Comparative In Vitro and In Vivo Absorption of 2-Hydroxy-4(Methylthio) Butanoic Acid and Methionine in the Broiler Chicken

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ABSTRACT Poultry diets are typically supplemented with DL-2-hydroxy-4(methylthio) butanoic acid (HMTBA, or the hydroxy analog of methionine) or DL-methionine (DLM). Although HMTBA and DLM provide methionine activity, they are structurally distinct molecules with different physiological characteristics until they are converted to L-methionine. The relative rates of intestinal HMTBA vs. DLM absorption have been controversial, and it has been claimed that HMTBA is not fully absorbed. We measured the uptake of HMTBA and DLM in an in vitro everted intestinal slice model. Sections of intestinal slices (jejunum and ileum) were incubated with 0.1 to 50 m*M* HMTBA that was radiolabeled or DLM that was radiolabeled, and absorption was measured by scintillation counting. The HMTBA uptake was equal to or greater than DLM absorption in each tissue and at every time point with one exception. Furthermore, the rates of HMTBA absorption were always equal to or significantly greater than DLM uptake. In a separate in vivo experiment, absorption of HMTBA and L-methionine was monitored along the entire gastrointestinal (GI) tract. Broilers were fed commercial-type corn-soy diets supplemented with 0.21 % HMTBA. Digesta was collected from crop, proventriculus, gizzard, duodenum, jejunum, ileum, large intestine, and cloaca and analyzed for the concentration of free HMTBA and free methionine in each compartment. These studies demonstrated that HMTBA is absorbed completely and along the entire GI tract, especially the upper GI tract. Furthermore, there was a higher concentration of free L-methionine than HMTBA in the digesta from every segment distal to the gizzard.

(*Key words*: Alimet, 2-hydroxy-4(methylthio) butanoic acid, methionine, absorption, broiler)

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INTRODUCTION

Methionine is typically the first-limiting amino acid with respect to growth performance in poultry diets. Therefore, diets are generally supplemented with either the hydroxy analog of methionine, liquid DL-2-hydroxy-4(methylthio) butanoic acid (HMTBA, Alimet[2] feed supplement) or dry DL-methionine (DLM). Both methionine sources are absorbed in the gastrointestinal (GI) tract of an animal, converted to L-methionine, and used in protein synthesis. Controversy has surrounded the relative bioefficacy of HMTBA vs. DLM in animals, and some of the argument has centered on the absorption of these 2 methionine sources.

The relative rates of HMTBA vs. DLM uptake, as well as the completeness of uptake, have been investigated previously. Several in vitro and in vivo experiments have demonstrated efficient uptake of both sources, with HMTBA uptake rates being equivalent to or greater than methionine uptake. For example, Knight and Dibner (1984) reported that the rate of HMTBA absorption is equal to or greater than L-methionine uptake in an in vivo intestinal uptake model. Furthermore, although in vitro uptake of HMTBA and DLM are comparable during thermoneutral conditions, HMTBA absorption is greater during periods of heat stress (Dibner et al., 1992). This difference was partially due a reduction in D-methionine uptake during periods of high temperature (Knight et al., 1994). Finally, Esteve-Garcia and Austic (1993) concluded that HMTBA and DLM are fully absorbed by the chicken in vivo, and Han et al. (1990) drew similar conclusions. In contrast to these findings, experiments with in vitro brush border membrane vesicle (BBMV) models have called into question the relative rates of absorption of the 2 methionine sources (Brachet and Puigserver, 1987, 1989; Maenz and Engele-Schaan, 1996a,b). Some papers report that L-methionine uptake is faster than that of DL-HMTBA (Brachet and Puigserver, 1987, 1989) or L-HMTBA (Brachet and Puigserver, 1989; Maenz and Engele-Schaan,

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Abbreviation Key: BBMV = brush border membrane vesicle; DLM = DL-methionine; GI = gastrointestinal; HMTBA = DL-2-hydroxy-4(methylthio) butanoic acid.

1996b) uptake in this system, although statistics to demonstrate significant differences are not reported. In contrast, other experiments indicate that absorption of L-HMTBA and L-methionine into BBMV is comparable (Maenz and Engele-Schaan, 1996a). In addition, differences between DL-HMTBA and DLM absorption are unclear. For example, Brachet and Puigserver (1989) report a numerical advantage for DLM in this system, but it is not significant.

One important disadvantage of using BBMV to measure HMTBA absorption is that BBMV tend to minimize HMTBA uptake by diffusion (Brachet and Puigserver, 1989), which is a major mechanism of uptake for HMTBA (Knight and Dibner, 1984; Brachet and Puigserver, 1987). To better understand the relative rates of HMTBA and DLM absorption, we measured uptake of these 2 sources in an in vitro everted intestinal slice system. Intestinal slices have been used to evaluate uptake of methionine and HMTBA (Lerner and Taylor, 1967; Lerner et al., 1968; Knight and Dibner, 1984; Brachet et al., 1987; Brachet and Puigserver, 1987; Dibner et al., 1992; Knight et al., 1994), as well as a variety of other nutrients including other amino acids (Agar et al., 1954; Schachter et al., 1961; Johnston and Borgstrom, 1964; Robinson and Felber, 1964, 1965; Feldman and Borgstrom, 1966; Lerner and Taylor, 1967; Nilsson and Borgstrom, 1967; Lerner et al., 1968; Olsen and Rosenberg, 1970; Elsas and MacDonell, 1972; Ziporin et al., 1973; Knight and Dibner, 1984; Osiecka et al., 1985; Porter et al., 1985; Brachet et al., 1987; Brachet and Puigserver, 1987; Dibner et al., 1992; Gonzalez and Vinardell, 1992, 1993; Knight et al., 1994; Stewart et al., 1995; Kettunen et al., 2001; Thiesen et al., 2003). Furthermore, in contrast to BBMV, intestinal slices allow efficient uptake of HMTBA by diffusion and carrier-mediated absorption (Knight and Dibner, 1984; Brachet and Puigserver, 1987). In addition, DLM absorption by active transport and carrier-mediated uptake, which represent the major mechanisms of amino acid transport, also occur in in vitro slices (Dibner et al., 1992; Knight et al., 1994). Therefore, everted intestinal slices are an appropriate model system to compare the relative rates of HMTBA and DLM uptake. The data described here reinforce the notion that HMTBA absorption in the small intestine occurs at rates equal to or greater than DLM absorption.

We also performed in vivo disappearance studies to evaluate free HMTBA and L-methionine concentrations along the GI tract. This was done to help determine the concentrations of HMTBA and DLM to be tested in the slices system, as well as to identify the major sites of HMTBA absorption in the GI tract. With these studies, we report the first data demonstrating that HMTBA absorption occurs along the entire GI tract of the bird.

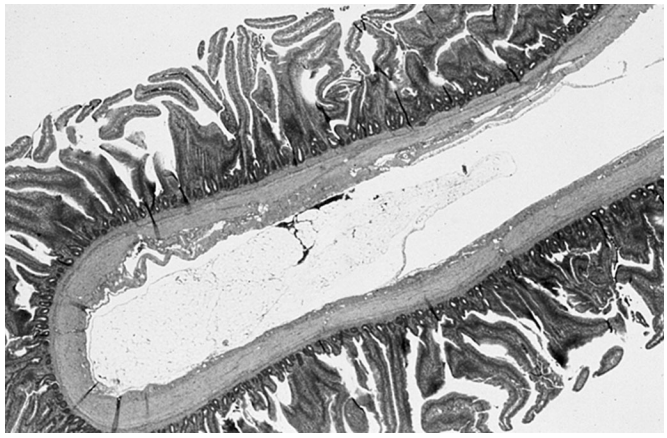

FIGURE 1. Histological examination of an inverted intestinal slice. An inverted slice of jejunum was prepared and processed as described in Materials and Methods. It was sectioned and stained with hematoxylin and eosin and evaluated to ensure proper morphology. A typical section is shown.

MATERIALS AND METHODS

Birds

Cobb broilers were hatched onsite, housed in cages at thermoneutral temperatures, and fed commercial type corn-soy diets as described below. Birds were allowed free access to food and water. Birds were euthanized by CO_2 inhalation.

In Vitro Absorption Experiments

Birds used for the in vitro uptake experiments were fed diets supplemented with equimolar amounts (0.1% each) of HMTBA (88% Alimet feed supplement) and DLM (99%). The jejunum or ileum was collected from birds (d 13 to 30), rinsed in Earle's balanced salt solution (EBSS) complete (1× EBSS supplemented with 0.1% dextrose, 25 m*M* HEPES, and 1 mL/L gentamicin; pH adjusted to 7), and then rinsed in ice-cold rinse buffer (10 m*M* HEPES, 300 m*M* mannitol, pH 7.3). Tissues were everted, sliced into circular sections (rings; >150mg), covered with rinse buffer, and kept on ice until needed. Uptake experiments were conducted in 6-well plates. Each well was filled with 3.95 mL (0.1 to 20 m*M* experiments) or 3.2 mL (50 m*M* experiments) transport buffer (10 m*M* HEPES, 100 m*M* mannitol, pH 7), 0.2 mL 0.5 *M* glucose, and 0.3 mL 1.25 *M* NaCl (3 wells) or 0.3 mL 1.25 *M* KCl (3 wells). Five random slices were placed into each well, and the plate was floated on a 37°C waterbath for 10 min. Periodically, an extra section was examined histologically to ensure structural integrity of the slices as described below (Figure 1). We added 0.05 mL of ^{3}H-inulin (0.5 μCi/mL) to each well as a nonspecific binding control. Finally, 0.5 mL (0.1 to 20 m*M* experiments) or 1.25 mL (50 m*M* experiments) DL-HMTBA or DLM was added to each well to a final concentration of 0.1, 0.5, 2, 5, 10, 20, or 50 m*M*, depending on the experiment. The final fluid volume was

[3]American Radiolabeled Chemicals, St. Louis, MO.
[4]New England Nuclear, Boston, MA.

5 mL per well. The HMTBA and DLM both contained methyl ^{14}C-HMTBA[3] or ^{14}C-DLM[4] tracers at 2.5 μCi/100 mmol, except for the 0.5 m*M* experiments (8.3 μCi/100 mmol) and the 0.1 m*M* experiments (20.8 μCi/100 mmol). One slice was removed out of each well at 5 times (0.5, 1, 1.5, 2, and 5 min). Each removed slice was placed in 5 mL of ice-cold rinse buffer and rinsed of unabsorbed radioactivity. Tissues were blotted gently with Kimwipes,[5] weighed, and transferred to individual scintillation vials. Two milliliters of 2.5 *N* NaOH was added to each vial and incubated overnight at room temperature. Three milliliters of 2 *N* HCl and 15 mL of scintillation fluid were added, and radioactivity was counted in a scintillation counter. Each treatment was performed in triplicate on a given day and repeated on at least 2 different days. Each paired comparison between HMTBA and DLM was replicated equally. ^{3}H-corrected HMTBA and DLM counts were calculated, and absorption of HMTBA or DLM per milligram of tissue was analyzed using the GLM procedure of SAS software,[6] as a 2 × 2 factorial design (2 methionine sources × 2 ions) using tissue weight as a covariate at each time point. No methionine source × ion interactions existed, and so data from KCl-containing wells and NaCl-containing wells were combined. The statistical model used was represented by the equation $Yijk = \mu + Ii + Sj + ISij + \gamma(Tijk - Tm) + \varepsilon ijk$, where $Yijk$ = uptake, μ = general mean, i = the effect of ion, j = the effect of methionine source, k = the effect of replication, $ISij$ = the interaction between i and j, γ = a covariate coefficient which is multiplied by the difference between the individual tissue weight ($Tijk$) and the average tissue weight (Tm) to account for the covariate effect, and ε_{ijk} = the error term. A probability level of $P = 0.05$ was considered statistically significant. Rates of uptake (uptake vs. concentration) were analyzed by GLM procedure of SAS software using a linear regression model whereby time (Xl) was the independent variable, and tissue weight was the covariate. The experimental model is represented by the equation $Yl = \beta(Xl) + \gamma(Tl - Tm) + \varepsilon lk$, where β = slope (or rate of uptake), Tl = individual tissue weight, and the other variables are described above. Differences in rate within each concentration were established by *t*-test.

Histology

Inverted intestinal slices were prepared as described above. After the 10-min incubation at 37°C, the section was fixed with NoTox,[7] embedded in paraffin, and sectioned using standard procedures. Sections were stained with hematoxylin and eosin and evaluated for proper morphology. Images were visualized and captured at 100× on an Olympus AX80 microscope.[8]

TABLE 1. Diets for the in vivo absorption experiment

Ingredients, % as is	Starter diet	Grower diet
Corn	59.5	57.8
Soybean meal	34.8	35.28
Soybean oil	1.67	3.34
Dicalcium phosphate	1.88	1.65
Limestone	0.96	0.83
Salt	0.58	0.5
Vitamins and minerals	0.35	0.34
Lysine	0.15	0.086
Threonine	0.10	0.097
HMTBA[1]	0 or 0.21	0 or 0.21
Nutrients (calculated)		
Metabolizable energy, Mcal/kg	2,998	3,100
CP, %	21.8	21.8
Digestible lysine, %	1.15	1.11
Digestible methionine, %	0.32	0.32
Digestible sulfur amino acids, %	0.63	0.63
Digestible threonine, %	0.76	0.76

[1]DL-2-hydroxy-4(methylthio) butanoic acid.

In Vivo Absorption

Cobb broilers were raised in battery cages and fed commercial-type corn-soy diets supplemented with or without 0.21 % HMTBA. Diets are shown in Table 1. Birds were fed the crumbled starter diet ad libitum up to 15 d of age. At 16 d, the birds were switched to the pellet grower diet ad libitum. On d 36, 4 birds per cage and 4 cages per treatment were fed 300 g per cage of HMTBA-supplemented feed or control feed, each with silica[9] added at 2% as a digestibility marker. Feed was removed after 6 h, and the unconsumed feed was weighed. Birds were euthanized, and digesta was taken from crop, proventriculus, gizzard, duodenum, jejunum, ileum, large intestine, and cloaca. Digesta samples from birds within a cage were combined and frozen for later analysis. This process yielded 4 samples from each GI compartment for each treatment (HMTBA or control). Digesta samples from each compartment and feed samples were analyzed for insoluble ash using described methods (Vogtmann et al., 1975 ; Scott and Boldaji, 1997).

The isotope-dilution technique (Calder et al., 1999) was used to determine concentrations of HMTBA and methionine in broiler digesta. Briefly, to known weights (1.0 and 0.5 g) of ice cold digesta, we added, respectively, internal standards of 0.050 and 0.030 g containing 10 nmol [1-^{13}C]methionine (99%[10]) plus 10 nmol [1-^{13}C]HMTBA (synthesized by Novus International) or 200 nmol [1-^{13}C]methionine. The reason for these 2 different additions was the wide range in methionine concentrations in the different digesta samples under different nutritional conditions. Immediately after addition of the internal standard, 2 mL of ice-cold 1.4 *M* HCl was added, the sample was mixed under vortex and centrifuged at 7,200 × *g* for 5 min, and the supernatant was applied to a 0.5-mL AG 50W × 8 (H+), 100 to 200 mesh resin column.[11] The initial effluent

[5]Kimberly-Clark, Roswell, GA.
[6]SAS User's Guide, 1999, version 8, SAS Institute Inc., Cary, NC.
[7]Scientific Device Laboratory, Des Plaines, IL.
[8]Hitschfel Instruments, Inc., St. Louis, MO.
[9]Sipernat 22, Degussa Corp., Kennesaw, GA.
[10]Isotec Inc., Miamisburg, OH.
[11]Bio-Rad Laboratories, Hemel Hempstead, UK.

plus the subsequent 0.5 mL of water wash was collected for HMTBA analysis.

For analysis of methionine, the column was washed with a further 2 × 2 mL of water (discarded), and the amino acids were eluted with 2 mL of 2 *M* NH_4OH followed by 1 mL of water. After freeze-drying, the sample was dissolved in 0.35 mL of 0.1 *M* HCl, transferred to a 1-mL vial, and evaporated to dryness at 90°C under a gentle stream of N. Derivatization and analysis by gas chromatography-mass spectrometry [T. J. Wester, A. G. Calder, and G. E. Lobley (Rowett Research Institute, Bucksburn, Aberdeen, UK) and M. Vázquez-Añon, J. Dibner, and D. S. Parker (Novus International), unpublished data]. The HMTBA and methionine values were corrected by insoluble ash values to estimate HMTBA and L-methionine concentrations, as described by Kadim and Moughan (1997). The 4 corrected HMTBA and free methionine values for each compartment from each treatment were averaged. Differences in HMTBA vs. methionine levels in each compartment were analyzed using the GLM procedure of SAS software.[6] A probability level of $P = 0.05$ was considered statistically significant.

RESULTS

In Vitro Uptake of 5 to 50 mM HMTBA and DLM

Luminal concentrations of amino acids exceeding 25 m*M* have been reported (Drew, 2004), and our data indicate HMTBA concentrations as low as approximately 0.1 m*M* in the ileum. Therefore, we tested the uptake of comparable concentrations of HMTBA and DLM in the in vitro slices system. Absorption of HMTBA and DLM was dependent on time and concentration.

Uptake of 5 m*M* HMTBA and DLM over a 5-min period is shown in Figure 2A. In general, absorption of both sources at 5 m*M* was comparable, and the only significant differences in uptake occurred at 1.5 and 5 min. At 1.5 min, DLM absorption in the ileum was significantly greater ($P = 0.05$) than uptake of HMTBA in the jejunum. This result was the only instance of greater uptake of DLM than HMTBA in these experiments. In contrast, at 5 min, DLM uptake in the jejunum was significantly less than HMTBA and DLM absorption in the ileum. At 10 m*M* (Figure 2B), absorption of HMTBA in the ileum at all time points was greater than that of HMTBA in the jejunum and DLM in either tissue. There were no significant absorption differences between HMTBA uptake in the jejunum and DLM in either tissue at this concentration. However, the greater absorption of HMTBA over DLM in the jejunum at 5 min was nearly significant ($P = 0.06$). At 20 m*M* (Figure 2C), absorption of HMTBA in both tissues was significantly greater than or equal to DLM absorption at all time points. In addition, several of the numerical differences between HMTBA and DLM uptake were near significance (Figure 2C). At 50 m*M* (Figure 2D), HMTBA uptake was always significantly greater than or equal to DLM uptake. Rates of uptake at each concentration were calculated for both sources in both tissues and are shown in Figure 3. At 5 m*M*, the rates of HMTBA absorption in both tissues were significantly greater than DLM absorption in the jejunum. At 20 m*M*, the rates of HMTBA absorption in both tissues were significantly greater than DLM uptake in the ileum. HMTBA uptake rates at 10 and 50 m*M* were significantly greater than DLM absorption in both tissues.

In Vitro Uptake of 0.1 to 2 mM HMTBA and DLM

Because the luminal concentration of both methionine sources can decline below 1 mM (see Figure 4), we decided to test uptakes of 0.1, 0.5, and 2 m*M* HMTBA and DLM in our model system. There were few significant differences among uptake rates in the jejunum vs. ileum in our previous experiments so we tested these lower concentrations only in the jejunum. Absorption rates are shown in Figure 5. Differences in rate were not significant between HMTBA and DLM.

In Vivo Disappearance of HMTBA and L-Methionine Out of the Digesta

Figure 4 illustrates the disappearance of HMTBA and free L-methionine from the digesta of birds fed diets supplemented with 0.21% HMTBA. Gas chromatography-mass spectrometry analysis demonstrated that the feed contained 17.8 m*M* HMTBA. No HMTBA was detected in control diets not supplemented with HMTBA (data not shown). Diets were not supplemented with DLM, and no free methionine was detected. Greater than 70% of the HMTBA disappeared from the digesta by the proventriculus, and 85% disappeared before the duodenum. By the ileum, the HMTBA levels were only 0.08 m*M*. Slightly higher levels were found in the large intestine and cloaca. In contrast to HMTBA, the highest levels of L-methionine were found in the duodenum (18 m*M*). There was a significantly higher concentration of free L-methionine than free HMTBA in the digesta of every segment distal to the gizzard, except in the cloaca, which was numerically greater for DLM. Concentration differences ranged from 9- to 14-fold in the intestines, depending on the compartment. Prior to the gizzard, there was a higher concentration of HMTBA than methionine in the digesta of the proventriculus. The crop values were not significantly different because only one methionine measurement could be taken from the crop digesta due to difficulties isolating sufficient quantities of digesta from this compartment.

DISCUSSION

Like other amino acids, DLM is absorbed primarily by active transport and to a lesser degree by carrier-mediated transport; both mechanisms occur principally in the small intestine (Lerner and Taylor, 1967; Stevens et al., 1982; Knight and Dibner, 1984; Brachet et al., 1987; Brachet and

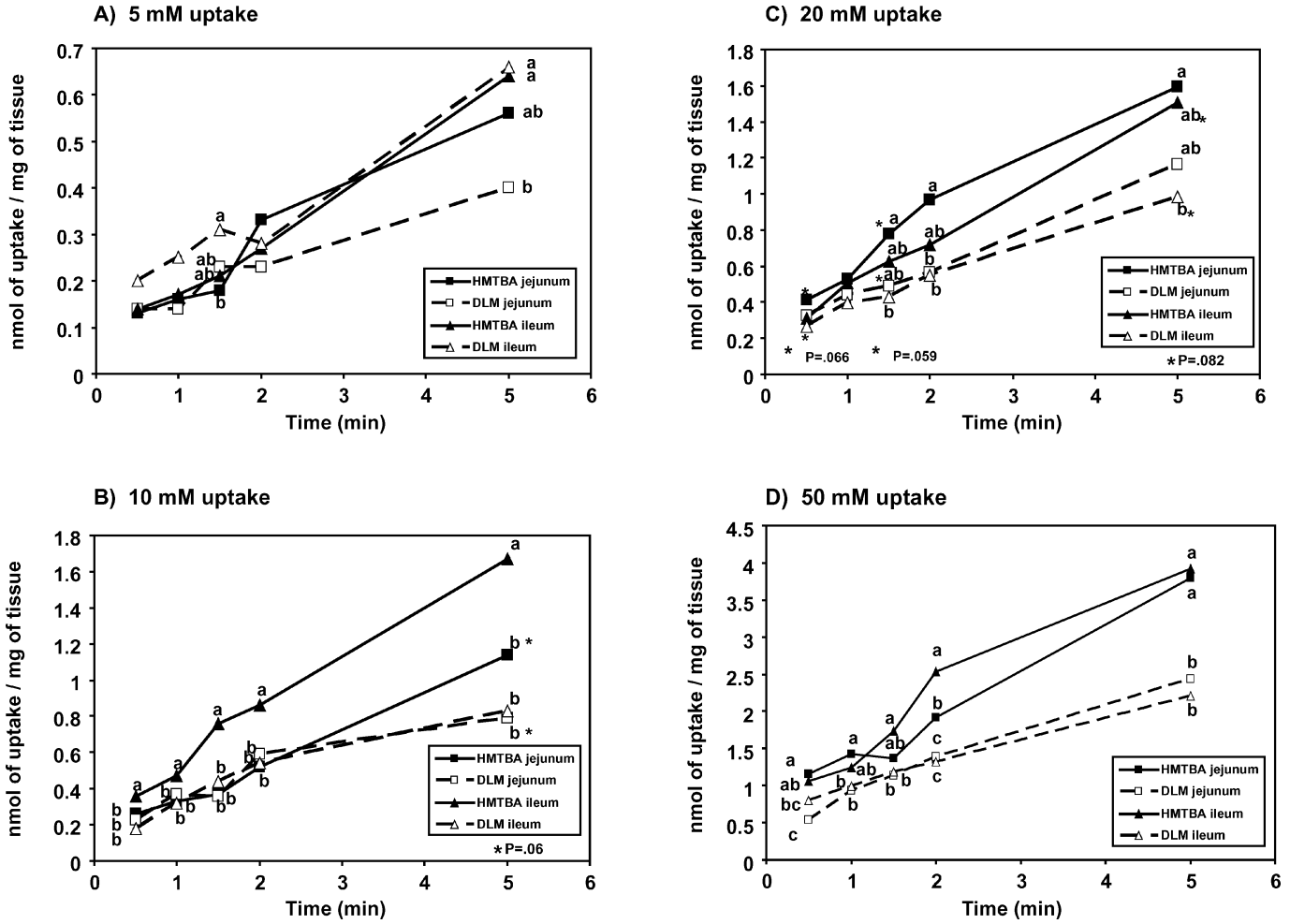

FIGURE 2. In vitro uptake of 5 to 50 m*M* DL-2-hydroxy-4(methylthio) butanoic acid (HMTBA) and DL-methionine (DLM) in the jejunum and ileum. Time courses of absorption of 5 to 50 m*M* HMTBA and DLM by inverted sections of jejunum and ileum are shown: A) 5 m*M*, B) 10 m*M*, C) 20 m*M*, and D) 50 m*M*. Uptake comparisons were assessed within each time point. Uptake values with no common superscripts differ ($P = 0.05$).

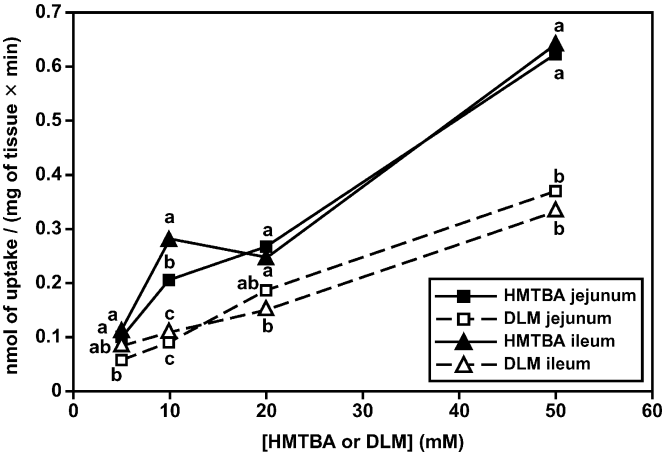

FIGURE 3. Rates of uptake of 5 to 50 m*M* DL-2-hydroxy-4(methylthio) butanoic acid (HMTBA) and DL-methionine (DLM) in vitro. The rates of 5, 10, 20, and 50 m*M* HMTBA and DLM absorption in jejunum and ileum were calculated from the data presented in Figure 2. Uptake rates were compared within each concentration. Rates with no common superscripts differ ($P = 0.05$).

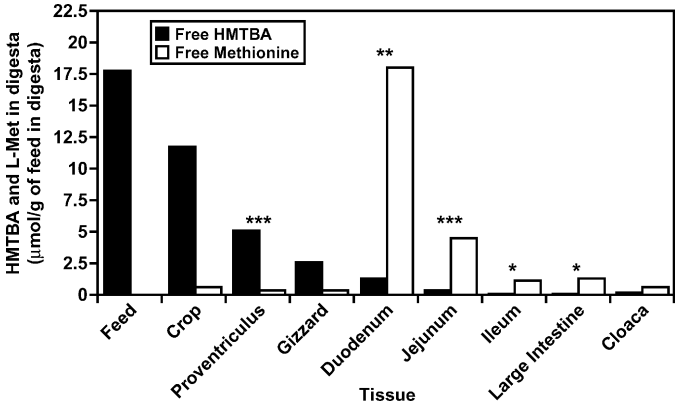

FIGURE 4. Disappearance of DL-2-hydroxy-4(methylthio) butanoic acid (HMTBA) and L-methionine from the digesta in vivo. Broilers were fed commercial type diets supplemented with 0.21% HMTBA. Feed, and digesta from the indicated compartments, were analyzed for the concentration of free HMTBA and free methionine as described in Materials and Methods. Asterisks denote significant differences in HMTBA vs. L-methionine concentrations within a given tissue: *$P = 0.05$; **$P = 0.001$; ***$P = 0.0001$.

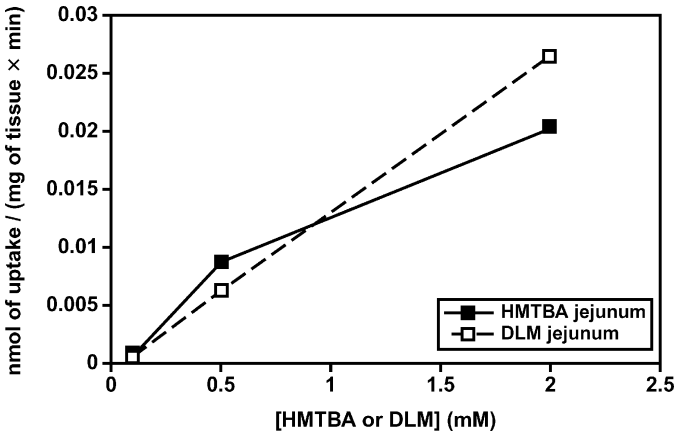

FIGURE 5. Rates of uptake of 0.1 to 2 m*M* DL-2-hydroxy-4(methylthio) butanoic acid (HMTBA) and DL-methionine (DLM) in the jejunum in vitro. The rates of 0.1, 0.5, and 2 m*M* HMTBA and DLM absorption in jejunum were determined. Uptake rates were compared within each concentration. There were no significant differences in uptake rates at these concentrations.

Puigserver, 1989; Wakeham and Webb, 1989; Dibner et al., 1992; Zheng et al., 1994; Maenz and Engele-Schaan, 1996b; Brandsch and Brandsch, 2003). Both mechanisms require receptors in the cell membrane to facilitate the transfer of the amino acid from the intestinal lumen across the membrane to the cytoplasm of the absorptive enterocyte (Brandsch and Brandsch, 2003). Receptor-mediated uptake is necessary because methionine is charged or polar at all pH levels and, therefore, unlikely to diffuse across the lipid bilayer (Nelson and Cox, 2000). Active transport expends cellular energy in the form of ATP hydrolysis, and both of these mechanisms exhibit saturation kinetics (Nelson and Cox, 2000; Brandsch and Brandsch, 2003). Moreover, the 2 isomers of DLM compete for many of the same transporters, and these transporters have a much lower affinity for the D-isomer than the L-isomer (Lerner and Taylor, 1967; Brachet et al., 1987; Knight et al., 1994; Maenz and Engele-Schaan, 1996b). As such, D-methionine and DLM absorption are substantially slower than L-methionine uptake (Brachet et al., 1987; Brachet and Puigserver, 1987, 1989; Zheng et al., 1994). This difference in D- versus L-methionine uptake rates is particularly great during periods of heat stress (Dibner et al., 1992; Knight et al., 1994).

The HMTBA is taken up differently than DLM. By using intestinal slices, Brachet and Puigserver (1987, 1989) found that HMTBA is taken up by a combination of diffusion and carrier-mediated uptake, and the carrier is the lactic acid carrier. At higher concentrations of HMTBA (7 m*M* and higher), uptake was mostly by diffusion, whereas at lower concentrations the lactic acid carrier became more important (Brachet and Puigserver, 1987). We have also reported that diffusion plays a major role in HMTBA uptake in intestinal slices (Knight and Dibner, 1984), and others have observed HMTBA diffusion into BBMV (Brachet and Puigserver, 1989; Maenz and Engele-Schaan, 1996a). That diffusion plays a major role in HMTBA uptake is consistent with the chemical structure of HMTBA as an organic acid (Dibner and Buttin, 2002). When undissociated, organic acids are able to diffuse through cell membranes into cells (Naupert and Rommel, 1975; Walter and Gutknecht, 1984; Von Engelhardt et al., 1989). This would be expected to occur primarily in the upper GI tract, where pH values are low. However, even in the distal GI tract where the luminal pH is neutral, an acidic microclimate exists at the enterocyte cell surfaces, which also allows diffusion of organic acids to occur (Hogben et al., 1959; Von Engelhardt et al., 1989; Shiau, 1990; Shiau et al., 1990). That HMTBA can be taken up by the lactic acid carrier is consistent with the observation that lactic acid and HMTBA are structurally similar (Dibner and Buttin, 2002).

Several publications using a variety of experimental models have shown HMTBA is absorbed at rates equal to or greater than DLM and that both methionine sources are absorbed completely by the animal (Saroka and Combs, 1983; Knight and Dibner, 1984; Esteve-Garcia, 1988; Han et al., 1990; Dibner et al., 1992; Esteve-Garcia and Austic, 1993). In contrast to these data, some papers utilizing an in vitro BBMV system have called into question the relative rates of uptake of these 2 methionine sources (see above). The BBMV provide a good model system to estimate uptake rates of actively transported molecules, including methionine. This is because these transporters can accumulate substrate into the BBMV against a concentration gradient. However, this model system is inappropriate to estimate rates of HMTBA uptake, because HMTBA absorption has such a strong diffusion component. First, diffusion is driven by the existence of a concentration gradient. In vivo, the HMTBA concentration gradient from the lumen to the absorptive epithelium is maintained in at least 3 ways: deprotonation of HMTBA in the near-neutral cytoplasm of the cell, conversion of HMTBA to L-methionine, and export out of the cell via the bloodstream (Dibner and Knight, 1984; Knight and Dibner, 1984; McCollum et al., 2000). The second and third mechanisms do not occur in the BBMV system, thereby disrupting the concentration gradient and minimizing diffusion. Second, most of the BBMV experiments with HMTBA were conducted with the outer buffer at pH greater than 7 (Brachet and Puigserver, 1987, 1989; Maenz and Engele-Schaan, 1996a,b). At this pH, most of the HMTBA (pKa 3.53) would be dissociated and ionized and, therefore, unable to diffuse efficiently across a lipid membrane. In fact, these papers often report that there is little or no diffusion of HMTBA into the BBMV at near-neutral pH. The inability of HMTBA to diffuse at near-neutral pH was offered as an explanation for the relatively low rate of HMTBA uptake by one group that performed these experiments (Brachet and Puigserver, 1989). Indeed, their data indicate that HMTBA diffusion into jejunal rings was 2.5-fold that of diffusion into BBMV (Brachet and Puigserver, 1987, 1989). Therefore, the BBMV model system minimizes uptake by diffusion and as such is an inappropriate model system to evaluate HMTBA uptake rates.

We wished to investigate the rates of HMTBA and DLM uptake in an in vitro system that did not have these shortcomings. In vitro intestinal slices have been used to evaluate uptake of methionine and HMTBA (Lerner and Taylor, 1967; Lerner et al., 1968; Knight and Dibner, 1984; Brachet et al., 1987; Brachet and Puigserver, 1987; Dibner et al., 1992; Knight et al., 1994) as well as a variety of other nutrients, including other amino acids and amino acid analogs (Agar et al., 1954; Robinson and Felber, 1964, 1965; Olsen and Rosenberg, 1970; Gonzalez and Vinardell, 1992, 1993; Stewart et al., 1995; Kettunen et al., 2001), lipids and fatty acids (Johnston and Borgstrom, 1964; Feldman and Borgstrom, 1966; Nilsson and Borgstrom, 1967; Thiesen et al., 2003), sugars (Olsen and Rosenberg, 1970; Elsas and MacDonell, 1972; Gonzalez and Vinardell, 1992), minerals (Schachter et al., 1961; Ziporin et al., 1973), vitamins (Olson, 1964), and drugs (Osiecka et al., 1985; Porter et al., 1985).

It is interesting to note that, with respect to drug uptake, everted intestinal slices are reported to give more consistent results than BBMV, correlate well with in vivo bioavailability, and are judged to be the best in vitro absorption model (Osiecka et al., 1985; Porter et al., 1985). Furthermore, the uptake rate of phenylalanine in everted intestinal rings was shown to be similar to the uptake rate measured by intestinal perfusion (Stewart et al., 1995). Similarly, Brachet and Puigserver observed that the uptake rates of D- and L-methionine in slices agreed with the rates they measured in vivo (Brachet et al., 1987). Finally, HMTBA and DLM have been shown to be absorbed via their major routes of uptake (diffusion and carrier-mediated uptake for HMTBA; active transport and carrier-mediated uptake for DLM) in in vitro intestinal slices, making this a very useful system to compare relative uptake rates of these methionine sources (Knight and Dibner, 1984; Brachet and Puigserver, 1987; Dibner et al., 1992; Knight et al., 1994).

Although BBMV tend to minimize diffusion, and, therefore, HMTBA uptake rates are underestimated (Brachet and Puigserver, 1989), in vitro intestinal slices clearly allow efficient HMTBA uptake by diffusion (Knight and Dibner, 1984; Brachet and Puigserver, 1987). This can be explained by a variety of reasons. First, this system would be better expected to maintain the concentration gradient of HMTBA. Although there is no blood flow to carry absorbed HMTBA out of the system, the concentration gradient could be maintained by the deprotonation of HMTBA that absorbs into the near-neutral cytoplasm of the intestinal epithelium and the conversion of a fraction of HMTBA to L-methionine (Dibner and Knight, 1984; Knight and Dibner, 1984; McCollum et al., 2000). Second, the experiments can be conducted at physiological pH. Although our experiments were conducted at pH 7, to mimic the pH of the intestinal lumen, the use of whole slices of tissue rather than membrane vesicles allows preservation of the acidic microclimate at the cell surface that facilitates HMTBA diffusion. In fact, the linearity of HMTBA uptake over a wide variety of concentrations was an indication that diffusion was not limited in this system (Figures 3 and 5). Our data show that absorption of both methionine sources was dependent on time and concentration. At all concentrations tested, the rate of intestinal HMTBA absorption was either not different than or was greater than the uptake of DLM. These data provide further evidence that HMTBA is absorbed efficiently.

Despite its drawbacks, the BBMV system has proven to be useful in describing other, nondiffusive mechanisms of HMTBA uptake. For example, Brachet and Puigserver demonstrated that HMTBA transport into BBMV was competitively inhibited by lactate, indicating that HMTBA can be taken up by the lactate carrier (Brachet and Puigserver, 1987, 1989). The lactate carrier belongs to the proton-linked monocarboxylate transporter family (Poole and Halestrap, 1993; Halestrap and Price, 1999). The monocarboxylate transporter proteins catalyze the facilitated diffusion of lactate and other monocarboxylic acid molecules with a proton. Therefore, it would be predicted that nondiffusive uptake of HMTBA would be proton dependent. In fact, BBMV experiments, as well as experiments using *Xenopus* oocytes injected with broiler intestinal mucosal cell mRNA, have demonstrated that this hypothesis is correct (Maenz and Engele-Schaan, 1996b; Pan et al., 2002).

Our data also provide the first demonstration that HMTBA is absorbed along the entire GI tract of the bird. Most papers describing methionine source absorption have focused on the small intestine, presumably because that is where methionine and other amino acids are absorbed (Brandsch and Brandsch, 2003). However, until it is converted to L-methionine, HMTBA is an organic acid, not an amino acid (Dibner and Buttin, 2002). As such, there is no reason to believe that HMTBA absorption would be limited to the small intestine. Diffusion into cells represents a major route of organic acid uptake, which occurs most rapidly at low pH when more of the acid will be protonated and lipophilic (Naupert and Rommel, 1975; Walter and Gutknecht, 1984; Von Engelhardt et al., 1989). Our data are consistent with this notion in that most of the dietary HMTBA (pKa 3.53) was absorbed out of the digesta in the acidic environment of the crop, proventriculus, and gizzard (Figure 4). The feed was analyzed to contain 17.8 m*M* HMTBA, whereas the in the gizzard it was only 2.6 m*M*. HMTBA levels in the jejunum and ileum were 0.4 and 0.08 m*M*, respectively, indicating 99.6% absorption by the ileum. Although the experiments reported here and elsewhere that demonstrate HMTBA is absorbed efficiently in the small intestine are informative, our data suggest that more attention should be paid to the upper GI tract, where the majority of HMTBA absorption occurs.

Our data also indicate that, as expected, L-methionine is absorbed primarily in the small intestine (Figure 4). Because the diet was supplemented with HMTBA and not DLM, very little free methionine was measured in the upper GI tract. What little there was might have been due to pepsin-mediated digestion (Hill, 1971). The pancreas secretes trypsin, chymotrypsin, and other proteases

into the duodenum, which explains the large peak of free methionine measured in this compartment (Hill, 1971). From here, free methionine is absorbed in the small intestine.

There have been recent reports claiming that HMTBA is degraded by microbes in the small intestine (Lemme et al., 2001; Drew et al., 2003; Drew, 2004). However, the chance for HMTBA uptake by fermentative microbes appears to be relatively small compared with that of methionine. Our data indicate that approximately 85% of dietary HMTBA is absorbed prior to the duodenum (Figure 4). Furthermore, free methionine concentrations in the small and large intestines were approximately 9 to 14 times greater than HMTBA levels on a molar basis (Figure 4). Therefore, it is unclear how any putative HMTBA degradation would be of significance relative to intestinal degradation of methionine. Indeed, the papers that report HMTBA degradation data and performance data show that performance of HMTBA-supplemented broilers is equal to that of DLM-supplemented birds (Lemme et al., 2001; Drew et al., 2003). This equal growth occurred despite the fact that HMTBA and DLM were supplemented on an equal weight rather than an equimolar basis, resulting in the HMTBA-fed birds being undersupplemented relative to the DLM-fed birds (Drew et al., 2003).

The experiments in this paper demonstrate that intestinal HMTBA absorption occurs at rates equal to or greater than that of DLM absorption. In addition, we provide the first evidence that HMTBA absorption occurs along the entire length of the GI tract and primarily in the proximal GI tract prior to the small intestine. These data support the conclusion that HMTBA is a highly effective source of supplemental methionine.

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