Hepatic Metabolism of 2-Hydroxy-4-Methylthiobutyrate in Growing Lambs

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ABSTRACT

This study was undertaken to determine how, and where, 2-hydroxy-4-methylthiobutyrate (HMTBA) can augment Met metabolism in lambs. Four lambs (initial body weight of 50 kg, SE = 2, and 6 mo of age) prepared with catheters in the mesenteric, portal, hepatic, and jugular veins plus the aorta, were fed at 1.5× maintenance on a grass hay, barley, fish meal, molasses/premix (5:3:1:1, as fed) diet, supplied as hourly meals. Lambs were infused for 10 h with [methyl-²H₃]Met (0.11 mmol/h) in a jugular vein and p-aminohippurate into the mesenteric vein. From 1 h onwards, successive 3-h infusions of saline (control), 0.55 mg/min (3.67 μmol/min), and 4.44 mg/min (29.6 μmol/min) of HMTBA were also infused into the mesenteric vein. Plasma, sampled continuously, was collected every 20 min during the last 60 min of each infusion. All infused HMTBA was recovered at the portal vein with 25% extracted subsequently by the liver. Portal appearance of total Cys and Met was unaltered by HMTBA infusion, but net splanchnic appearance of Cys increased (0.04, 0.08, 0.23 mmol/h, SEM = 0.05), whereas Met decreased (0.14, -0.01, -0.21 mmol/h, SED = 0.05). Despite this, arterial Met increased (27.0, 30.7, 51.5 μM, SEM = 2.1) as did Met irreversible loss rate (27.6, 28.7, 40.1 μmol/h, SEM = 0.51), equivalent to 40% of the HMTBA reentering the plasma after conversion to Met. These data indicate that, in ruminants, HMTBA is probably converted to Met within peripheral tissues; that is, where the metabolic need for Met exists.

Key words: 2-hydroxy-4-methylthiobutyrate, methionine, cysteine, lamb

INTRODUCTION

L-Methionine is an essential AA for production animals, but is limiting in a number of protein sources

used commercially (NRC, 2001). In ruminants, supplements in the form of protected Met are often added to diets. Protection is necessary to prevent or reduce microbial degradation of Met in the rumen. Commercial supplements available contain a racemic mixture of DL-Met, and at least part of the increases in plasma Met observed in cattle are due to the accumulation of the D-isomer (Lobley et al., 2001b; Vázquez-Añón et al., 2001). In contrast, the hydroxy analog of Met [2-hydroxy-4-methylthiobutanoic acid (**HMTBA**), Alimet, Novus International Inc., St. Louis, MO], although supplied as the DL racemic mixture, yields only L-Met in ruminants (Lobley et al., 2001b; Vázquez-Añón et al., 2001). One apparent disadvantage with HMTBA, however, is that, following dietary administration, increases in plasma Met concentrations are less than expected (Vázquez-Añón et al., 2001; Koenig et al., 2002). This may be due to 2 reasons, either poorer absorption or differences in how HMTBA and Met are handled by body tissues. In dairy cows, 50% rumen bypass and absorption rates have been reported (Koenig et al., 1999) but this may depend critically on rate of passage (Vázquez-Añón et al., 2001). In terms of tissue metabolism, data from anesthetized chickens indicate that the liver has considerable capacity to remove HMTBA, even at amounts in excess of normal levels of supplementation (Wang et al., 2001). Furthermore, although this extraction did result in increased Met appearance in the hepatic vein, this was much less than when the equivalent amount of DL-Met was supplied (Song et al., 2001). Thus, the anabolic responses observed with HMTBA in pigs (Knight et al., 1998) and poultry (Garlich, 1985) may be because of availability as nonfree Met forms or metabolites; for example, Cys, either free or as glutathione, or Met present in plasma export proteins. Nonetheless, plasma Met does increase in cattle following dietary supplementation with HMTBA (Koenig et al., 1999), indicating that conversion does occur in ruminants, but the site and extent of this metabolism is unknown.

The question of the fate of absorbed HMTBA is addressed in this study and the companion paper (Lobley et al., 2006). In view of the general paucity of information on metabolic interconversions of HMTBA *in vivo*, use was made of conscious lambs. Furthermore, the

Received April 29, 2005.

Accepted November 2, 2005.

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initial emphasis was on hepatic metabolism in view of the observations in poultry that the liver is the major site of removal (Wang et al., 2001) and metabolism (Dibner and Ivey, 1992) of HMTBA. In addition to monitoring splanchnic movements of HMTBA, Met, and Cys, the impact of HMTBA supplementation on whole-body Met flux was evaluated. This allowed the impact of alterations in body Met metabolism on plasma Met concentration to be investigated. Parts of this study have been presented in abstract and review form (Wester et al., 2000; Lobley et al., 2001b).

MATERIALS AND METHODS

Animals and Design

This experiment was approved by the Ethical Review Committee of the Rowett Research Institute and conformed to UK legislation of the Animals (Scientific Procedures) Act of 1986. Four Suffolk crossbred lambs (initial BW of 50 kg, SE = 2, and 6 mo of age), obtained from the Rowett Research Institute flock, were surgically prepared with silicone rubber catheters in the hepatic, portal, and mesenteric veins, and the abdominal aorta (Lobley et al., 1995). Lambs were allowed 2 wk of recovery in heated floor pens after surgery before transfer into metabolism crates where they were fed hourly (by automatic feeder) a grass hay/barley-based concentrate diet (50% grass hay, 30% barley, 9% fish meal, 10% molasses, and 1% vitamin and mineral mix; ingredients mixed as fed; 9.3 MJ of ME/kg of DM) at 1.5× maintenance intake (i.e., 675 kJ of ME/kg of BW^{0.75} per d). A temporary polyvinyl chloride catheter (0.8 mm i.d. × 1.2 mm o.d., Critchley Engineering, Auburn, NSW, Australia) was inserted into a jugular vein on the day preceding the infusions.

The experimental protocol is shown in Figure 1. On the day of experiment, 7.3 mM L-[methyl-²H₃]Met (99 atom %; Isotec Inc., Miamisburg, OH) was infused for 10 h into the jugular vein (solution infusion rate, 0.25 g/min; 1.85 μmol/min [methyl-²H₃]Met). The [methyl-²H₃]Met was dissolved in physiological saline (154 mM NaCl) containing heparin (700 IU/g; Leo Laboratories Ltd., Princes Risborough, UK). Starting 1 h after the initiation of the [methyl-²H₃]Met infusion, 2 solutions, one of 0.267 M sodium p-aminohippurate (pAH) in 0.05 M sodium phosphate, pH 7.4, and the other, 0.15 M PBS, pH 7.4, were infused into the mesenteric vein for 3 h, each at a rate of 0.25 g/min. After 2 h, 6-mL blood samples were collected continuously over ice by peristaltic pump for three 20-min periods (Lobley et al., 1995) from the aorta plus the portal and hepatic veins.

After the last blood sample was collected, solutions of 14.7 mM HMTBA in 0.15 M PBS, pH 7.4, and 0.267 M pAH in 0.05 M sodium phosphate, pH 7.4, were in-

fused (15 g/h) into the mesenteric vein for 3 h. The HMTBA was infused at a rate of 0.55 mg/min (3.68 μmol/min). Again, three 20-min continuous collections from the aorta, and hepatic and portal veins were taken over the last hour. Then the concentration of HMTBA was increased to 118.4 mM (4.44 mg/min, 29.6 μmol/min) for the final 3 h, with three 20-min samples taken continuously during the last hour of infusion.

Analyses

Plasma was prepared from blood by centrifugation at 1,000 × g for 15 min at 4°C. Plasma flow was calculated from downstream dilution of pAH by the gravimetric procedure (Lobley et al., 1995). The isotope-dilution technique (Calder et al., 1999) was used to determine concentrations of Met, Cys, and HMTBA in plasma. Briefly, to a known weight (0.9 g) of plasma, was added 0.1 g of a mixture of 0.1 M dithiothreitol, 0.2 mM [1-¹³C]Met (99 atom %; Isotec Inc.), 0.8 mM [1-¹³C]Cys (99 atom %; Isotec Inc.), and 0.2 mM [1-¹³C]HMTBA (see below for synthesis). The plasma sample was vortex mixed and allowed to stand at room temperature for 30 min. Plasma proteins were then precipitated with 200 μL of 38% (wt/wt) sulfosalicylic acid. The sample was centrifuged at 7,200 × g for 5 min and the supernatant applied to a 0.5-mL AG 50W × 8 (H⁺), 100–200 mesh resin column (BioRad Laboratories, Hemel Hempstead, UK). The initial effluent plus the subsequent 0.5-mL water wash was collected for HMTBA analysis. For analysis of Met and Cys, the column was washed with a further 2 × 2 mL of water (discarded), and the AA eluted with 2 mL of 2 M NH₄OH 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 nitrogen. To the dry residue was added 80 μL of 2 M NH₄OH plus 20 μL of 0.1 M dithiothreitol; the solution was left to stand at room temperature for 30 min. The sample was then evaporated to dryness, 70 μL of a 1:1 (vol:vol) mixture of N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide and acetonitrile was added, and the t-butyldimethylsilyl derivative was formed by heating at 90°C for 20 min.

For HMTBA analysis, the initial column effluent and 0.5-mL water wash was extracted with 2 × 2 mL of ethyl acetate, centrifuged at 3,000 × g, and the pooled organic layers dried over sodium sulfate for 10 min. The pooled ethyl acetate was evaporated to dryness at 40°C under nitrogen. To the dry residue was added 70 μL of N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide:acetonitrile, as above, and the t-butyldimethylsilyl derivative of HMTBA was formed by heating at 90°C

Figure 1. Experimental protocol. Plasma was sampled continuously during each collection period from the aorta, and portal and hepatic veins. See *Materials and Methods* for greater detail of 2-hydroxy-4-thiomethylbutyrate (HMTBA), p-aminohippurate (pAH), and [methyl-²H₃]Met and heparin infusions.

for 15 min. Calibration curves were prepared from standards taken through the above procedures.

The separation of HMTBA and the AA Met and Cys was effected on a 25 m × 0.25 mm × 0.25 μm SE-30 CB capillary column (Alltech, Carnthorn, UK) using the following temperature programs: HMTBA, 180°C for 2.5 min, then 25°C/min to 260°C for 10 min; Met and Cys, 160°C for 5 min, then 15°C/min to 270°C for 10 min. Injections (1 μL) were made with a 50:1 split mode. The mass spectrometer was operated in the electron impact mode under selective ion monitoring conditions with the M-57 ions at *m/z* 321 and 322 monitored for natural and [1-¹³C]HMTBA, respectively (see Appendix). Methionine was monitored at *m/z* of 320 and 321 for the natural and [1-¹³C] product, and *m/z* of 292 and 295 for the natural and [methyl-²H] forms; Cys was monitored at *m/z* of 406 and 407 for the natural and [1-¹³C] derivative.

Synthesis of [1-¹³C]HMTBA

First, 150 μmol of [1-¹³C]Met (98 atom %; Isotec Inc.) was converted enzymatically to the oxo-acid using L-amino acid oxidase, according to the method of Nissen et al. (1982). The resulting suspension was centrifuged at 5,000 × *g* for 5 min, and the supernatant applied to

0.3 mL of AG 50 (H⁺) resin. The eluate plus 0.5-mL water wash was collected, made basic with 5 M NaOH, and the oxo-acid reduced to [1-¹³C]HMTBA using sodium borohydride according to the method of Mamer et al. (1986). A stock solution of the [1-¹³C]HMTBA was prepared in water and stored at 4°C. This stock solution was analyzed for isotopic purity (97 atom %) and found to contain no Met.

Sensitivity and Precision of HMTBA Analysis

Sensitivity and precision of the isotope dilution approach to determine concentrations were determined from calibration slopes where known amounts of unlabelled HMTBA (10 to 80 nmol) were added to 0.9 g of pooled ovine plasma that did not contain any natural HMTBA but with a nominal 20 nmol of [1-¹³C]HMTBA present. The regression of ¹²C HMTBA against the true 321/322 ratio gave an equation of 0.06290x + 0.010809, with an R² = 0.99997. A plasma sample containing 73.21 nmol/g was analyzed in quadruplicate and gave a coefficient of variation of 0.53%. Similar sensitivity and precision were obtained at lower concentrations. For example, the reverse procedure, whereby 2 nmol of ¹²C HMTBA was added to 0.9 g of plasma containing additions of 1 to 5 nmol of [1-¹³C]HMTBA, gave a regression

equation for ^{13}C HMTBA against the true 322/321 ratio of $0.35749x + 0.00767$, with an $R^2 = 0.99995$. Similarly, with this calibration, a plasma sample containing 3.90 nmol/g was analyzed in quadruplicate with a coefficient of variation of 0.52%. The lower limits of detection within plasma were not fully determined but, from the various calibration curves, values of between 0.5 and 1.0 nmol/g would be easily quantified with the necessary degree of precision.

Calculations

Plasma flows were determined by gravimetric analysis of pAH concentrations as described previously (Lobley et al., 1995). Metabolite net and total flows ($\mu\text{mol}/\text{min}$) across the digestive tract and liver for HMTBA, Met, and Cys were calculated as follows:

Total portal vein appearance [absorption plus that recirculated via the arterial supply to the portal-drained viscera (**PDV**)] = $[P] \times PF_p$

Total hepatic supply (absorption plus recirculation entering liver via portal vein and hepatic artery) = $([P] \times PF_p) + ([A] \times PF_a)$

Net portal vein appearance (absorption plus infusion) = $([P] - [A]) \times PF_p$

Total splanchnic appearance (total liver outflow) = $[H] \times PF_h$

Net splanchnic appearance (absorption plus infusion minus liver removal) = $([H] - [A]) \times PF_h$

Net hepatic uptake (absorption plus infusion removed by liver) = $([H] - [A]) \times PF_a + ([H] - [P]) \times PF_p$

% Net portal appearance removed by liver = (net hepatic uptake / net portal appearance) $\times 100$

% Total hepatic inflow removed by liver = (net hepatic uptake / total liver appearance) $\times 100$

where $[A]$, $[P]$, and $[H]$ represent concentrations in arterial, portal vein, and hepatic vein plasma respectively, and PF represents plasma flow in the hepatic artery (a) and portal (p) or hepatic (h) veins. Positive numbers represent net appearance across a tissue bed whereas negative values indicate net uptake. Values were calculated for each 20-min sample collection and the mean then used for statistical analysis.

Met irreversible loss rate (**ILR**; $\mu\text{mol}/\text{min}$) was calculated from

$$(99/E_a - 1) \times \text{infusion rate [methyl-}^2\text{H}_3\text{]Met}$$

where 99 is the molar percent of the infusate and E_a is the enrichment (molar percent excess) of the arterial plasma [methyl- $^2\text{H}_3$]Met.

The tissue contributions to Met ILR ($\mu\text{mol}/\text{min}$) were calculated as follows. For PDV [representing the total gastrointestinal tract (**GIT**)]

$$= ([A] \times E_a - [P] \times E_p)/E_a \times PF_p$$

and for the combined splanchnic tissues (i.e., GIT plus liver)

$$= ([A] \times E_a - [H] \times E_h)/E_a \times PF_h.$$

Liver Met ILR was the difference between combined splanchnic tissues and PDV values. The enrichment of arterial plasma Met was selected as precursor to allow assessment of the relative contributions of the tissues to whole body Met ILR.

Statistical Analyses

Data were compared using ANOVA (GenStat for Windows, version 6, release 6.1; Lawes Educational Trust, Rothamsted, UK) with sheep as a random effect and level of HMTBA as treatment (fixed effect). For analyses involving Met and Cys, this involved 6 residual degrees of freedom, and where the treatment effect was significant ($P < 0.05$ or better), comparisons between treatments were assessed by a protected Fisher's least significance difference test (P set at 0.05 for 2-tailed analysis). When HMTBA measurement was a component of the response variable (e.g., plasma HMTBA concentration), only the 2 treatments with HMTBA (i.e., the 0.55 and 4.44 mg/min infusions) were analyzed; this yielded 3 degrees of freedom, with main effects at the same significance as the treatment differences. Analyses were based on the mean of the 3 samples taken for the last hour of each period, these having been first checked by inspection to ensure that concentrations and arterio-venous differences were constant. Recovery of infused HMTBA in the portal vein (i.e., the difference in recovery/infused from unity) was assessed by a posthoc t -test based on the ANOVA.

RESULTS

HMTBA Transfers

Plasma flows in the portal vein (draining the total GIT) and hepatic vein (draining the GIT and liver) were unaltered ($P > 0.35$) by HMTBA infusion (Table 1). Recovery of infused HMTBA between the mesenteric and portal veins did not differ from unity for either dose,

Table 1. Plasma 2-hydroxy-4-thiomethylbutyrate (HMTBA) concentrations and transfers across the splanchnic tissues of lambs infused into the mesenteric vein with 2 levels of HMTBA

	Infusion level of HMTBA, $\mu\text{mol/min}$			SEM	P^1
	0	3.68	29.6		
Plasma flow, g/min					
Hepatic artery	391	218	267	80	NS ²
Portal vein	1,591	1,465	1,486	85	NS
Hepatic vein	1,983	1,683	1,753	139	NS
Plasma concentrations, nmol/g					
Arterial plasma	—	12.5	114.3	6.4	0.002
Portal vein	—	15.3	138.7	6.5	<0.001
Hepatic vein	—	14.2	130.4	6.5	0.001
Total flows, $\mu\text{mol/min}$					
Portal appearance	—	22.4	205.7	13.6	0.002
Hepatic inflow	—	24.9	233.9	10.5	<0.001
Splanchnic appearance	—	23.9	226.6	9.8	<0.001
Net flows, $\mu\text{mol/min}$					
Portal appearance	—	3.8	35.2	1.98	0.002
Hepatic removal	—	-1.0	-7.2	0.90	0.016
Splanchnic appearance	—	2.8	27.9	1.78	0.002

¹By ANOVA with sheep treated as a random effect. For plasma flow measurements, 6 residual degrees of freedom. For HMTBA measurements, during control periods no HMTBA could be detected (<0.2 nmol/mL) and so all comparisons are for the 2 levels of HMTBA. Again, sheep were treated as a random effect with 3 residual degrees of freedom.

²NS = Nonsignificant.

based on a posthoc *t*-test (mean = 1.04, SEM = 0.06, $P = 0.53$). 2-Hydroxy-4-methylthiobutyrate was not detected in plasma (<0.2 μM) during the control periods, but increased in all plasma samples during the HMTBA infusions. Similar values between each of the 20-min samples taken over the last hour of infusion indicated that a metabolic steady state had been achieved (data not shown). Arterial plasma HMTBA concentrations were raised approximately 9-fold ($P < 0.001$) in response to an 8-fold increase in the dose infused. This was because only 24% of the infused HMTBA was extracted by the liver. Although the absolute amount extracted varied between the 2 doses (1 vs. 7 $\mu\text{mol/min}$, $P = 0.016$), it remained constant as a fraction of the dose (27 vs. 21%, not significant). The HMTBA that bypassed the liver was returned via recirculation and increased the hepatic inflow in both the portal vein and hepatic artery. Consequently, total hepatic inflow was 6.7- to 7.9-fold greater ($P < 0.001$) than the dose infused. When expressed as a fraction of this total inflow, only 3 to 4% was removed by the liver; this was similar at both doses of HMTBA.

Met and Cys Transfers

Arterial plasma Met concentrations increased at both HMTBA infusions ($P < 0.05$), but was particularly elevated ($P < 0.001$) at the higher dose (Table 2). In contrast, plasma concentrations of Cys remained unaltered (higher dose) or were slightly decreased (lower dose, $P < 0.05$). In consequence, total portal appearance of Met

(net absorption plus recirculation) increased ($P = 0.001$) at the higher dose of HMTBA, but appearance of Cys was unaltered. Net absorption of Met was unaltered during the HMTBA infusion and accounted for 9 to 11% of total portal appearance. Net absorption of Cys was also unaltered by HMTBA infusion and represented less than 2% of total portal appearance.

The liver removed both Met and Cys under control infusions; this represented 73 and 75% of net absorption, respectively. When compared with total hepatic inflow, however, the extractions decreased to 10 and 1%, respectively. During HMTBA infusion, the absolute amount of Met removed by the liver increased at the higher dose ($P = 0.004$). Consequently, hepatic removal of Met at this HMTBA dose exceeded net absorption of Met (-10.81 vs. 7.33 $\mu\text{mol/min}$, SEM = 1.41, $P = 0.003$), such that net splanchnic release (i.e., the amount available for peripheral tissues) was negative for all sheep. Nonetheless, the fraction of total Met inflow to the liver removed remained constant (10 to 11%), and the increase in absolute removal was a function of the increased amount recirculated as part of the elevated arterial concentrations.

In contrast, as the HMTBA dose increased there was a nonsignificant export of Cys from the liver, rather than uptake. Therefore, net availability of Cys beyond the liver (splanchnic appearance) increased ($P = 0.024$) with HMTBA supply. Again, the proportion of total hepatic supply of Cys removed remained low (approximately 1%) and was unaffected by HMTBA infusion.

Table 2. Plasma Met and Cys concentrations and transfers across the splanchnic tissues of lambs infused into the mesenteric vein with 2 levels of 2-hydroxy-4-thiomethylbutyrate (HMTBA)

	Infusion level of HMTBA, $\mu\text{mol/min}$			SEM	P^1
	0	3.68	29.6		
Plasma Met, nmol/g					
Arterial plasma	27.0 ^a	30.7 ^b	51.5 ^c	0.9	<0.001
Portal vein	32.6 ^a	34.3 ^a	56.3 ^b	1.1	<0.001
Hepatic vein	28.2 ^a	30.5 ^a	49.5 ^b	0.9	<0.001
Plasma Cys, nmol/g					
Arterial plasma	97.3 ^a	92.7 ^b	95.8 ^{ab}	0.9	0.028
Portal vein	99.2	93.9	97.3	1.4	0.097
Hepatic vein	97.6	93.5	97.9	1.1	0.061
Total transfers, $\mu\text{mol/min}$					
Met					
Portal appearance	51.5 ^a	50.7 ^a	83.8 ^b	3.9	0.001
Hepatic inflow	62.1 ^a	57.1 ^a	97.8 ^b	5.2	0.003
Splanchnic appearance	55.6 ^a	51.5 ^a	86.9 ^b	4.9	0.004
Cys					
Portal appearance	156.4	136.6	144.3	9.2	NS ²
Hepatic inflow	196.9	157.4	170.9	15.5	NS
Splanchnic appearance	194.5	157.0	172.4	14.5	NS
Net transfers, $\mu\text{mol/min}$					
Met					
Portal appearance	8.88	5.39	7.33	0.99	NS
Hepatic appearance	-6.50 ^a	-5.58 ^a	-10.82 ^b	0.70	0.022
Splanchnic appearance	2.38 ^a	-0.20 ^a	-3.48 ^b	0.80	0.006
Cys					
Portal appearance	3.07	1.76	2.24	1.34	NS
Hepatic appearance	-2.34	-0.39	1.48	1.08	NS
Splanchnic appearance	0.74 ^a	1.37 ^a	3.76 ^b	0.58	0.024

^{a-c}Significant differences between values within rows were tested by the Fisher's protected least significant difference test for multiple comparisons and unlike superscripts indicate $P < 0.05$ or better.

¹By ANOVA with sheep treated as a random effect, 6 residual degrees of freedom.

²NS = Nonsignificant.

Whole-Body Met Flux

Under control infusions, whole body Met ILR (1.8 mmol/h, Table 3) was compatible with other studies under similar conditions (Lobley et al., 1996). This is equivalent to 300 g/d of protein flux, without correction

for oxidative losses. In response to the higher HMTBA infusion, Met flux increased by 45% ($P < 0.001$), equivalent to 40% (range 36 to 42%) of the infused HMTBA. Met ILR (an index of protein synthesis) across the PDV (representing the total GIT) was unaltered by HMTBA infusion. In contrast, Met ILR across the liver was in-

Table 3. Whole-body Met ILR and tissue protein ILR in lambs infused with HMTBA¹

	Infusion level of HMTBA, $\mu\text{mol/min}$			SEM	P^2
	0	3.68	29.6		
Whole-body ILR, $\mu\text{mol/min}$	27.6 ^a	28.7 ^a	40.1 ^b	0.5	<0.001
Tissue ILR, $\mu\text{mol/min}$					
PDV	8.4	9.9	9.2	1.2	NS ³
Liver	8.7 ^a	5.8 ^a	12.6 ^b	1.0	0.007
TSP	17.2	15.6	21.8	1.8	NS
Tissue ILR: whole-body ILR					
PDV	0.31	0.35	0.23	0.04	NS
Liver	0.32	0.20	0.31	0.04	NS
TSP	0.62	0.56	0.54	0.06	NS

^{a,b}Significant differences between values within rows were tested by the Fisher's protected least square difference test for multiple comparisons and unlike superscripts indicate $P < 0.05$ or better.

¹ILR = Irreversible loss rate; HMTBA = 2-hydroxy-4-thiomethylbutyrate; PDV = portal-drained viscera; TSP = total splanchnic tissues.

²By ANOVA with sheep treated as a random effect, 6 residual degrees of freedom.

³NS = Nonsignificant.

creased ($P = 0.007$) at the higher HMTBA dose. Despite these changes, the contributions of both the PDV and liver to whole-body Met ILR were unaltered by HMTBA infusion at 0.30 and 0.28, respectively.

DISCUSSION

The primary objective of this paper was to examine the fate of absorbed HMTBA in the conscious lamb. Based on findings in poultry (Dibner and Ivey, 1992; Wang et al., 2001), the initial focus was on the liver as the principal site of HMTBA removal and metabolism. It quickly became apparent, however, that in lambs, the liver was not the principal site of HMTBA metabolism, and so monitoring of whole-body use of Met became necessary. Thus, in addition to tracing the splanchnic movements of HMTBA, Met, and Cys, the impact of HMTBA supplementation on whole-body Met flux was evaluated. This allowed comparisons between change in plasma Met concentrations and alteration in body Met metabolism.

Before in vivo experiments could be performed, analytical methods to measure arterio-venous differences of physiological HMTBA concentrations needed to be refined. The development of analytical procedures for HMTBA based on isotope dilution, rather than conventional HPLC, considerably increased the sensitivity and precision of the assay. The original HPLC method (Ontiveros et al., 1987) was able to detect HMTBA at concentrations of 0.5 mM, and even with recent improvements (McCollum et al., 2000), the detection limit was approximately 20 μ M. In terms of detection, the isotope dilution procedure is 100-fold more sensitive than the original method and 20-fold better than the improved technique, and allows much better precision. For example, the HPLC method has a coefficient of variation of approximately 4% (Ontiveros et al., 1987), whereas that for the isotope dilution approach is <1%. These technical improvements allowed the quantification of arterio-venous differences for HMTBA at accuracy similar to those already developed for Met and Cys (Calder et al., 1999). This was important because plasma HMTBA concentrations expected under commercial conditions will probably be below 20 μ M. For example, when scaled to body weight, the current infusions would match 10 and 80 g of HMTBA/d for a dairy cow and, at the lower dose with a putative absorption of 50% (Koenig et al., 1999), this would yield arterial plasma concentrations of approximately 6 μ M. The consequence of the improved analytical precision is demonstrated by the excellent recovery of the 2 infused doses at the portal vein.

The most striking observation was that the liver removed less than 30% of the dose infused into the mesenteric vein. This fractional removal of net supply re-

mained constant across the 8-fold difference in rate of HMTBA infusion and would suggest that neither uptake nor metabolism by the liver are saturated across the amounts likely to be used in commercial practice. Furthermore, these data are similar to the 37% net hepatic extraction in dairy cows infused with 36 g/d of HMTBA in the jugular vein (Lapierre et al., 2002b). Therefore, in both sheep and dairy cows, most absorbed HMTBA will be available to posthepatic tissues. The relatively low affinity of the liver for HMTBA was shown even more clearly when the total hepatic supply (i.e., infused plus that recirculated via arterial supply) is considered, with only 4% extracted per pass. Furthermore, these values observed for fattening lambs at a moderate intake are similar to the 5% removal of total hepatic HMTBA inflow observed for the lactating cow (Lapierre et al., 2002b) and would indicate that fractional extraction by the liver is not related to production status. In contrast, the fraction of total inflow of Met removed by the liver was considerably greater, 10 to 11%, across a 2-fold range in plasma Met concentration. These values compare well with previous studies in sheep in which Met, along with the other essential AA, showed constant fractional removals even when total supply to the liver was altered 5-fold (Lobley et al., 2001a). The fraction of total hepatic inflow of Cys removed was lower than for either HMTBA or Met and considerably below that normally reported (e.g., 15 to 16% in steers; Koeln et al., 1993). This is because the current estimates include all the available Cys in plasma; that is, free Cys, free cystine, and protein-associated Cys. The latter can be substantial in sheep and raises "apparent" Cys concentrations from the 10 to 20 μ M obtained with conventional analytical approaches (Koeln et al., 1993) to the 60 to 90 μ M range (Lee et al., 1993), nearer to the values observed in the present study. Much of the protein-associated Cys exchanges rapidly with the "free" Cys forms and, therefore, is dynamically available to the animal (Lee et al., 1993) so must be included in the measurements.

The observed fraction (4%) of total HMTBA inflow extracted by the liver contrasts markedly with the 86% removal reported for the chicken (Wang et al., 2001). Although there are marked differences in the experimental approaches adopted between the 2 studies, including anesthetized birds vs. conscious sheep and estimated rather than measured blood flows, it is difficult to envisage that these would affect metabolism sufficiently to account for such contrasting responses. Furthermore, although the HMTBA challenge was considerably greater in the chicken (2.2 to 22 mg/min per kg of BW) compared with the sheep (0.01 to 0.09 mg/min per kg of BW), in both species, hepatic extraction remained linear over the respective ranges, indicating

that transport and metabolic pathways were unlikely to be saturated. Thus, HMTBA metabolism appears to genuinely differ between species, but whether this represents an avian vs. mammalian contrast or perhaps ruminant vs. nonruminant, is difficult to say. Much of the understanding of HMTBA metabolism arises from avian studies (e.g., Dibner and Knight, 1984; Dibner and Ivey, 1992), but the current data suggest that not all such interpretations readily extend to ruminants. Indeed, in practice, there appear to be both similarities and differences between the ovine and poultry data. In both species, HMTBA infusion increased arterial Met concentrations, but to a much lesser extent than infusion of equimolar amounts of DL-Met (Song et al., 2001). In birds, however, infusion of HMTBA into the portal vein resulted in net release of Met from the liver (Song et al., 2001) whereas, in sheep, hepatic removal of Met was increased, resulting in lower net availability to peripheral tissues. Thus, although HMTBA lead to increases in Met concentrations in both species, they differed in the origin of this Met increase, the liver being dominant in poultry but playing a lesser role in the lambs.

The HMTBA extracted by the liver can undergo several fates: catabolism (oxidation); conversion to Met and export or use by the cell (thus sparing dietary Met); or conversion to other metabolic products, such as Cys and glutathione (these involve transfer only of the S-moiety). The mass movements determined from the arterio-venous approaches adopted here and in the chicken (Song et al., 2001; Wang et al., 2001) monitor net transfers only and not changes in rates of both influx and efflux from liver cells. Nonetheless, in chickens, most of any excess HMTBA provision is removed by the liver (Wang et al., 2001) with a small output as Met (Song et al., 2001); thus, under the supra-commercial doses used in those studies, most of the extracted HMTBA was presumably catabolized within the liver. In contrast, although there was no apparent net Met output from HMTBA across the sheep liver, there was an increase in splanchnic Cys release and this was equivalent to 41% of HMTBA hepatic removal. This Cys may have arisen from the increased net Met removed by the liver, rather than from the HMTBA extracted. Cysteine (along with sulfate and taurine) is an end product of Met catabolism through the transsulfuration pathway and, in sheep, requires to be synthesized de novo from Met to support important processes such as glutathione synthesis and wool growth (Liu et al., 2000). It is not clear if the remainder of the HMTBA (equivalent to 15% of the dose) extracted by the liver was oxidized.

Despite the fact that net Met supply to peripheral tissues was reduced in sheep during HMTBA infusion,

ILR of Met increased. Normally, for an essential AA, ILR represents either inflows from absorption plus release from protein breakdown or outflows to protein synthesis plus oxidation. These conventional ILR approaches need to be modified, however, when HMTBA is infused as this adds another potential inflow via synthesis de novo. On the inflow side, the changes in Met ILR were not due to altered absorption so must be attributable to either a change in protein degradation or increased synthesis de novo. In fattening lambs, protein synthesis and protein degradation will be nearly equal and studies involving other AA as tracers have shown that 35 to 50% of whole-body protein synthesis is associated with GIT and liver metabolism (e.g., Lobley et al., 1996; Macrae et al., 1997; Lapierre et al., 2002a). These are similar to the ILR contributions for these tissues in the current study and suggest that protein synthesis (and degradation) is unaltered by HMTBA infusion. This then leaves synthesis de novo as the probable cause of the increased ILR and with 40% of the HMTBA being converted to Met, which is then recycled through the plasma compartment. This is a minimum estimate for the quantity of HMTBA converted to Met and any Met synthesized within a tissue and used for anabolic purposes (or oxidized) therein would not contribute to the ILR change.

So where in the body does synthesis of Met from HMTBA occur? The ILR across the GIT was unaltered, as was apparent Met absorption (i.e., net absorption from the diet plus any synthesis from HMTBA by the GIT tissues). In chickens, however, the GIT may synthesize small quantities of Met from HMTBA (Song et al., 2001). Hepatic ILR was increased by 4 $\mu\text{mol/min}$, but this could be attributed to the extra 4 $\mu\text{mol/min}$ of Met removed by the liver. By difference, therefore, this leaves one or more postsplanchnic tissues as the probable source of plasma Met produced from HMTBA.

This kinetic evidence for postsplanchnic metabolism of HMTBA is also supported by the net transfer data. At the highest dose of HMTBA, the liver removed more Met than was actually absorbed, suggesting that Met from peripheral sources was also removed. This would be derived either from tissue mobilization; that is, the sheep would move into negative N balance, or from Met synthesized de novo by peripheral tissues. Mobilization of body tissues is unlikely because concentrations of other plasma AA did not alter. Furthermore, in dairy cows posthepatic supply of Met during HMTBA infusion was only 35% of that needed to support observed milk protein output, again indicating synthesis by peripheral tissue(s) (Lapierre et al., 2002b). As shown in chickens (Dibner and Knight, 1984) and sheep (McCollum et al., 2000), tissues other than the liver can convert HMTBA to Met. These include the rumen (sheep), brain

and muscle (chicken), and small intestine and kidney (both species), so considerable potential for extrahepatic conversion exists. In terms of the magnitude of conversion in the current study, if it is assumed that Met requirements to support production and growth were 2.4 $\mu\text{mol}/\text{min}$ (the amount available beyond the liver under control conditions) then, because net splanchnic supply changed to $-3.5 \mu\text{mol}/\text{min}$ during the higher HMTBA infusion, a minimum of 5.9 $\mu\text{mol}/\text{min}$ of Met must have been synthesized to maintain the same production status. This is within the maximal capacity of organs such as the ovine kidney (McCollum et al., 2000). Such Met synthesis would represent a 21% conversion of postsplanchnic net HMTBA supply, but less than the 12.5 $\mu\text{mol}/\text{min}$ increase in Met ILR. Under the current conditions, therefore, tissue synthesis of Met from HMTBA exceeded Met absorption from the diet. Indeed, this led to an oversupply of Met (absorbed plus synthesized) such that increased anabolism must have occurred or that some of the excess Met was catabolized in posthepatic tissues. Neither of these latter parameters was measured in this study.

Overall, a clear picture of the fate of absorbed HMTBA in sheep (and probably other ruminants; Lapierre et al., 2002b) has emerged. Approximately 25% is removed by the liver, but this does not result in increased hepatic Met release. Instead, most of the absorbed HMTBA is available to peripheral tissues, one or more of which converts this to Met (Lobley et al., 2006). At appropriate levels of absorbed HMTBA, this extrasplanchnic synthesis of Met exceeds Met absorption from the diet, all of which is removed by the liver and presumably catabolized. As Met synthesis *de novo* can also be in excess of needs to support production, some of this is also catabolized. Synthesis of Met within peripheral tissues and, thus, the potential to be retained therein for anabolic purposes would also mean that changes in plasma Met concentrations, resulting from conversion of HMTBA to Met, would be less than observed with DL-Met products that are transferred to the target tissues via the blood.

CONCLUSIONS

The absorbed methionine hydroxy analog, HMTBA, can increase Met availability within ruminants. Contrary to the situation with nonruminants, the ovine liver does not appear to be a major site of Met synthesis and instead it is probable that synthesis occurs in the peripheral target tissues, with any excess Met produced being returned to the liver for catabolism. This mechanism will result in lower concentrations of plasma Met than when an equivalent amount of supplemented Met

is given. Suitable doses of HMTBA can replace completely the need for dietary Met.

ACKNOWLEDGMENTS

The advice offered on statistical matters by G. Holtrop of Biomathematics and Statistics Scotland is gratefully acknowledged.

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APPENDIX

Figure A1. Electron impact spectra of t-butyltrimethylsilyl-2-hydroxy-4-thiomethylbutyrate (tBDMs-HMTBA) derivative. For determination of plasma 2-hydroxy-4-thiomethylbutyrate (HMTBA) concentrations, ion intensities for the M-57 fragment at m/z 321 (natural abundance) and 322 (^{13}C HMTBA) were monitored.