 μL of 15% perchloric acid to precipitate protein, and the samples were then boiled for 10 min. Following centrifugation at 1854 × g for 10 min, 400 μL of the supernatant was added to 200 μL of sodium acetate (4.0 M) and 25 μL of acid phosphatase 2% [20 mg phosphatase per mL of ammonium acetate buffer (50 mM, pH 4.0)]. Samples were then incubated for 18 h in a water bath at 37°C to convert flavin-adenine dinucleotide into riboflavin. After centrifugation for 10 min at 1854 × g, samples were injected into the HPLC. A Microsorb-MV 100-5 C18 column (250 × 4.6 mm; Varian) was used, with a mobile phase composed of 80 mL of ammonium acetate buffer (pH 4.0), 800 mL of ultrapure water, and 200 mL of acetonitrile at a flow rate of 1.0 mL/min. The fluorimetric detection system was adjusted to 520 and 450 nm for emission and excitation, respectively. Recovery rates averaged 101.9% for the duodenal and 94.2% for the ileal samples. Mean rate of conversion of flavin-adenine dinucleotide into riboflavin was 96.2%. The mean interassay variation coefficient was 15.3%. A coefficient of variation of less than 10% was accepted between duplicates in an assay.

Niacin. Concentrations of nicotinic acid (NA) and nicotinamide (NAM) were determined in duplicate according to a method adapted from Mawatari et al. (1991), Lahély et al. (1999), and Ndaw et al. (2002). In summary, 0.1 g of feed or digesta was added to 7 mL of HCl (0.1 M) in 15-mL polypropylene conical tubes. All the samples were then autoclaved for 50 min at 121°C. After cooling, the tubes were diluted to a final volume of 10 mL with ultrapure water and were centrifuged for 10 min at $1854 \times g$ and 4°C. Approximately 2 mL of supernatant was collected, of which 20 μ L was injected into the HPLC. Samples were run through a Pursuit C18 column (150 mm $\times$ 4.6 mm i.d., 5 μ ; Varian) preceded by a guard column (MetaGuard Pursuit, 4.6 mm i.d., 5 μ ; Varian). The postcolumn photochemical reaction was carried out in a polytetrafluoroethylene tube (10 m $\times$ 0.5 mm i.d.) that was wound around a black light (Black-Ray model XX-20BLB, 300 to 400 nm with a filter excluding the 254 nm line, 115 V, 60 Hz, 60 A; UVP, Upland, CA). The mobile phase consisted of potassium dihydrogen phosphate (0.07 M), hydrogen peroxide (0.075 M), and copper II sulfate (5.1 μ M). The flow rate was adjusted to 1.5 mL/min and the fluorimetric detector operated at an excitation wavelength of 322 nm and an emission wavelength of 380 nm. Recovery rates for NA and NAM averaged respectively, 93.9 and 102.7% for the duodenal samples and 98.4 and 96.7% for the ileal samples. Interassay coefficients of variation averaged 7.7 and 11.2% for NA and NAM, respectively.

As reported by Ndaw et al. (2002), hydrolysis of the samples in HCl led to partial conversion of NAM into NA. This conversion was observed with standard solutions of NAM, but it is not known if it occurred in the same manner and to the same magnitude in samples. Accordingly, no correction was made to adjust the values, which suggests a slight overestimation of NA concentrations coupled with an underestimation of NAM concentrations for the samples, nevertheless, resulting in a correct evaluation of total niacin.

Vitamin B₆. Vitamin B₆ concentrations were determined according to a method adapted from Matte et al. (1997) and Srivastava and Beutler (1973). Samples were analyzed in duplicate for 3 different forms of vitamin B₆ simultaneously: pyridoxamine (PAM), pyridoxal (PAL), and pyridoxine (PYR); pyridoxal-5-phosphate (P5P) was converted to PAL for analysis. Briefly, 9 mL of sulfuric acid (0.01 M) was added to 0.2 g of sample and autoclaved for 15 min at 121°C. After cooling, the sample was diluted to a final volume of 20 mL with ultrapure water. The samples were then centrifuged for 10 min at $15,142 \times g$. From the supernatant, 1 mL was incubated with 25 μ L of acid phosphatase 2% (phosphatase in ammonium acetate buffer; 50 mM,

pH 4.0) for 18 h in a water bath at 37°C to convert P5P into PAL. The samples were then centrifuged again 10 min at $15,142 \times g$ before being injected into the HPLC, using the same column as for riboflavin analyses. The mobile phase used for vitamin B₆ consisted of 2.4 mL of sulfuric acid per 1000 mL of ultrapure water, and had a flow rate of 1 mL/min. Emission and excitation wavelengths on the fluorimetric detection system were 290 and 395 nm, respectively. Rates of recovery were (respectively for duodenal and ileal samples): 100.6 and 68.7% for PAM; 94.8 and 79.6% for PAL; and 99.1 and 87.3% for PYR. The composition of the ileal samples (color and viscosity) seemed to interfere with analysis. The lower recovery rates for these vitamins suggest slight underestimation of the values reported for the ileal samples in the present experiment. Average conversion rate from P5P into PAL was 86.6%. The lower conversion rate of this vitamin could be due to its high sensitivity to light and other environmental conditions. Interassay coefficients of variation averaged 14.9, 14.5, and 27.1% for PAM, PAL, and PYR, respectively. Concentrations of some forms of this vitamin were close to the level of detection of the method used. Consequently, a coefficient of variation of 20% or less was accepted between duplicates in the same assay.

Biotin. Biotin concentrations were quantified in duplicate by ELISA according to an adaptation of the techniques described by Wellenberg and Banks (1993) and Bayer et al. (1990). Coating of a 96-well plate (flat-bottomed immuno plate, Maxisorp; Nalge Nunc International, Rochester, NY) was done by incubating 100 μ L of biotin-conjugated antibody (antimouse IgG biotin conjugate, antibody developed in goat; Sigma) in each well for 3 h at 37°C. The wells were then rinsed 4 times with 300 μ L of a washing solution (0.05%) made of PBS and Tween 80 in the ratio 2:1. Following rinsing, 300 μ L of BSA was added and the plate was incubated for 1 h at 37°C. The wells were then rinsed again 4 times with the washing solution, and 100 μ L of avidin solution (per plate: 12.0 mL of PBS and 40 μ L of avidin) were added to each well before the final incubation at 23°C for 45 min.

Approximately 0.125 g of sample was dissolved in 25 mL of ultrapure water. Dried pancreas extract (pancreas acetone powder, bovine; Sigma) was added to the samples. This extract contained biotinidase, the only enzyme known to degrade the bound form of the vitamin biocytin (biotin bound to lysine) (Le Grusse and Watier, 1993) and to consequently release free biotin for analysis. Precisely 1000 mg of dried pancreas extract was added; the tubes were mixed carefully and were then incubated in the ultra-sound bath for 15 min and in a water bath at 37°C for 18 h. Following incubation, samples were autoclaved for 10 min at 121°C to stop

all enzymatic activity. After cooling, volumes were diluted to 50 mL with ultrapure water, and tubes were centrifuged for 10 min at $1538 \times g$ and 4°C . Samples were diluted 1:70 in PBS to achieve a final dilution of 1:3500. This dilution rate had been previously tested in our laboratory to be optimal regarding viscosity (conferred by the pancreas extract and interfering with the analyses) and vitamin concentration, to get precise and repeatable values. Each plate also had wells containing only pancreas extract (without sample) to quantify the concentration of biotin in the pancreas, which was then subtracted from the final concentration of biotin per gram for each sample.

A volume of 100 μL of each diluted sample was put in the wells, and the plate was incubated for 45 min at 23°C . The wells were then rinsed 4 times with 300 μL of the washing solution, and 100 μL of peroxidase solution was added to each well (per plate: 12.0 mL of PBS and 4 μL of labeled biotinamidocaporyl peroxidase). Samples were incubated again at 23°C for 30 min and rinsed 4 times as before. A volume of 100 μL of a substrate solution [per plate: 12.25 μL of acetate buffer, 200 μL of 3,3'-5,5'-tetramethylbenzidine solution (6 mg per mL of dimethylsulfoxide), and 50 μL peroxide 10%] was added to each well, and the plate was protected from light for 15 to 30 min, until the coloration was clearly visible but not saturated. The reaction was then stopped with 50 μL of sulfuric acid (1.8 M). The plate was agitated for 8 s before being read at 450 and 650 nm. After subtraction of the 2 values obtained (650 nm – 450 nm), a 4-parameter regression curve was used to quantify the concentrations. Without treatment with pancreas extract, biocytin was not detected by the assay but the rate of conversion of biocytin into biotin following the enzymatic treatment was 91.9%. Less than 8% of the total biotin found in feed and duodenal samples could be detected when no biotinidase treatment was applied to the samples (unpublished data). Recovery rates were 98.8 and 95.1% for total biotin in duodenal and ileal samples, respectively. A coefficient of variation of 10% or less was accepted between samples in a same assay.

Folates. Folate concentrations were measured in duplicate as described by Girard et al. (1994). A sample of 0.1 g of solid was homogenized in a grinder tube with 8 mL of McIlvaine's buffer (per L of buffer: 28.392 g of 0.2 M Na_2HPO_4 and 0.5 g of ascorbic acid dissolved in distilled water, pH adjusted to 4.6 with 1.0 M citric acid, diluted to 1000 mL with distilled water). The homogenate was then transferred to conical polypropylene tubes and autoclaved for 10 min at 121°C . The pH was adjusted to 7 with NaOH (3.3 M), and the volume was diluted to 10 mL with distilled water. The solutions were centrifuged at $1854 \times g$ for 10 min, and the super-

natants were diluted 1:2 with BSA (7%). Folates were then analyzed by radioassay (Quantaphase Folate, BioRad Laboratories, Mississauga, Ontario, Canada). Recovery rate averaged 104.2%. Interassay coefficients of variation were below 10%.

Vitamin B_{12} . Only the biologically active form of vitamin B_{12} (true vitamin B_{12}) was analyzed in the present experiment. Hydrolysis of 0.1 g of sample was done using 24 mL of extractive solution (per L: 13 g of Na_2HPO_4 , 12 g of citric acid, and 10 g of sodium metabisulfite in distilled water). Samples were then autoclaved at 121°C for 10 min. After cooling, NaOH (3.3 M) was added to bring the pH to approximately 6.2 to 6.5, and the final volume was diluted to 30 mL with distilled water. From that solution, 200 μL was used to determine the concentration of the vitamin. Vitamin concentrations were determined using a commercial kit (Quantaphase B_{12} ; BioRad Laboratories Ltd.). Rate of recovery in duodenal and ileal digesta samples averaged respectively, 101.3 and 104.3%. Interassay coefficients of variation were always below 10%.

Chromic oxide. Chromic oxide concentrations were determined by atomic absorption as described by Sidons et al. (1985) with air-acetylene being used instead of N_2O -acetylene for better combustion conditions. A coefficient of variation of 2.5% or less was accepted between duplicates.

Calculations and Statistical Analyses

Daily values of intestinal flows of vitamins for each cow were averaged over the 4 d of the same period, and the mean for each animal was used for further calculations.

Apparent daily ruminal synthesis during the CON periods was calculated as the intake of vitamin in the feed subtracted from the duodenal flow and averaged for the 4 cows.

Disappearance before the duodenal cannula during the TRT periods in both studies was calculated individually for each cow, and the mean of the 4 cows was then reported for each vitamin. It was calculated as the amount of vitamins appearing at the duodenum relative to the quantity given:

$$\text{Disappearance (\%)} = \frac{100 \times ([\text{Duo TRT}] - [\text{Duo CON}])}{\text{Amount of vitamin supplemented (mg/d)}}$$

where [Duo TRT] is the duodenal flow of vitamin during the TRT period (mg/d); and [Duo CON] is the duodenal flow of vitamin during the CON period (mg/d).

Average apparent intestinal disappearance for the 4 cows was calculated as the amount disappearing be-

tween the 2 intestinal cannulas compared with the amount arriving at the duodenum, for both the CON and the TRT periods, in studies 1 and 2:

$$\text{Intestinal disappearance (\%)} = \frac{100 \times ([\text{Duo}] - [\text{Ile}])}{[\text{Duo}]}$$

where [Duo] is the duodenal flow of vitamin (mg/d); and [Ile] is the ileal flow of vitamin (mg/d).

A paired *t*-test was performed to compare the mean intestinal disappearance rates between the CON and TRT periods of each study, to verify if supplementation of vitamins negatively affected their apparent absorption. Means were assumed to be different from each other at $P \leq 0.05$ and tend to differ at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Although the design used results in complete confounding between period and treatment, this design was chosen preferentially to a crossover design to overcome the problems related to the major role played by the enterohepatic cycle in the folate and vitamin B₁₂ metabolism, and to the fact that concentrations of those vitamins increased in bile with vitamin intake (Steinberg et al., 1979; Schneider, 1987; Le Grusse and Watier, 1993). To prevent a period effect between the 2 periods of one study, the amount of feed offered to the cows was restricted to 95% of their ad libitum intake and was maintained throughout the project.

Average duodenal and ileal daily flows of the different vitamins are reported in Table 3. Large variation was noted between animals. However, our data suggest a change in intestinal flows in response to supplementation for most of the vitamins studied, as well as substantial amounts apparently disappearing from the small intestine. Average intestinal flows of vitamins reported in the present experiment are similar to the values presented by Schwab et al. (2004a,b), obtained from lactating dairy cows fed different forage and nonfiber carbohydrate concentrations.

Apparent Ruminal Synthesis

Average amounts of vitamins being produced daily in the rumen of the 4 cows for studies 1 and 2 are reported in Table 4. Estimates of ruminal synthesis obtained in the present experiment do not account for destruction or use in the rumen, nor for absorption, which could take place through the walls of the 4 stomach compartments or at the level of the duodenum, before the cannula. The values reported probably un-

derestimate the net amount of vitamins being produced by ruminal bacteria.

Duodenal flows of most vitamins were higher than intake levels, suggesting that ruminal production was occurring. Vitamin B₁₂, which is synthesized solely by microorganisms and only under adequate cobalt supply (McDowell, 2000), showed the greatest increase in flow relative to the amount consumed daily. Biotin, according to our results, does not seem to be synthesized in the rumen. However, this vitamin might be destroyed or used at the same rate as it is produced, suggesting no apparent synthesis, as reported by Frigg et al. (1993a).

Daily ruminal synthesis of niacin was more than 2.2 g in the present experiment. Niacin exerts its major functions in its coenzyme forms (NAD and NADP), which are especially important in reactions that provide energy to the animal (McDowell, 2000). Results from the present study indicate that both NA and NAM were synthesized in substantial amounts in the rumen of dairy cattle, which is in opposition to results from Campbell et al. (1994). These authors reported that only NA could be detected in ruminal and duodenal fluids, but they did not measure niacin concentrations in total ruminal or duodenal contents (which also includes feed particles and bacterial cells). It is therefore likely that both forms of niacin were synthesized in the rumen, but that most of NA and all NAM was present within the bacterial cells.

The negative value obtained for PYR is mainly due to the conversion of this form of the vitamin into PAM and PAL. Pyridoxine, the predominant form in plants (McDowell, 2000), represented almost 75% of total vitamin B₆ in the feed, whereas it accounted for approximately 20% of the vitamin at the duodenal level. Accordingly, PAM and PAL (the forms usually found in animal products) represented, respectively, 8 and 18% in the feed, and increased to proportions around 40 and 35% of the duodenal flow of vitamin B₆ during the control periods. The slightly negative value for total vitamin B₆ suggests that no net synthesis of this vitamin occurred, and that the increases in PAM and PAL were solely due to interconversions between the forms of the vitamin. Our results indicate that most of this conversion took place in the rumen, since daily flows of the other forms of vitamin B₆ were not altered by the post-ruminal infusion of PYR (study 2).

Disappearance Before the Duodenum

Study 1. Average rates of disappearance of dietary supplemented vitamins before the small intestine are reported in Table 5. As indicated by these results, substantial proportions of the supplemented vitamins were destroyed or used before the duodenum. Riboflavin, nia-

Table 3. Intestinal flows of B-vitamins in studies 1 and 2 (mg/d).^{1,2}

	Study 1		Study 2	
	CON	TRT	CON	TRT
Thiamin				
Duodenal flow	66 ± 3	163 ± 30	68 ± 7	78 ± 9
Ileal flow	16 ± 5	85 ± 27	19 ± 6	25 ± 4
Riboflavin				
Duodenal flow	361 ± 29	371 ± 27	375 ± 26	380 ± 24
Ileal flow	233 ± 12	244 ± 27	239 ± 27	237 ± 16
Niacin ³				
Duodenal flow	2777 ± 416	2724 ± 365	2578 ± 249	2946 ± 279
Ileal flow	418 ± 25	410 ± 23	416 ± 78	422 ± 60
Nicotinic acid (NA)				
Duodenal flow	1420 ± 257	1259 ± 182	1334 ± 125	1815 ± 114
Ileal flow	343 ± 28	324 ± 25	361 ± 50	355 ± 55
Nicotinamide (NAM)				
Duodenal flow	1276 ± 198	1458 ± 187	1242 ± 182	1140 ± 116
Ileal flow	77 ± 12	83 ± 3	84 ± 23	64 ± 17
Vitamin B ₆ ⁴				
Duodenal flow	150 ± 11	656 ± 73	153 ± 19	171 ± 18
Ileal flow	47 ± 6	99 ± 14	43 ± 6	46 ± 4
Pyridoxamine (PAM)				
Duodenal flow	66 ± 6	77 ± 9	60 ± 7	62 ± 6
Ileal flow	8 ± 2	13 ± 3	6 ± 1	7 ± 1
Pyridoxal (PAL)				
Duodenal flow	48 ± 3	70 ± 9	56 ± 12	47 ± 6
Ileal flow	22 ± 2	28 ± 3	22 ± 3	19 ± 1
Pyridoxine (PYR)				
Duodenal flow	31 ± 5	503 ± 76	26 ± 3	65 ± 11
Ileal flow	20 ± 6	60 ± 8	13 ± 3	18 ± 3
Biotin ⁵				
Duodenal flow	120 ± 6	136 ± 9	152 ± 12	132 ± 13
Ileal flow	90 ± 8	85 ± 11	81 ± 6	83 ± 7
Folates				
Duodenal flow	27 ± 2	106 ± 2	28 ± 2	111 ± 7
Ileal flow	40 ± 3	102 ± 16	38 ± 3	102 ± 11
Vitamin B ₁₂				
Duodenal flow	75 ± 5	261 ± 38	70 ± 6	72 ± 5
Ileal flow	67 ± 2	223 ± 35	62 ± 6	59 ± 4

¹Mean ± SE. CON = No vitamin supplementation; TRT = with vitamin supplementation in feed (study 1) or infused in duodenum (study 2).

²Mean intake on basal ration (mg/d): thiamin: 41 ± 1; riboflavin: 101 ± 3; niacin: 465 ± 16; NA 465 ± 16; NAM 0; vitamin B₆: 166 ± 6; PAM: 13 ± 0.4; PAL: 30 ± 1; PYR: 123 ± 4; biotin: 138 ± 5; folates: 7 ± 0.2; vitamin B₁₂: 0.2 ± 0.01.

³Sum of the molar concentrations of NA and NAM; expressed as NA-equivalents.

⁴Sum of the molar concentrations of PAM, PAL, and PYR; expressed as PYR-equivalents.

⁵Means calculated on 3 cows for the TRT period of study 1.

cin, and folates almost completely disappeared before the duodenal cannula (99.3, 98.5, and 97.0%, respectively), whereas less than half of the supplemented dose of vitamin B₆ and biotin was destroyed or used (41.0 and 45.2% respectively). Disappearances of thiamin and vitamin B₁₂ were intermediate (67.8 and 62.9%, respectively). Zinn et al. (1987) had previously reported extensive disappearance of supplementary B-vitamins. These authors reported similar rates of ruminal disappearance for riboflavin (98.8%), niacin (93.8%), and folates (97.3%) than the results presented here. However, they observed lower ruminal disappearance rate for thiamin (47.7%), and higher values for vitamin B₁₂ (90%). In addition, these authors suggested that vita-

min B₆ and biotin were not destroyed in the rumen (respectively, 101.0 and 132.5% of the supplementary doses escaping the rumen). The animals used by Zinn et al. (1987) were steer calves with lower intake levels and fed a diet richer in concentrates than the cows used in our experiment, which probably influenced bacterial population as well as transit time, which in turn could affect ruminal use (and synthesis) of B-vitamins. Other authors reporting ruminal disappearance of supplementary B-vitamins obtained values slightly different from ours. Frigg et al. (1993b) noted no significant ruminal degradation of biotin in Brown Swiss heifers, and Smith and Marston (1970) estimated that in sheep, approximately 80% of oral vitamin B₁₂ disappeared be-

Table 4. Apparent ruminal synthesis of B-vitamins during the control periods (average of studies 1 and 2).

Vitamins	Apparent ruminal synthesis (mg/d)	SE
Thiamin	26	3
Riboflavin	267	17
Niacin ¹	2213	224
Nicotinic acid (NA)	912	132
Nicotinamide (NAM)	1259	125
Vitamin B ₆ ²	-14	7
Pyridoxamine (PAM)	51	4
Pyridoxal (PAL)	24	6
Pyridoxine (PYR)	-94	6
Biotin	-1	9
Folates	21	1
Vitamin B ₁₂	73	4

¹Sum of the molar concentrations of NA and NAM; expressed as NA-equivalents.

²Sum of the molar concentrations of PAM, PAL, and PYR; expressed as PYR-equivalents.

fore the small intestine. These values are closer to those of Zinn et al. (1987) than ours are, but the different methods of analysis used may explain part of the discrepancy between the levels of vitamins detected. Microbiological assays are less specific than the chemical methods used in the present experiment and may not take into consideration the different forms of the vitamins and the analogues that may be present.

In the present experiment, preintestinal disappearance rates could have been slightly overestimated, and apparent ruminal synthesis was probably underestimated, because absorption through the 4 stomach compartments or in the duodenum before the cannula cannot be excluded.

Using isotope labels, Smith and Marston (1970) demonstrated that neither thiamin nor B₁₂ was absorbed in appreciable amounts through the ruminal wall. Similarly, results from Rérat et al. (1958a,b; 1959) indicated that, although the rumen wall is permeable to free vitamins, no significant absorption takes place in normally

fed animals. The lack of absorption in the rumen is likely because most of the vitamins are present in the bacterial fraction of ruminal content, and therefore not available in their free form. However, an accumulation of vitamins in the ruminal wall has been reported, making the vitamins available for later release in the blood or the rumen cavity (Rérat et al., 1958b; Smith and Marston, 1970).

Although probably minimal in the present experiment, absorption through the ruminal wall cannot be totally excluded, because levels of free vitamins were considerably increased during the dietary supplementation period. This is the case mainly for NAM, of which 12 g was supplemented daily. Erickson et al. (1991) observed that NAM was absorbed in the rumen at a rate of 0.98 g/h, whereas NA did not seem to be absorbed over a 1-h period. Because almost no niacin escaped the rumen in the present experiment, it may be suggested that the numerous beneficial effects reported for supplementary niacin may be either due to its roles in the rumen, or simply because the supplemented vitamin reaches the circulation through diffusion across the gastrointestinal wall before the duodenal cannula. Rérat et al. (1958b) showed that thiamin, riboflavin, niacin, pantothenic acid, and vitamin B₁₂ could be absorbed through the rumen wall when large quantities of these vitamins are present in their free form. Accordingly, Girard et al. (2001) and Girard and Rémond (2003) noted that although no net positive flows were found under normal conditions, folates, and vitamin B₁₂ were able to reach the blood circulation through the rumen wall of normally fed cows and sheep following an infusion of high doses of these vitamins. However, the efficiency of this process is very low.

Study 2. Study 2 was designed to verify the hypothesis that disappearance of dietary vitamins before the small intestine takes place in the rumen, and that digestion and absorption following the forestomach is similar in ruminant and nonruminant animals. Accordingly, only negligible amounts of infused vitamins were lost before the duodenum in this study, except for niacin and folic acid. Duodenal flows of NAM were not increased following the postruminal infusion of this form of niacin. However, the infusion of NAM resulted in an increase of NA [average increase for the 4 cows (mean $\pm$ SE): 481 $\pm$ 100 mg/d]. Conversion of the amide into the acid form has already been reported in extraction methods involving acids (Ndaw et al., 2002). This conversion could also take place in the acidic environment of the abomasum of dairy cows. Accordingly, Campbell et al. (1994) reported increased duodenal NA concentrations for cows supplemented with NAM (12 g/d). However, these authors only considered duodenal fluid, and

Table 5. Disappearance rates of B-vitamins before the duodenal cannula following dietary supplementation in study 1.

Vitamins	Ruminal disappearance (%)	SE
Thiamin	67.8	8.9
Riboflavin	99.3	1.7
Niacin (as nicotinamide)	98.5	1.8
Vitamin B ₆ (as pyridoxine)	41.0	8.9
Biotin ¹	45.2	37.7
Folic acid	97.0	0.5
Vitamin B ₁₂	62.9	7.5

¹Mean calculated on 3 cows.

Table 6. Apparent intestinal disappearance during the CON and TRT periods for studies 1 and 2.^{1,2}

Vitamins	Study 1 Intestinal disappearance (%)		<i>P</i>	Study 2 Intestinal disappearance (%)		<i>P</i>
	CON	TRT		CON	TRT	
Thiamin	77 ± 7	55 ± 13	0.09	73 ± 6	67 ± 5	0.47
Riboflavin	35 ± 3	36 ± 4	0.88	37 ± 4	37 ± 4	0.79
Niacin ³	84 ± 4	84 ± 2	0.87	84 ± 2	85 ± 3	0.76
Nicotinic acid (NA)	73 ± 6	73 ± 4	0.99	73 ± 4	80 ± 4	0.18
Nicotinamide (NAM)	94 ± 2	94 ± 1	0.68	94 ± 1	94 ± 1	0.63
Vitamin B ₆ ⁴	69 ± 4	85 ± 2	0.02	72 ± 4	73 ± 1	0.60
Pyridoxamine (PAM)	89 ± 3	83 ± 2	0.08	90 ± 2	88 ± 2	0.15
Pyridoxal (PAL)	55 ± 5	60 ± 3	0.46	59 ± 7	57 ± 4	0.66
Pyridoxine (PYR)	46 ± 12	88 ± 2	0.09	50 ± 14	72 ± 2	0.23
Biotin ⁵	28 ± 9	37 ± 5	0.38	46 ± 6	36 ± 6	0.07
Folates	Neg ⁶	10 ± 5	—	Neg	16 ± 5	—
Vitamin B ₁₂	11 ± 4	15 ± 4	0.40	11 ± 2	16 ± 7	0.55

¹Mean ± SE. CON = No vitamin supplementation; TRT = with vitamin supplementation in feed (study 1) or infused in duodenum (study 2).

²Percentage of duodenal flow.

³Sum of the molar concentrations of NA and NAM; expressed as NA-equivalents.

⁴Sum of the molar concentrations of PAM, PAL, and PYR; expressed as PYR-equivalents.

⁵Means calculated on 3 cows for study 1.

⁶Neg = Negative value.

therefore did not account for niacin, which could have been found in the solid fraction of intestinal content.

Between 18 and 36% (mean ± SE: 25 ± 4) of the infused dose of folic acid was not recovered at the duodenum. Folate absorption takes place in the proximal duodenum (Le Grusse and Watier, 1993; McDowell, 2000), and it is likely that some absorption may have occurred before the duodenal cannula. This would imply that the rate of ruminal disappearance of folic acid was overestimated, and that a proportion of the supplemented vitamin was not used or destroyed in the rumen, but rather absorbed before the duodenal cannula. These results further suggest that ruminal synthesis of folates may be underestimated, because although the vitamin is bound to other material in the rumen, it is released upon passage through the abomasum, and could therefore be available for absorption early in the small intestine.

Although duodenal flows of most vitamins were increased by the postruminal infusions, the levels of riboflavin, biotin, and vitamin B₁₂ infused were too small to induce a change in concentrations in the duodenal flows of these vitamins.

Apparent Intestinal Disappearance Rate

Average apparent intestinal disappearance rates for the 4 cows are reported in Table 6. Apparent absorption of thiamin and PAM in study 1 tended to be reduced ($P \leq 0.09$) during the treatment periods. For all other

vitamins, the treatment had either a positive or no significant effect on apparent intestinal absorption.

Thiamin. Average intestinal disappearance rates obtained for thiamin in the present experiment are similar to those reported by Zinn et al. (1987) and Miller et al. (1986), which were both approximately 75%. Although the proportion of this vitamin disappearing from the small intestine seemed to be reduced during the TRT periods, especially in study 1, absolute amounts being absorbed by the cow were higher than in the CON periods.

Riboflavin. Because less than 1% of the supplemented dose of riboflavin reached the small intestine, absolute amounts of this vitamin disappearing between the 2 intestinal cannulas were not increased in the TRT period in study 1. Moreover, amounts infused in study 2 were not sufficient to increase duodenal flows of riboflavin. Previous studies reported slightly lower intestinal disappearance rates than the values obtained in the present study, ranging from 25 to 28% of the amount of vitamin arriving at the duodenum (Miller et al., 1986; Zinn et al., 1987). However, these estimations were calculated from studies with steers with lower intake levels than the animals used in the present experiment.

Niacin. Apparent intestinal absorption of NA and NAM were 73 and 94%, respectively, and averaged 84% of duodenal flows of total niacin. These values for total niacin agree well with those reported by Zinn et al. (1987) and Miller et al. (1986) for beef cattle (79 and 70% of duodenal levels, respectively). In study 1, apparent

absorption rates for the CON and TRT periods were identical due to the substantial disappearance of this vitamin before the small intestine. The slightly increased absorption rate observed for NA in the treatment period of study 2 (80%) can be attributed to the conversion of NAM into NA following postruminal infusion, as discussed earlier.

Vitamin B₆. The slightly reduced apparent absorption rate for PAM did not affect total vitamin B₆ absorption, which was significantly increased by the dietary supplementation in study 1 ($P = 0.02$). Absorption of vitamin B₆ mainly occurs in one of the dephosphorylated forms; PAM, PAL, and PYR having equal activities in animals and being interconvertible in the tissues (McDowell, 2000). This suggests that the sum of the forms of vitamin B₆ being taken up by the intestine is more important for the dairy cow than the proportions of the different forms. Intestinal disappearance rates for vitamin B₆ are close to the estimation of 79% obtained with growing steers (Zinn et al., 1987).

Biotin. Biotin apparent absorption rates during the CON periods are slightly lower than those reported by Frigg et al. (1993a), who estimated that 50 to 60% of dietary biotin was taken up by the small intestine. However, these authors mentioned that their analysis may not be specific, and could therefore have included biotin analogues. Concerning biotin in the TRT period of study 2, a greater quantity of this vitamin should have been infused to the animals to clearly see the effects of the postruminal infusion. However, our results clearly showed that absorption of this vitamin was occurring in the small intestine of dairy cows, unlike what was suggested for steers by Miller et al. (1986). The intestinal synthesis reported by these authors is probably due to the method of analysis used. In the upper digestive tract, most of biotin is bound to proteins, and only a small proportion is in the free form. Proteins are hydrolyzed under the action of proteases and acid in the stomach (or abomasum), and biotin is then released in the form of biocytin, which consists of the vitamin bound to lysine. Normal stomach proteases cannot degrade biocytin. This biotin-lysine bond can only be degraded by a very specific enzyme (biotinidase), which is found in the pancreatic secretions and brush-border membrane, releasing biotin in its free form (Le Grusse and Watier, 1993). Miller et al. (1986) reported no biotinidase treatment of their samples and therefore probably only analyzed free biotin, which resulted in a significant underestimation of the duodenal levels of this vitamin. Due to this underestimation of the duodenal flows of biotin, their results suggested that synthesis of this vitamin was occurring in the small intestine. Similar results had been observed in our laboratory before the use of dried pancreas extract containing biotinidase for

the extraction of duodenal samples: biotin concentrations in duodenal samples were lower than in ileal samples, suggesting intestinal synthesis. With the addition of pancreas extract, duodenal levels of biotin increased several fold, but no effect was noted on ileal samples (unpublished results), revealing that most of duodenal biotin was in the form of biocytin whereas ileal biotin was mainly free vitamin. In fact, under the action of endogenous biotinidase in the small intestine, most biotin present in the ileal samples had been converted to free biotin, and could therefore be measured. Analysis of total biotin therefore revealed that absorption rather than synthesis was occurring in the small intestine, as for the other B-vitamins.

Folates. Results from the present experiment suggest that supplementary folates are extensively destroyed or used before they reach the small intestine (study 1) and that some absorption could occur before the duodenal cannula (study 2). In addition, folic acid absorption during the control periods was negative, with concentrations being higher at the ileal than the duodenal level. Intestinal absorption of folic acid has been reported to take place in the proximal duodenum and jejunum (Le Grusse and Watier, 1993; McDowell, 2000). Girard et al. (2001) reported rapid appearance of folates in the portal blood following ingestion of 2.6 g of folic acid. In their study, 108.7 mg of folates reached the portal blood within 6 h after supplementation. This quantity represented 98% of the total amount of folates being absorbed in 24 h. In addition, these authors noted that folates could be measured in the portal circulation as soon as 30 min after ingestion of a dietary supplement. From our results, approximately 10 to 16% of supplemented folic acid reaching the duodenum was absorbed between the 2 cannulas for the TRT periods. This value, relatively small compared with apparent absorption rates of most of the other vitamins, is probably underestimated, because compounds recycled through the enterohepatic cycle are released between the 2 cannulas, therefore increasing ileal concentrations. Folic acid is known to be extensively recycled through this cycle. Following absorption early in the intestine and liver uptake, methylated folates are rapidly excreted into bile, whereas nonmethylated forms are either methylated and added to the bile or included in the hepatic pool. Bile folates are then reabsorbed in the small intestine and available for distribution to the liver or the tissues (Steinberg et al., 1979; Steinberg, 1984; Le Grusse and Watier, 1993). Using labeled folates, Steinberg et al. (1979) demonstrated that approximately 30% was taken up by tissues and then returned to the liver for remethylation and recirculation through the bile and small intestine, proving the importance of

the enterohepatic cycle even once folate has reached the tissues.

Vitamin B₁₂. At normal physiological doses, vitamin B₁₂ is absorbed through an extremely specific mechanism in the distal portion of the ileum. If very high doses of this vitamin are provided to the animal, approximately 1% can be absorbed by passive diffusion along the whole length of the small intestine (Le Grusse and Watier, 1993). In sheep, Smith and Marston (1970) reported that approximately 5% of ruminally produced B₁₂ was absorbed in the small intestine, whereas Sutton and Elliot (1972) found that between 1 and 35% of duodenal B₁₂ disappeared between the 2 intestinal cannulas. These authors also suggested that considerable amounts of analogs could be absorbed. In the present experiment, analogs were not measured, and true vitamin B₁₂ absorption rates were 11% during the CON periods and 15 to 16% during the TRT periods. These values are lower than the ones reported by Zinn et al. (1987), which averaged 48%. Here again, the differences in the animals and methods of analysis used may explain the discrepancy between our results and the literature.

CONCLUSION

Results from the present studies indicate that some dietary supplemented B-vitamins are extensively destroyed or used before the small intestine. Postruminal infusions proved that most of this disappearance was occurring in the rumen, except for niacin and folic acid. A considerable proportion of folates seemed to be absorbed in the proximal duodenum before the cannula, whereas it appears that niacin was converted to other forms or apparently absorbed through the walls of the gastrointestinal tract. Although net values of ruminal use and intestinal absorption of B-vitamins could not be obtained from the present studies, supplementation clearly lead to an increased supply of vitamins at the duodenal level and to greater amounts apparently disappearing from the small intestine, except for riboflavin and niacin. The results obtained in the present experiment are essential for further studies looking at the effects of B-vitamin supplementation on production or metabolic parameters in dairy cows.

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