J. Dairy Sci. 93:260–264
doi:10.3168/jds.2009-2200

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) O_2:CO_2 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 cm^2. 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

Received March 10, 2009.
Accepted September 20, 2009.
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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 cm^2. 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% O_2:5% CO_2. The rumen-side buffer solution contained (mmol/L): NaCl 57.0, KCl 5.0, $CaCl_2 \cdot 2H_2O$ 1.2, $MgCl_2 \cdot 6H_2O$ 1.2, $NaHCO_3$ 21.0, $Na_2HPO_4 \cdot 2H_2O$ 1.2, $NaH_2PO_4 \cdot 1H_2O$ 1.6, Na acetate$\cdot 3H_2O$ 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, $CaCl_2 \cdot 2H_2O$ 1.2, $MgCl_2 \cdot 6H_2O$ 1.2, $NaHCO_3$ 21.0, $Na_2HPO_4 \cdot 2H_2O$ 1.4, $NaH_2PO_4 \cdot 1H_2O$ 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 transepithelial short-circuit currents ($\mathbf{I_{sc}}$) as a measure of net electrogenic ion transport and tissue conductances ($\mathbf{G_t}$) 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 μEq/cm^2 per hour and 5.5 and 7.0 mS/cm^2, 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-μm 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 μL 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 N_2 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-