Apparent ruminal synthesis and intestinal disappearance of vitamin B₁₂ and its analogs in dairy cows¹

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ABSTRACT

The aim of the project was to calculate the apparent synthesis or destruction of cobalamin (vitamin B₁₂) and its analogs in the rumen as well as their apparent intestinal disappearance in dairy cows. Four lactating cows were fed a diet supplemented with cobalt alone (0.76 mg/kg of DM; control) or with cobalt and vitamin B₁₂ (cyanocobalamin, 500 mg/d; treated). In addition to cobalamin, the only biologically active molecule for the cow, 7 analogs were identified in duodenal and ileal digesta: cobinamide, which lacks the base, ribose, and phosphate groups; and 6 other molecules in which the base, 5,6-dimethylbenzimidazole, is replaced by cresol, 2-CH₃-adenine, adenine, 2-CH₃-S-adenine, or 5-OH-benzimidazole, or an unidentified cobamine. Small amounts of cobalamin and cobinamide were detected in the total mixed ration, but apparent synthesis of all forms took place in rumen. During the control period, cobalamin represented 38% of the total amounts of corrinoids produced in rumen. Approximately 11% of the average daily intake of cobalt was used for apparent ruminal synthesis of corrinoids, of which only 4% was incorporated into cobalamin. Only 20% of the supplement of cyanocobalamin was recovered at the duodenal level; cobinamide appeared to be the major product of degradation of supplementary cyanocobalamin in the rumen. During the control and treatment periods, there was an apparent intestinal disappearance of cobalamin and 5-OH-benzimidazole cobamide only; only the apparent intestinal disappearance of cobalamin differed between the 2 periods. Although cobalamin was not the major form synthesized by ruminal microflora and, even if supplementary cyanocobalamin was extensively destroyed by ruminal microflora, based on calculations of apparent intestinal disappearance, cobalamin seems to be the major form absorbed in the small intestine.

Key words: dairy cow, vitamin B₁₂, ruminal synthesis, intestinal absorption

INTRODUCTION

Unlike other B-complex vitamins, vitamin B₁₂ (cobalamin; **CBL**) is synthesized almost exclusively by bacteria and is therefore present only in foods that have been bacterially fermented or are derived from animals that have obtained this vitamin from their gastrointestinal microflora or their diet (Combs, 1998). As one atom of cobalt is part of the molecule of CBL, it is generally assumed that ruminant requirements for the vitamin equate with ruminal bacteria requirements for cobalt (McDowell, 2000). However, ruminal microflora also use dietary cobalt to produce different molecules, called analogs, closely related to CBL but without biological activity for the host (Ford et al., 1953; Dawbarn et al., 1957; Gawthorne, 1970; Dryden and Hartman, 1971). Increasing dietary cobalt supply increases production of these analogs in rumen at the expense of the biologically active forms of the vitamin (Kawashima et al., 1997). The biological importance of these analogs for the host animal has not been clearly elucidated. Most analogs are inactive for the host, but a few studies indicate that some analogs could have deleterious effects. Growth and development of chickens and embryos are impaired by CBL analogs (Coates et al., 1960). In vitro, some analogs have a higher affinity for the enzyme methylmalonyl CoA mutase prepared from sheep kidney than for CBL itself (Lengyel et al., 1960).

Recent studies showed that dairy cows in early lactation could benefit from an increased supply of CBL, even when the dietary supply in cobalt is adequate. In primiparous cows, intramuscular injections of CBL increased concentrations of the vitamin in milk and milk yields of solids, fat, and lactose compared with cows fed only supplementary folic acid (Girard and Matte, 2005). Intramuscular injections of CBL also increased blood hemoglobin and decreased serum methylmalonic acid (Girard and Matte, 2005). In multiparous cows, oral or parenteral combined supplements of folic acid and CBL given in early lactation increased milk and milk component yields by improving efficiency of energy metabolism (Graulet et al., 2007; Preynat et al., 2009). However, increasing the dietary supply of cobalt

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Table 1. Ingredients and chemical composition of the basal diet

Item	Quantity
DM (%)	40.0
Ingredients (% DM basis)	
Grass-legume silage	43.6
Corn silage	14.7
High-moisture corn	33.6
Soybean meal	0.8
Corn gluten meal	1.1
Extruded soybean	1.0
Wheat distillers grain	1.0
Canola meal	1.0
Corn distillers grain	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	1.2
NDICP ³	2.3
ADF	20.0
NDF	28.2
Nonstructural carbohydrates	44.3
Fat	2.6
Ash	7.2
Total dietary Co (mg/kg of DM)	2.5

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

²ADICP = acid detergent insoluble CP.

³NDICP = neutral detergent insoluble CP.

from 0.19 to 0.93 mg/kg of DM from parturition to 120 d of lactation had no effect on plasma concentrations of vitamin B₁₂ or on milk production and milk component yields (Kincaid and Socha, 2007).

Therefore, in a first step to gain understanding on factors affecting vitamin B₁₂ supply to the dairy cow, duodenal and ileal digesta samples collected from lactating cows fed a diet supplemented with cobalt alone or with cobalt and CBL during another experiment (Santschi et al., 2005) were used to identify corrinoids present in the gastrointestinal tract. Apparent synthesis or destruction of each corrinoid in rumen and their apparent intestinal disappearance were calculated.

MATERIALS AND METHODS

The experimental procedures of this study were approved by the Institutional Animal Care Committee of the Dairy and Swine Research and Development Centre, Sherbrooke, Quebec, and followed the guidelines of the Canadian Council on Animal Care (1993).

Animals and Diets

Four multiparous lactating Holstein cows (average milk production: 27.7 ± 1.4 kg/d) equipped with can-

nulas in the rumen, the proximal duodenum (approximately 30 cm from the pylorus), and the distal ileum (approximately 60 cm from the ileo-cecal valve) were used. The cows were fed 12 times daily a TMR containing chromic oxide as digesta passage marker (Table 1). Chromic oxide was included in the protein supplement pellets. Cobalt as cobalt carbonate was added at the level of 0.76 mg of Co/kg of DM. 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 periods (average DMI: 19.8 ± 0.5 kg/d). The cows were milked twice daily at 0900 and 2100 h. Feed intake and milk production were recorded daily. Feed samples were taken weekly and frozen at -20°C for later analyses of chemical composition and corrinoid concentrations.

Treatments and Collection of Samples

The experiment consisted of a control period (no vitamin supplementation) followed by the treatment period. During the latter period, the cows received a daily dietary supplement of 500 mg of vitamin B₁₂ (cyanocobalamin; Hoffmann-LaRoche, Cambridge, Ontario, Canada) as previously used by Girard et al. (2001). The duration of each period was 9 d. Cobalamin supplementation took place for 5 d before and during the 4 d of collection in the treatment period.

Duodenal and ileal samples (approximately 350 mL) were simultaneously collected twice daily over 4 consecutive days in both 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).

Laboratory Analyses

Digesta samples were frozen at -20°C until being freeze-dried and ground through a 1-mm screen. The samples were then pooled per cow and collection site. Analyses of corrinoids (CBL and its analogs) and chromic oxide concentrations were performed on the pooled digesta samples and on the feed. Chromic oxide concentrations were determined by atomic absorption as described by Siddons et al. (1985) with air-acetylene being used for better combustion conditions instead of N₂O-acetylene. A coefficient of variation of 2.5% or less was accepted between duplicates. Sample preparation and analysis of corrinoids by liquid chromatography-mass spectrometry were performed as described by Allen and Stabler (2008). Stable isotope-labeled standards for CBL, the biologically active form in mammals, were used: cobinamide (**COB**), a corrin ring without the base, ribose, and phosphate groups; or substitution of 5,

Table 2. Concentrations in TMR and duodenal and ileal fluxes of corrinoids in lactating dairy cows fed (treated) or not fed (control) a supplement of 500 mg/d of cyanocobalamin

Item	Corrinoid ¹							
	CBL	COB	CRE	MADE	ADE	MSADE	OHBZA	UN
TMR ² (µg/kg of DM)	6.5 ± 0.7	7.0 ± 0	ND ³	ND	ND	ND	ND	ND
Duodenal flux (mg/d)								
Control	49.8 ± 6.4	1.9 ± 0.6	21.9 ± 7.3	32.1 ± 4.5	5.0 ± 1.2	1.6 ± 0.2	14.3 ± 1.0	2.8 ± 0.5
Treated	151.5 ± 26.6	185.4 ± 67.3	20.0 ± 3.9	36.5 ± 8.9	6.8 ± 2.2	1.7 ± 0.3	13.7 ± 0.9	2.9 ± 1.3
<i>P</i> -value	0.002	0.006	0.36	0.14	0.11	0.10	0.25	0.44
Ileal flux (mg/d)								
Control	27.4 ± 6.3	5.1 ± 2.5	19.7 ± 8.5	27.2 ± 6.1	6.7 ± 1.0	1.7 ± 0.6	9.1 ± 1.8	2.0 ± 0.4
Treated	114.4 ± 22.2	177.1 ± 80.5	17.9 ± 2.1	33.0 ± 10.9	8.3 ± 2.4	2.1 ± 0.2	9.8 ± 1.4	2.0 ± 1.4
<i>P</i> -value	0.001	0.01	0.35	0.26	0.20	0.16	0.30	0.49

¹CBL = cobalamine, biologically active form in mammals; COB = cobinamide, a corrin ring without the base, ribose, and phosphate groups; cobamides with substitution of 5, 6-dimethyl benzimidazole by: CRE = cresol; MADE = 2-CH₃-adenine; ADE = adenine; MSADE = 2-CH₃-S-adenine; OHBZA = 5-OH-benzimidazole; and UN = unidentified cobamine. Data are presented as mean values with their standard deviations for 4 cows per group.

²Concentrations in the basal diet (n = 8). During the treatment period, the cows received a dietary supplement of 500 mg of cyanocobalamin (CBL).

³ND = not detected.

6-dimethylbenzimidazole by adenine (**ADE**); benzimidazole (**BZA**); cresol (**CRE**); 2-CH₃-adenine (**MADE**); 5-CH₃-benzimidazole (**MBZA**); 5-CH₃O-benzimidazole (**MOBZA**); 5-CH₃O, 6-CH₃-benzimidazole (**MOMBZA**); 2-CH₃-S-adenine (**MSADE**); naphthimidazole (**NZA**); 5-OH-benzimidazole (**OHBZA**); and phenol (**PHE**). All data are presented as cyano-CBL equivalents.

Calculations and Statistical Analyses

Although the design used (control period followed by the treatment period) results in a complete confounding between periods and treatments, this design was chosen preferentially to a crossover design to overcome the problems related to the substantial enterohepatic recycling of CBL, biliary excretion of CBL increasing with its intake (Combs, 1998; Schneider and Stroinski, 1987).

During the control period, apparent daily ruminal synthesis was calculated as the intake of corrinoids in the feed subtracted from the duodenal flow. During the treatment period, apparent daily ruminal synthesis/destruction was calculated as the intake of corrinoids in the feed plus CBL supplement subtracted from the duodenal flow. Apparent intestinal disappearance was calculated as the difference between duodenal and ileal flows for each corrinoid (Santschi et al., 2005).

A *t*-test was performed to determine if apparent ruminal synthesis/destruction and apparent intestinal disappearance differ from zero for each corrinoid. A paired *t*-test was also performed to compare apparent ruminal synthesis/destruction and intestinal disappearance between the control and treatment periods. Means were assumed to be different from each other at $P \leq 0.05$.

RESULTS

Small amounts of CBL and COB were detected in the TMR (Table 2). In addition to CBL, the biologically active form of the vitamin, 7 CBL analogs were detected in duodenal and ileal digesta of dairy cows: COB, CRE, MADE, ADE, MSADE, OHBZA, and an unidentified cobamine.

The duodenal and ileal fluxes of CBL were 3- and 4-fold higher, respectively, following ingestion of supplementary cyanocobalamin ($P \leq 0.001$; Table 2). The only analog to increase with supplementary CBL was COB ($P \leq 0.01$); it increased massively as shown in Table 2.

Cobalamin and its 7 analogs were synthesized by ruminal microflora (Table 3). However, only apparent ruminal synthesis or destruction of CBL and COB differed between the control and treatment periods ($P \leq 0.006$).

During the control and treatment periods, apparent intestinal disappearance of CBL ($P = 0.01$ and $P = 0.001$ for control and treatment periods, respectively) and OHBZA ($P = 0.03$ and $P = 0.002$ for control and treatment periods, respectively) differed from zero. There was no apparent intestinal disappearance of the other analogs during the 2 periods (means different from zero, $P \geq 0.1$). Only the apparent intestinal disappearance of CBL was increased during the treatment period compared with the control one (Table 3; $P = 0.05$).

DISCUSSION

Concentrations of vitamin B₁₂ measured with a commercial radioassay developed for human plasma and using purified hog intrinsic factor as binding protein in the same duodenal and ileal digesta samples as those used

Table 3. Apparent ruminal synthesis/destruction and apparent intestinal disappearance of corrinoids in lactating dairy cows fed (treated) or not fed (control) a supplement of 500 mg/d of cyanocobalamin

Item	Corrinoid ¹							
	CBL	COB	CRE	MADE	ADE	MSADE	OHBZA	UN
Apparent ruminal synthesis/destruction (mg/d)								
Control	49.7 ± 6.4	1.8 ± 0.6	21.9 ± 7.3	32.1 ± 4.5	5.0 ± 1.2	1.6 ± 0.2	14.3 ± 1.0	2.8 ± 0.5
Treated	-348.6 ± 26.6	185.2 ± 67.3	20.0 ± 3.9	36.5 ± 8.9	6.8 ± 2.2	1.7 ± 0.3	13.7 ± 0.9	2.9 ± 1.3
P-value	0.0001	0.006	0.36	0.14	0.11	0.10	0.25	0.44
Apparent intestinal disappearance (mg/d)								
Control	22.5 ± 7.6	-3.2 ± 1.9	2.2 ± 5.2	4.9 ± 10.2	-1.7 ± 1.9	-0.1 ± 0.5	5.2 ± 2.7	0.8 ± 0.8
Treated	37.1 ± 6.2	8.3 ± 23.0	2.2 ± 1.9	3.6 ± 5.2	-1.5 ± 2.4	-0.3 ± 0.3	3.9 ± 0.7	0.9 ± 1.1
P-value	0.05	0.21	0.49	0.43	0.48	0.28	0.23	0.44

¹CBL = cobalamine, biologically active form in mammals; COB = cobinamide, a corrin ring without the base, ribose, and phosphate groups; cobamides with substitution of 5, 6-dimethyl benzimidazole by: CRE = cresol; MADE = 2-CH₃-adenine; ADE = adenine; MSAD = 2-CH₃-S-adenine; OHBZA = 5-OH-benzimidazole; and UN = unidentified cobamine. Data are presented as mean values with their standard deviations for 4 cows per group.

in the present experiment have been reported elsewhere (Santschi et al., 2005). The correlation between values obtained with the radioassay (Santschi et al., 2005) and CBL concentrations in the present study was high ($r^2 = 0.986$ and 0.995 for duodenal and ileal digesta, respectively). However, compared with CBL concentrations measured in the present study, the radioassay method overestimated vitamin B₁₂ concentrations by 158 and 188% for duodenal and ileal digesta, respectively, which affected calculations of apparent ruminal synthesis and apparent intestinal disappearance.

Only small amounts of CBL and COB were detected in the TMR, probably because of the presence of fermented materials such as corn and grass-legume silages and corn and wheat distillers grain. Moreover, it is possible that, under some conditions, legume plants can produce small amounts of CBL (Combs 1998). Ford et al. (1954) detected CBL, pseudovitamin B₁₂ (ADE), factors A (MADE), B (COB), and C in silage and rumen contents of dairy cow, CBL representing 35% of total corrinoids in silage. Similarly, Dryden and Hartman (1971) observed that CBL represents between 10 to 38% of total corrinoids in hay, silage, and corn.

Nine corrinoids were reportedly detected in rumen content of sheep (Gawthorne 1969) and although the nomenclature was different, 4 of them were identified as the equivalent of CBL, COB, MADE, and ADE in the present experiment. Only 8 corrinoids were detected in rumen content of dairy heifers (Dryden and Hartman 1971); in addition to those described previously in sheep, OHBZA (analog U) was also identified. Three forms were not identified, although Dryden and Hartman (1971) named them factors C₁, C₂, and E.

Concentrations of corrinoids in the rumen content are higher than in the diet (Ford et al., 1954; Dryden and Hartman, 1971), which confirms that synthesis of these molecules takes place in the rumen. During the

control period, approximately 129 mg of corrinoids was apparently produced daily by the ruminal microflora, of which 50 mg was CBL, the biologically active form of the vitamin. Apparent ruminal synthesis of CBL and MADE represents 38 and 25% of the total amounts of corrinoids produced in rumen, respectively. Similarly, Bigger et al. (1976), using digesta samples from different experiments, calculated that CBL and MADE (factor A) were the major fractions produced in rumen of sheep.

Cobalt represents roughly 4.4 and 5.8% of the molecular weight of cobamines and cobinamides, respectively. Based on this estimation, during the control period, 5.6 mg of cobalt was incorporated into the total amount of corrinoids reaching the duodenal cannula. Average daily intake of cobalt was 50 mg with 15 mg provided by the mineral premix as cobalt carbonate. Therefore, 11% of the average daily intake of cobalt was used for apparent ruminal synthesis of total corrinoids, of which only 4% was incorporated into CBL. This figure is in the range reported for sheep. In sheep, the efficiency of production of CBL from cobalt decreased as cobalt intake increased, from 15% for a diet not supplemented with cobalt to 3% for a diet supplemented with 1 mg of cobalt/d (Smith and Marston, 1970). In dairy cows, Stemme et al. (2008) calculated that efficiency of cobalt utilization for CBL production varied between 7.1 and 9.5% with cobalt intakes approximately 10 times lower than in the present experiment. However, although the efficiency of cobalt utilization was higher, daily ruminal production of CBL reported by these authors was lower (3.7 and 8.6 mg/d with a cobalt supply of 2.27 and 3.97 mg/d, respectively) than the daily production of CBL observed in the present study.

A large proportion of the amount of cyanocobalamin provided by the supplement fed to the dairy cows disappeared before the duodenal cannula; only

20% of the supplement was recovered at the duodenal level as CBL. However, apparent ruminal synthesis of COB increased by 185.2 mg/d, an increase 100 times the amount measured during the control period. On a weight basis, it represented 37% of the amount of cyanocobalamin supplement, but closer to 48% on a molecular basis. These results are in agreement with Smith and Marston (1970), who reported that, in sheep, a dietary supplement of cyanocobalamin is rapidly destroyed in rumen and that factor B (COB) appears to be the major product of degradation of supplementary cyanocobalamin in the rumen. In humans, ingestion of CBL supplements increased not only fecal excretion of COB but also excretion of MADE, ADE, and CRE (Allen and Stabler, 2008).

Intrinsic factor has been detected in cows (Schneider and Stroński, 1987). Consequently, intestinal absorption is likely to be similar to that in humans, the binding of the complex CBL-intrinsic factor with specific receptors at the ileal level being an essential step for the absorption of the vitamin. In rabbits, Kolhouse and Allen (1977) observed that a high affinity for the intrinsic factor is essential for intestinal absorption. Only CBL and cobamides containing benzimidazole bind efficiently to intrinsic factor (Kolhouse and Allen, 1977; Schneider and Stroński, 1987). This may explain why only apparent intestinal disappearance of CBL and OHBZA was observed in the present experiment.

The efficiency of apparent intestinal disappearance decreased with the amount of CBL present at the duodenum level. In cows fed the control diet, 45% of the amount of CBL reaching the duodenal cannula disappeared before the ileal cannula. In cows fed a supplement of 500 mg of cyanocobalamin, only 24.5% of this amount appeared to have been absorbed in the small intestine. This efficiency is similar to the values of 48% observed in steers (Zinn et al., 1987). In sheep, Sutton and Elliot (1972) observed highly variable values for apparent intestinal disappearance, varying from 1 to 35%. Smith and Marston (1970) calculated that approximately 5% of the vitamin B₁₂ produced in the rumen of sheep was absorbed in the small intestine, whereas this efficiency decreased to 1 to 3% for a dietary supplement of cyanocobalamin. Kolhouse and Allen (1977) demonstrated in rabbits that only CBL analogs with a high affinity for intrinsic factor are taken up by the ileal cells. However, they also identified the presence of another mechanism at the ileal level that prevents release from the ileal cells and entry into the portal circulation of most analogs, except CBL. In dairy cows, during the 24 h following the ingestion of a dietary supplement of 500 mg of cyanocobalamin, the flow of CBL through the portal-drained viscera was only 1.3 mg (Girard et al., 2001). This amount represents 4.1%

of the amount of corrinoids (CBL and OHBZA) disappearing from the small intestine. These observations seem to indicate that in dairy cows, as in rabbits, there is a mechanism present at the ileal level that favors the entry of CBL at the expense of the other vitamin B₁₂ analogs that cannot be used by the mammalian cells.

CONCLUSIONS

With the diet used in the present experiment, apparent ruminal synthesis of CBL is higher than values previously reported for sheep and dairy cows, although it represents only 38% of the total amount of corrinoids produced in rumen. Moreover, only 4% of dietary cobalt was used for CBL synthesis. Use of a dietary supplement of cyanocobalamin is not an efficient means to increase vitamin B₁₂ supply to cows, because 80% of the supplement disappeared before reaching the duodenal cannula. However, although the efficiency of apparent intestinal disappearance of CBL decreased as the amount reaching the duodenal cannula increased, apparent intestinal disappearance of CBL was still higher in absolute values when the cows were fed the dietary supplement of cyanocobalamin.

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