Short communication: Transport of 2-hydroxy-4-methyl-thio-butanoic isopropyl ester by rumen epithelium in vitro

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ABSTRACT

Our objective was to evaluate the potential of rumen epithelium to transport 2-hydroxy-4-(methylthio)-butanoic isopropyl ester (HMBi) using the Ussing chamber technique. Rumen tissues were obtained from a nearby slaughterhouse, separated from the muscle and serosal layer as quickly as possible after exsanguination, placed in buffer, and gassed with 95:5 (vol/vol) O₂:CO₂ before tissue mounting. Two levels of HMBi (0.44 and 0.88 mg/mL) and 2 incubation times (120 and 180 min) were used in 12 chambers with 3 replicates per treatment with an exposed surface area of 2 cm². Four separate experiments were conducted (n = 16). Concentrations of HMBi and methionine hydroxy analog (HMB) were measured by HPLC in rumen-side and serosal-side buffers. Data are expressed as percentage of added HMBi. Initial time samples were taken for comparison with incubated samples. Adding the HMBi-buffer mixture to the rumen side caused an immediate release of HMB (mean = 6.3%). Breakdown of HMBi to HMB at initial time was due to hydrolysis reactions at the epithelial surface. Overall, a small and variable amount of HMBi was transferred to the serosal buffer (mean of 0.58% across both times and both concentrations). A larger amount of HMB (8.94%) was isolated in the serosal buffer. Increasing incubation time increased the amount of HMB in the ruminal buffer (34.0% at 120 min vs. 43.4% at 180 min) and decreased the amount of HMBi (37.9% at 120 min vs. 28.1% at 180 min). These data indicate that very limited amounts of HMBi may cross the rumen epithelium. The amount of HMB isolated on the serosal side was about 10 times higher than HMBi. The hydrolysis of HMBi to HMB required the presence of rumen tissue or perhaps microbes attached to the tissue. Based on this in vitro system, direct transport from the rumen would not explain rapid blood methionine increases observed when HMBi is fed.

Key words: 2-hydroxy-4-(methylthio)-butanoic isopropyl ester, methionine hydroxy analog, rumen epithelium, Ussing chamber

The rumen epithelium is a highly absorptive organ in spite of being a keratinized, squamous epithelial tissue (Graham and Simmons, 2005). Small organic molecules such as VFA, lactate (Graham and Simmons, 2005), niacinamide (Erickson et al., 1991), carnosine (Remond et al., 2000), and histamine (Aschenbach and Gabel, 2000) have all been shown to be absorbed directly from the rumen across the rumen epithelium. This is assumed to be a largely passive process, with transport driven by electrochemical gradients. However, these molecules may also be actively absorbed (Kirat et al., 2006). Although there are reports that several amino acids can be absorbed directly across the rumen wall (Liebholtz, 1971; Michnova and Faix, 2001), this route would appear to make a very small contribution to the overall amino acid supply in ruminants (Remond et al., 2000). Ruminal absorption of amino acid analogs has not been studied. The Ussing chamber has been used to study the absorption of metabolites across the gastrointestinal tract, including the rumen epithelium, and the results obtained in this manner approximate what has been observed in vivo (Breves et al., 1988; Schröder et al., 1997).

Methionine has been proposed as the limiting amino acid in many North American dairy cattle diets (NRC, 2001). Because a Met source must be able to escape degradation by rumen microorganisms, supplemental Met must be added as a protein with high Met content and low rumen degradability or as a rumen-protected Met source. Methionine hydroxy analog [2-hydroxy-4-(methylthio)-butanoic] isopropyl ester (HMBi) has been proposed as a novel Met supplement for ruminant animals (Robert et al., 2000). Those investigators observed about 60% protection from microbial degradation for the isopropyl ester, but noted that methyl, ethyl, butyl, and cyclohexyl esters were more readily converted to the methionine hydroxy analog (HMB or methionine hydroxy analog free acid) in the rumen

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environment and subsequently used by microorganisms. This observation suggests that the isopropyl ester confers a steric hindrance of bacterial esterase enzymes that is not conferred by other organic esters. A significant increase in the Met content of blood serum has been reported within minutes of HMBi being introduced into the rumen (Robert et al., 2000). It was suggested by Robert et al. (2000) that HMBi is not a rumen bypass source of Met, but rather it is rapidly and quantitatively transported across the rumen tissue where it is then hydrolyzed to HMB and isopropanol. They suggest that HMB is then rapidly converted to Met. This implies that there might be an active transport mechanism localized in the rumen epithelium for HMBi. This mechanism has not been demonstrated. However, it has been demonstrated that the rumen epithelium has active sites for monocarboxylate transporter 1 (**MCT-1**; Kirat et al., 2006), which raises the possibility that there may be export of HMB from the epithelium after the highly polar HMBi molecule has entered the epithelial cell. The objective of the present study was first to determine if an HMBi transport mechanism exists within rumen epithelium by using the Ussing chamber technique, and then to characterize the nature of this transport.

The HMBi product, MetaSmart Dry, was purchased from Adisseo (Antony, France). Our analysis indicated that the product was 57.0% HMBi. Two stock solutions of rumen-side buffer were constructed to provide 0.44 and 0.88 mg/mL of HMBi or 8 and 16 mg of HMBi per incubation. This was calculated to be equivalent to 30 and 60 g of the MetaSmart product per cow per day, or 12.5 and 25 g of Met equivalent per day (Graulet et al., 2005).

In vitro studies with isolated, intact rumen wall were carried out with the Ussing chamber technique as described earlier (Schröder et al., 1997). On 4 separate occasions, coinciding with each of the 4 experiments, rumen wall tissues from the ventral sac were obtained from a single animal immediately after exsanguination (the same slaughterhouse was used on each occasion). Pieces were immediately immersed in rumen buffer solution at 38°C, where the epithelium was stripped from the underlying muscle layers and the serosa. Stripped epithelial samples were mounted between the 2 halves of incubation chambers with an exposed area of 2 cm². Twelve Ussing chambers were connected simultaneously to reservoirs containing 18 mL of rumen or serosal buffer solution on the respective sides. The solutions were kept at 38°C and were continuously stirred by the use of a gas lift system that supplied 95% O₂:5% CO₂. The rumen-side buffer solution contained (mmol/L): NaCl 57.0, KCl 5.0, CaCl₂·2H₂O 1.2, MgCl₂·6H₂O 1.2, NaHCO₃ 21.0, Na₂HPO₄·2H₂O 1.2, NaH₂PO₄·1H₂O 1.6,

Na acetate·3H₂O 36.0, Na propionate 15.0, Na butyrate 9.0, and Na gluconate 1.2 (adjusted with HCl to pH 7.4 at 38°C and gassed and adjusted to 300 mOsm/L with mannitol). The composition of the serosal-side buffer was (mmol/L): NaCl 57.0, KCl 5.0, CaCl₂·2H₂O 1.2, MgCl₂·6H₂O 1.2, NaHCO₃ 21.0, Na₂HPO₄·2H₂O 1.4, NaH₂PO₄·1H₂O 1.4, glucose 5, and Na gluconate 61.0. The tissues were allowed to equilibrate for 30 min with continuous gassing. At the end of 30 min, the contents of the ruminal side of the chamber were replaced by 18 mL of 1 of the 2 HMBi stock solutions. Incubations proceeded for either 120 or 180 min, resulting in a 2 × 2 factorial arrangement of treatments. Samples (5 mL) were taken at the beginning of the incubation periods (time = 5 min) for comparison of HMB and HMBi concentrations and after 120 and 180 min of incubation. Under these conditions, the functional viability of the epithelia was intact over the entire incubation period. This was monitored by continuous recording of trans-epithelial short-circuit currents (I_{sc}) as a measure of net electrogenic ion transport and tissue conductances (G_t) indicating potential effects on transcellular or paracellular permeabilities. Irrespective of different incubation conditions, no significant changes in I_{sc} or G_t values could be detected. The I_{sc} and G_t values varied between 0.2 and 0.5 μ Eq/cm² per hour and 5.5 and 7.0 mS/cm², respectively. These results are comparable with data from other studies using ruminal epithelia from sheep and goats (Schröder et al., 1997, 1999) and from calves and cattle (Sehested et al., 1999; Breves et al., 2002) for transport measurements as well as characterization of short-chain fatty acid metabolism. At the conclusion of the incubations, the remaining ruminal and serosal solutions (13 mL) were collected and kept at -20°C until analysis, as were rumen epithelial samples.

Samples were analyzed for HMBi and HMB using HPLC with known additions of HMB and HMBi as reference points. Reference samples of HMB and HMBi were obtained from Carl Roth GmbH (Karlsruhe, Germany) and were guaranteed to be 98.5% and 99% purity, respectively. Samples were placed on an Inertsil ODS-3V, RP-18, 5- μ m column (GL Sciences Inc., Torrance, CA) of 250 mm and maintained at 30°C. Mobile phase was 825 mL water (purified by Millipore system) + 125 mL of 0.2 M phosphoric acid + 50 mL of acetonitrile maintained at a flow rate of 1 mL/min. Samples were introduced in 20 μ L of mobile phase. Frozen epithelia were weighed and 15 mL of water:acetonitrile (9:1 vol/vol) was added and ground with a mortar and pestle in an atmosphere of N₂ until the tissue was solubilized.

To determine whether the degradation of HMBi to HMB was the result of buffer hydrolysis or interaction with the epithelium, 3 additional experiments were conducted as follows: 1) replicates of HMBi buffer solu-

Table 1. Least squares means for effects of amount of added 2-hydroxy-4-methyl-thio-butanoic isopropyl ester (HMBi) and time of incubation on site and amount of HMBi and methionine hydroxy analog (HMB) recovered (% of added basis)

Site	Form	Amount and incubation time				±SE	Significance of effects ($P < $)	
		8 mg	16 mg	120 min	180 min		Amount	Time
Rumen buffer	HMBi (%)	30.3	35.6	37.9	28.0	3.42	0.02	0.01
	HMB (%)	40.5	37.0	34.0	43.4	2.71	0.08	0.01
	Mean HMB (mg)	3.24	5.92	4.08	5.21	1.05		
Tissue	Mean tissue weight (mg)	351	322	372	372	45.0	NS	NS
	HMBi (%)	ND ¹	ND	ND	ND			
	HMB (%)	2.64	2.00	2.58	1.88	0.55	NS	0.01
Serosal buffer	HMBi (%)	0.3	0.9	0.6	0.6	0.11	0.01	NS
	HMB (%)	10.5	7.3	7.9	10.0	0.78	Amount $\times$ time $P < 0.01$	
	Mean HMB (mg)	0.84	1.17	0.95	1.27	0.02		
Summary	Total HMBi + HMB recovered	84.2	82.8	83.0	83.9	0.61	NS	0.10
	HMBi transferred as HMB (%)	10.5	7.3	7.9	10.0			

¹ND = not detected.

tions were maintained at incubation temperature for 24 h; 2) unmounted epithelial tissue was incubated in 3 flasks containing buffer supplemented with 16 mg of HMBi for 180 min; 3) 3 replicates of 16 mg of HMBi were incubated with a mounted, nonpermeable, non-biologic membrane (Parafilm) for 180 min. At the end of each of these experiments, buffers were analyzed for relative amounts of HMBi and HMB using the methods described above.

Statistical analysis was by Proc Mixed of SAS (version 8.0, SAS Institute, Cary, NC) using a split plot design with a 2×2 arrangement of treatments. The observational unit was the mean recovery of HMBi and HMB from the 4 replicate incubations except for rumen tissue where the replicate samples were pooled.

There was no HMB or HMBi in the serosal buffer at initial time, nor was there any HMB isolated in the stock solutions. The initial time sample was taken after about 5 min of mixing time ($t = 5$) and after all rumen-side chamber contents were replaced with the respective buffer. For all samples at $t = 5$, a mean of $6.3 \pm 0.46\%$ of HMBi had been converted to HMB and a mean of $9.2 \pm 1.04\%$ of HMBi was not recovered as either HMBi or HMB. This indicates rapid hydrolysis of HMBi to HMB in the incubation system. At $t = 5$, doubling the amount of HMBi resulted in slightly less conversion of HMBi to HMB (6.8 and 5.8% for 8 and 16 mg, respectively; $P < 0.01$) and increased the percentage total recovery, defined as the amount of HMBi + HMB (90.2 and 91.3%, respectively; $P < 0.10$). Recoveries of total added HMBi were greater in the rumen-side buffer than in the serosal-side buffer ($P < 0.01$) for both HMBi and HMB (Table 1). At 120 min of incubation, 8.5% of added HMBi could be found

in the serosal buffer predominantly as HMB, whereas at 180 min of incubation, this value increased to 10.6% (1.27 mg). This equates to an average transfer of HMBi as both HMBi and HMB of between 3.5 and 4.25%/hr. There was no interaction between incubation time and amount of added HMBi that remained as HMBi or HMB in the rumen-side buffer, but there was for HMB in the serosal buffer (Table 1). The 10% loss of product of HMBi + HMB at $t = 5$ increased to an approximate 20% loss by 120 min of incubation and remained more or less constant through 180 min. Methionine could not be detected in any buffer solution, nor was there a measurable increase in acetone or isopropanol, known breakdown products of HMBi (European Commission, 2003).

In the rumen-side buffer, isolated HMBi was greater for the higher addition ($P < 0.02$, Table 1). The percentage of HMBi in the rumen buffer isolated as HMB tended to be larger with addition of 8 mg compared with addition of 16 mg ($P < 0.08$), although the total milligrams isolated as HMB was greater for the higher concentration. Increasing the incubation time from 120 to 180 min decreased the amount of HMBi isolated as HMBi in rumen-side buffer, but increased the amount of HMB recovered by about 10% ($P < 0.01$), indicating ongoing hydrolysis equivalent to 1.13 mg of added HMBi.

A small amount of HMBi was isolated in the serosal buffer after both incubation times. Doubling the concentration of HMBi in the rumen buffer tripled the amount of HMBi in the serosal-side buffer (Table 1). Increased incubation time had no effect on percentage of HMBi isolated in serosal buffer. Greater amounts of HMB than HMBi appeared on the serosal side of

the rumen epithelium. This appearance was affected by the interaction of HMBi addition and incubation time. With addition of 8 mg and incubation of 120 min, 8.5% of the total addition was isolated as HMB in the serosal buffer. When the same addition was incubated for 180 min, this value increased to 12.6%; with addition of 16 mg, 7.5% was isolated in serosal buffer regardless of incubation time (data not shown).

We did not detect HMBi in tissue samples. Increasing the incubation time tended to increase HMB recovery in tissue ($P < 0.10$; Table 1). Neither the amount of added HMBi nor tissue weight affected HMB recovered in tissue. In HMBi buffer solutions incubated without the presence of a membrane, no HMB was detected, and HMBi recovery averaged 105%. This is in contrast to the incubations using mounted tissue or Parafilm, where recovery was 80 to 85%. At the end of 180 min of incubation of unmounted epithelial tissue, 36.7% of added HMBi was recovered as HMB, and 61.8% was isolated as HMBi. Slight hydrolysis of HMBi to HMB (0.30%) occurred in ruminal buffer when incubated in the presence of Parafilm. There was no transfer to the serosal buffer, but total recovery was limited to 81.3%. Because there were no enzymes in this system, conversion to Met was not possible, which suggests that HMBi loss was not due to increased Met.

These experiments indicate that very little HMBi passes directly through the rumen epithelium. Because only 10% of the supplemented HMBi appears to be able to enter the blood as HMB, it is doubtful that direct rumen absorption of HMBi could be the source of the increased blood Met within the timeframe observed by Robert et al. (2000). Rather, it appears that part of HMBi is hydrolyzed to HMB at the tissue surface and during its transport across the rumen epithelium. Because no esterases have been reported for rumen epithelium, this may occur as the result of microbial action from organisms on the epithelial surface (Graham and Simmons, 2005). In this closed system, approximately 10% of added HMBi (Table 1) was transferred across rumen tissue as HMB. This process may be mediated either by passive diffusion, similar to VFA absorption, or via the action of the MCT-1 transporter (Kirat et al., 2006). In either case, the driving force is provided by the concentration gradient between the mucosal and serosal sides of rumen tissue. This is supported by the finding that the rate of transport was the same in the final hour as in the first 2 h. In a functional rumen with its volume, motility, and associated bacterial population that degrade HMB and incorporate it into their metabolism (Patterson and Kung, 1988), it is very likely that there would not be enough HMB at the rumen wall for massive absorption. However, a targeted site of hydrolysis that confers protection of HMB and

allows greater transport to occur cannot be excluded. Lobley et al. (2006) have established that HMB is converted to Met not as blood passes through the liver in ruminants but rather when it enters other tissue. There is a time lapse before HMB can be converted to Met, mix with the peripheral blood, and cause a significant increase in jugular Met concentration. The European Commission (2003) has determined that effects of HMB are mitigated only through action on the rumen microflora. Our work suggests that at least a fraction of the HMB action may be as a directly absorbed Met supplement. However, the extent to which these in vitro experiments approximate the in vivo situation is not certain and may not be enough to account for the observations made by Robert et al. (2000). The loss of HMBi when incubated with either a rumen tissue or Parafilm membrane is unresolved. Resolution of this will require more experimentation, as will the exact manner in which HMBi contributes to Met status in the dairy cow.

We conclude that HMBi is partially hydrolyzed to HMB at the rumen epithelial surface. Moreover, HMBi does not appreciably pass the rumen wall intact. After entering the epithelium, HMBi is converted to HMB and appears to exit the epithelium via passive diffusion or possibly at low rates of MCT-1 transport.

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