Intestinal absorption and renal excretion of dietary methionine sources by the growing chicken

Enrique Esteve-Garcia and Richard E. Austic

Department of Animal Science and Division of Nutritional Sciences, Cornell University, Ithaca, NY USA

The biopotency of DL-methionine (MET) and 2-hydroxy-4-[methylthio]butanoic acid (DL-HMB) for broiler chicks was determined using a semipurified diet. Based on growth rate and efficiency of feed utilization, DL-HMB was 72% as potent as DL-MET, while the potencies of L-MET and DL-MET were not different. Studies were conducted to determine if differences in intestinal absorption or urinary excretion of these compounds account for differences in bioavailability. ^{14}C-labeled methionine sources were fed to 2- to 3-week-old broiler chicks and the extent of their absorption determined using Cr_2O_3 as a marker of digestibility. All three sources were completely absorbed in the small intestine. Urinary excretion was studied in 4- to 6-week-old broiler chicks. Saline solutions containing inulin, and 20 mmol/L L-MET, 20 mmol/L D-MET, or 5 or 20 mmol/L DL-HMB were infused intravenously. Blood and urine samples were taken at regular intervals over a 50-min period. The plasma concentrations of L-MET, D-MET, and DL-HMB increased during the course of infusion to 0.5 mmol/L. Urinary excretion of L-MET and D-MET remained less than 1% and 2.2% of the filtered load, respectively. Excretion of HMB increased from about 6% to about 18% of the filtered load as plasma concentration increased to about 0.3 mmol/L, and increased abruptly at higher plasma concentrations. Based on these data and plasma HMB concentrations determined in chicks fed DL-HMB, urinary losses of HMB would be less than 1% of the daily intake. These experiments indicate that differences of biopotency between methionine sources are not due to variations in intestinal absorption or urinary excretion. (J. Nutr. Biochem. **4**:576–587, 1993.)

Keywords: L-methionine; D-methionine; methionine hydroxy analog; 2-hydroxy-4[methylthio]butanoic acid

Introduction

Synthetic sources of methionine have been available for more than 30 years in two forms: DL-methionine (DL-MET) and DL-2-hydroxy-4-[methylthio]butanoic acid (DL-HMB). DL-HMB is also known as methionine hydroxy analog. Since these products became available, many experiments have been conducted to establish their bioefficacies in diets of monogastric species, particularly poultry, for which they are used extensively.[1] There is considerable controversy about whether the two sources have the same bioefficacy.[1–3]

Intestinal absorption and urinary excretion are two of the processes that may limit the utilization of methionine sources. Balance studies using adult male chickens indicated that the net retention of DL-MET was nearly 100%,[4,5] whereas that of DL-HMB ranged from 83–86%[4] and 96–99%.[5] Such studies do not reveal the separate digestive and urinary contributions to the retention values, however, because feces and urine are voided together as a common excretory product in avian species.[6]

Transport systems for D-MET[7] and DL-HMB[8,9] have been investigated. It appears from these studies that the relative rates of absorption from the intestine may differ between sources, but it is not clear whether these

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Current address for Dr. Esteve-Garcia is Institut de Recerca i Tecnologia Agroalimentàries, Centre Agropecuari Mas Bové, Apartat 415, 43200 Reus, Spain.
Address reprint requests to Dr. Richard E. Austic at the Department of Animal Science, 248 Morrison Hall, Cornell University, Ithaca, NY 14853 USA.
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differences actually limit the utilization of these compounds. Gordon and Sizer[10] and Knight and Dibner[11] gave oral doses of radioactive DL-HMB or L-MET and found that the rate of accumulation of radioactivity in plasma was equal or greater in chicks given DL-HMB. This was taken as evidence that DL-HMB is absorbed at least as rapidly as DL-MET. The interpretation of such studies, however, is complicated because the plasma concentration is influenced not only by the rate of absorption, but also by the rate at which these compounds disappear from plasma, either by excretion in the urine or by metabolism.

L-amino acids, and in particular L-MET, seem to be very efficiently reabsorbed from renal tubules.[12] Studies by Crampton and Smyth[13] indicated that at physiologic levels, D-MET is efficiently reabsorbed in cats. However, Gordon[14] suggested that although D-MET did not appear in the urine of chickens after an oral dose, it was lost in the urine as 2-keto-4-[methylthio]-butanoic acid (KMB), while DL-HMB behaved in a fashion similar to L-MET. Saunderson[15] reported that the losses of carbon-14 from DL-[1-^{14}C]HMB in excreta after intraperitoneal injection into chicks was greater than that from L-[1-^{14}C]MET, while the loss of carbon-14 from DL-[1-^{14}C]MET was intermediate. However, both investigations involved the use of radiolabeled compounds, and only in Saunderson's study[15] was the radiochemical purity tested. In this study, it was stated that DL-[1-^{14}C]HMB was 90% pure. Because the recovery of carbon-14 in excreta was about 20% of the dose, it was not clear how much of what was recovered was due to the 10% of the dose that was not HMB. Furthermore, in both studies the quantities of each MET source administered were not the same, and the specific activities of the radioactive amino acids and analogs in plasma and tissues were not measured. Because the effects of pool dilution are not known, the results have to be interpreted with caution.

The objectives of the present studies were to determine whether there are differences in the bioefficacies of different methionine sources, and if so, to determine whether these could be attributed to differences in the intestinal absorption or renal excretion of the sources.

Methods and materials

Male broiler Hubbard × Hubbard (Hubbard Farms, Walpole, NH USA) chicks received a practical diet from the day of receipt (2 days after hatching) to the day of the experiment unless otherwise indicated. The chicks were housed in thermostatically controlled cages with raised wire floors. Feed and water were provided ad libitum except as otherwise specified, and the chicks were exposed to 15 hours of light daily from 700 to 2200 hr.

Growth assay

Experiment 1 One-week-old chicks were given diets based on the formula shown in *Table 1*, which satisfied the requirements for all nutrients, except sulfur amino acids, as estimated by the National Research Council.[16] The diet was calculated to have 2940 kcal of metabolizable energy per kg and 21.7% protein. Methionine and cystine concentrations were calculated to be 0.19% and 0.30%, respectively. Additions of L-MET, DL-MET, and calcium salt of DL-HMB (DL-HMB-Ca) were made at the expense of corn starch and were isosulfurous to L-MET. DL-HMB-Ca was considered to be 83% equivalent to L-MET on a weight basis.[17]

Table 1 Composition of the basal diet (experiment 1)

	g/kg
Peanut meal (42% protein)	500.0
Corn starch	381.9
Corn oil	20.0
Tallow	35.7
$CaHPO_4 \cdot 2H_2O$	22.2
L-lysine·HCl	4.4
L-threonine	1.9
L-tryptophan	0.1
Vitamin mixture[1]	10.0
Choline chloride[2]	2.0
Mineral mixture[3]	4.0
$NaHCO_3$	3.7
$KHCO_3$	3.7
$CaCO_3$	9.6
NaCl[3] (iodized)[4]	0.8

[1]Provided the following amounts per kg of diet: thiamin HCl, 15 mg; riboflavin, 15 mg; nicotinic acid, 50 mg; folic acid, 6 mg; pyridoxine HCl, 6 mg; biotin, 0.6 mg; d-calcium pantothenate, 20 mg; menadione sodium bisulfite, 1.5 mg; DL-α-tocopheryl acetate, 50 IU; cholecalciferol, 4500 IU; retinyl acetate, 4500 IU; ethoxyquin, 100 mg; cyanocobalamin, 0.3 mg.
[2]Premix (52% choline), Nutrius, Brecksville, OH USA.
[3]Provided the following in mg/kg of diet: $MnSO_4 \cdot H_2O$, 350; $FeSO_4 \cdot 7H_2O$, 5000; $MgSO_4$, 3000; $CuSO_4 \cdot 5H_2O$, 30; ZnO, 13; $CoCl_2 \cdot 6H_2O$, 1.7; $NaMoO_4 \cdot 2H_2O$, 8.3; Na_2SeO_3, 0.2.
[4]Inorganic iodine content of the diet was 2.9 mg/kg (by analysis).

To the basal diet were added (in g/kg of diet) 0.5, 1.0, 1.5, and 2.0 L-MET or DL-MET and 0.6, 1.2, 1.8, and 2.4 DL-HMB-Ca. Samples of diets were stored at −20° C for later analysis. Duplicate samples of diets were analyzed for crystalline methionine additions by extracting 0.5-g samples of diets for 15 min at 37° C on a shaking water bath with 10 mL of a solution of 4% sulfosalicylic acid containing 0.25 μmoles/mL of taurine as an internal standard. After the supernatants were transferred to 25 mL volumetric flasks, two successive 15 min 37° C extractions of the residue were carried out using 5 mL aliquots of extracting solution. Extracts of each sample were pooled, brought to 25 mL volume with extracting solution, filtered, and analyzed by ion exchange chromatography using a minhydrin detection system. DL-HMB-Ca was analyzed according to the method of Ontiveros et al.[18] Two sets of analyses involving two and three replications were carried out and averaged.

Five replicates of five chicks, which had been fed a practical diet during the first week of age, were fed the experimental diets for 2 weeks. At the end of the experiment, after an overnight fast, one chick per pen was analyzed for carcass water and fat content according to the method described by Pfaff.[19]

Statistical analysis of the results was performed using nonlinear regression based on the model $y = a + b[1 - e^{-c1 \times 1 - c2 \times 2 - c3 \times 3}]$; where y is the response; a is the response at 0 supplementation; b, c1, c2, and c3 are the regression coefficients; and x1, x2, and x3 are the dietary levels of the methionine sources.[20] In this equation, the ratio between c_2 or c_3 and c_1 represents the amount of methionine source x_1 that is replaced by one unit of source x_2 or x_3. The standard

error of c_2 and c_3 is used in the calculation of confidence intervals for relative efficiency.

Intestinal absorption

The method utilized in all the experiments involving intestinal absorption consisted of feeding two- to three-week-old chicks (which had been fasted overnight) diets containing L-[methyl-^{14}C]MET, DL-[1-^{14}C]MET, or DL-[1-^{14}C]HMB and 0.5% Cr_2O_3 as a marker of digestibility. The radioactive materials were dissolved with the nonradioactive methionine source in water and mixed with the diet in a proportion of 10 mL of solution per 100g of diet. In a preliminary experiment it was determined that 2½ hours were required for the feed to pass through the digestive tract, and this was the time selected to collect intestinal samples. Chicks were euthanized by intracardiac injection of euthanasia solution T-61 (Hoechst American Corp., Sommerville, NJ USA), the intestine was immediately excised, and the contents of each segment were obtained by flushing with 10 mL of distilled water. One mL of 6% sulfosalicylic acid (SSA) was added to prevent bacterial degradation of the methionine sources. Samples were centrifuged, and the radioactivity of the supernatant determined by scintillation counting. Feed was also extracted with water, and the radioactivities of each diet determined. The chromic oxide concentrations of feed and luminal contents were determined by the method of Fenton and Fenton.[21] The percent of the dietary methionine sources unabsorbed at the specific section of the tract was calculated from the carbon-14 to chromate ratio of the feed and luminal contents, where percentage of methionine sources recovered (i.e., unabsorbed) was calculated as shown in Equation 1.

$$\% \textit{ unabsorbed} = 1 - \left[\frac{^{14}C/Cr_{feed} - {}^{14}C/Cr_{lumen\ contents}}{^{14}C/Cr_{feed}}\right] \times 100 \quad (1)$$

Experiment 2 The purpose of this experiment was to determine the extent of absorption of methionine sources in different parts of the small intestine. The basal diet (*Table 2*) formulated from practical ingredients was supplemented to contain 0.1% L-MET, DL-MET, or the molar equivalent of DL-HMB, and 0.070, 0.067, and 0.080 μCi per gram of diet, respectively, of the carbon-14 labeled sources. Six chicks per treatment were used. The intestine was cut into six segments. The last was a 5-cm segment proximal to the ileocecal junction. The other five segments were five equal portions of the remainder of the small intestine starting from the caudal end of the duodenum. Experiment 2 was repeated using the same design except that the small intestine (excluding the duodenum) was sampled in four segments of equal length.

A statistical analysis of the results was performed using a split-plot design[22] to account for the fact that all six intestinal segments were taken from the same chicken. Diet, chicken, segment, and the interactions diet × segment and diet × chicken were included in the model. A set of orthogonal contrasts was used to estimate differences between treatment means. Each segment was compared with the average of the following segments to the end of the intestine to determine whether significant disappearance of radioactivity occurred from segment to segment. Diets were compared against each other with single degree-of-freedom linear contrasts, using the interaction chicken × diet as the error term. The individual observations, expressed as a percent of dietary methionine source recovered, were transformed to arc sin of the square root of each value.

Another group of four chicks was fed the same basal diet as in experiment 2, but containing 0.1% DL-MET and 1 microcurie of DL-[1-^{14}C]MET per gram of diet. The higher level of radioactivity was used to allow sufficient recovery of ^{14}C-from luminal contents for analysis of radioactivity in D- and L-MET. Eight-cm long segments of the distal portion of the intestine were excised, and the intestinal contents extracted with cold distilled water. A portion of the extract was incubated with L-amino acid oxidase to destroy L-MET (see experiment 5 for procedures), and both incubated and unincubated extracts were acidified with SSA and chromatographed using a Technicon TSM Amino Acid Analyzer (Technicon Instrument Corporation, Tarrytown, NY USA). The fraction corresponding to methionine was collected and the radioactivity determined in a liquid scintillation counter.

Other experiments (not shown) determined the effect of dietary level of sources (equivalent to 0.04%–0.24% L-MET), adaptation time (none, or 1 week of diet containing methionine source), type of chicken (Leghorn or commercial broiler), and type of diet (practical or semipurified) on absorption of sources in the most caudal 5 cm of small intestine.

Table 2 Composition of basal diets (experiments 2–6)

	g/kg
Yellow corn	525.0
Soybean meal, 48.5%	255.0
Corn gluten meal	50.0
Fish meal, menhaden	75.0
Dried distillers gr. and sol.	50.0
Alfalfa meal, dehydrated	10.0
Corn oil	10.0
Dicalcium phosphate	12.5
Limestone	5.0
Iodized salt	2.5
Mineral-vitamin premix[1]	5.0

[1]Supplied the following in mg per kg of diet: retinyl acetate, 11500 IU; cholecalciferol, 1300 IU; DL-α-tocopheryl acetate, 5.5 IU; menadione sodium bisulfite, 2; riboflavin, 3; nicotinic acid, 33; d-calcium pantothenate, 11; cyanocobalamin, 0.007; choline chloride, 224; ethoxyquin, 80; ZnO, 110; $MnSO_4$, 220; and corn meal, 4117.

Experiment 3 The digestibility of HMB using a nonradioactive source was determined. Two-week-old chicks were fed the same diet as in experiment 2 (*Table 2*), except it was supplemented with 1% pharmaceutical grade DL-HMB-Ca. Another group of four chicks received the same diet without any DL-HMB added. After 2 days the chicks were killed and the contents of the last 20 cm of the small intestine collected and suspended in 2 mL of 10% acetonitrile. The samples obtained were centrifuged at 16000*g* for 5 min, and the supernatants diluted one to one with distilled water. A 50 μL aliquot of extract of luminal contents from a chicken receiving no HMB was combined with 20 μL of 20 mmol/L DL-HMB. This resulted in a final concentration of DL-HMB equivalent to what would be found in the extracts from chicks receiving 1% DL-HMB if the amount of DL-HMB unabsorbed was 10% of that present in the diet. Twenty microliter aliquots were analyzed according to the liquid chromatographic procedure of Lawson and Ivey,[23] with the exception that the flow rate of eluting buffer through the Zorbax NH_2 column was 1.1 mL per min. The experiment was repeated, using four equal segments of the small intestine caudal to the distal end of duodenum.

Renal excretion

In the following experiments, four- to seven-week-old chicks weighing 0.8–2.0 kg that had been fed a practical diet since the time of hatching, were anesthetized with a mixture of 5% halothane in oxygen administered through a plastic head cone at a rate of 2 L/min. The concentration of halothane was decreased to 1–2% and maintained at this concentration throughout the experimental period. The animals were prepared according to the method described by Austic and Cole.[24] The openings of the ureters were sutured into a small plastic tube to permit quantitative urine collection. Both wing veins (*l.* and *r. vena cutanea ulnaris*) were cannulated: one was used for the collection of blood samples; the other for infusion of the test substance. After a priming dose of 1 mL of 10% inulin had been administered through the wing vein, the cannula was connected to a peristaltic infusion pump. A solution containing 2.8% inulin, 0.38 M mannitol, and 0.154 M sodium chloride was infused at the rate of 0.5 mL/min to maintain diuresis.

After 15 min of infusion, a 5-min urine sample was taken. A 1.5 mL blood sample from the wing vein was taken during the midpoint of the urine collection. Following this first sample collection, the chicks were infused with a solution that contained the same components as before, plus the methionine source being tested. Five-minute urine samples and 1.5 mL blood samples were taken during the midpoint of the urine collection at intervals of 10 min. The total number of collections was normally six, and the time of infusion was usually about 75 min. Inulin in plasma and urine was analyzed by use of an autoanalyzer (Technicon) by the resorcinol method of Schreiner.[25] Plasma samples were treated with glucose oxidase to eliminate interference by glucose prior to cadmium sulfate precipitation. A fresh solution of 5 mg of glucose oxidase (Type II, 43,000 units/g in oxygen saturated media: Sigma Chemical Co., St. Louis, MO USA) per mL in 0.4 mmol/L potassium phosphate, pH 5.6, was prepared for each analysis. One-half milliliter of glucose oxidase solution was incubated with 0.5 mL of plasma in an oxygen atmosphere at room temperature for 0.5 hr. Urine samples were diluted with phosphate buffer without glucose oxidase.

Experiment 4 This experiment was carried out to determine the efficiency of retention of L-MET and DL-MET by the kidney. The infusion solution contained 20 mmol/L L- or DL-MET. L-MET was analyzed using the amino acid analyzer. Plasma and urine samples were treated with half volume of 6% SSA to precipitate the proteins and acidify the samples for analysis. Plasma D-MET was determined by the same procedure, after incubation of 100 μL samples with 25 μL of a solution containing 10 mg (5 units)/mL of L-amino acid oxidase (Type I, from *Crotalus adamantus* venom, Sigma) in 0.2 M Tris buffer, pH 7.4. Samples were incubated at 37° C for 30 min. The reaction was stopped by the addition of 75 μL of 6% SSA. This treatment was shown to reduce the methionine concentration of a solution of 1 mmol/L DL-MET by one-half and eliminate several amino acids from plasma samples. One hundred μL urine samples were treated with 10 μL of 0.1 N NaOH and 15 μL of the L-amino acid oxidase solution and incubated under the same conditions as plasma samples.

Experiment 5 The purpose of this experiment was to verify the results of the previous experiment and determine whether significant quantities of products from D-MET were excreted. D-[1-^{14}C]MET was prepared from DL-[1-^{14}C]MET as follows: 100 μCi of DL-[1-^{14}C]MET was dissolved in 200 μL of 0.2 M Tris buffer, pH 7.4. One hundred microliters of this solution was mixed with 25 μL of a L-amino acid oxidase solution (10 mg/mL) in 0.2 M Tris buffer, pH 7.4. After a 30-min incubation, the oxidation of L-MET was stopped by the addition of 75 μL of 6% SSA. The resulting suspension was centrifuged, and the supernatant applied to the amino acid analyzer. The fraction corresponding to methionine was collected and mixed with the infusion solution to provide 0.1 μCi per mL. The experiment was conducted as described in experiment 4. The concentration of D-MET in the infusion solution was 20 mmol/L. Five chicks were utilized for the experiment. Plasma and urine samples were analyzed for total radioactivity and for radioactivity contained in D-MET. D-MET was isolated by treatment of samples with L-amino acid oxidase as in experiment 4 and collection of the fraction corresponding to methionine from the amino acid analyzer.

Experiment 6 This experiment was conducted to determine the efficiency of retention of DL-HMB by the kidney. HMB does not separate completely from other UV-absorbing substances present in the urine using the chromatographic method of Lawson and Ivey.[23] Therefore, this procedure could not be utilized for HMB analysis of urine samples by measurement of optical density. Because no other analytical procedure was available, it was necessary to use radioactive HMB. The materials and methods used were the same as described at the beginning of this section. Two groups of five chicks were used. The first group was infused with 20 mmol/L DL-HMB containing 0.3 μCi per mL. The second group was infused with 5 mmol/L DL-HMB containing 0.2 μCi per mL, to determine the retention of HMB at low plasma concentrations, which would be expected when feeding practical levels of DL-HMB.

Plasma samples were centrifuged in CF 50 A Amicon Centriflo membrane cones (Amicon Corp., Lexington, MA USA) at 1000*g* to remove protein. Urine samples were centrifuged at 14000 rpm. One hundred microliters of the sample was injected into a Zorbax NH_2 column and the fraction corresponding to DL-HMB collected in a scintillation vial, which was subsequently counted. The amount of radioactivity paralleled the size of the peaks measured by optical density at 215 nm in plasma and urine samples containing high concentrations of HMB, suggesting that the radioactivity measured was only HMB.

To determine the plasma HMB concentrations that would be achieved upon feeding DL-HMB, 2-week-old chicks were fed diets containing DL-HMB. The basal diet (*Table 2*) was the same one used in experiments 2 and 4. The concentrations of DL-HMB-Ca present in the diets were 0.06, 0.12, 0.24, 0.36, and 1.00% of the diet, equivalent to 0.05, 0.1, 0.2, 0.3, and 0.82% methionine on a molar basis. The diets were fed over a period of 2 days, and blood samples collected from four chicks per treatment at 1300 hr on the second day.

The plasma samples were centrifuged as before through membrane filters and 20 μL samples were analyzed by the chromatographic procedure of Lawson and Ivey[23] modified as described below. HMB concentrations were determined using pharmaceutical grade DL-2-hydroxy-4-[methylthio]butanoic acid as a standard.

Radiochemical purity of radioisotopes

L-[1-^{14}C]-MET (New England Nuclear, Boston, MA USA) was supplied by the manufacturer and stored in ethanol:water (7:3, vol/vol) in glass ampules containing 50 μCi. DL-[1-^{14}C]MET and DL-[1-^{14}C]HMB (calcium salt) (Amersham, Intl. Plc., Buckinghamshire, UK) were supplied and stored

in lyophilized form in glass ampules containing 100 μCi each. Storage of the radioactive materials was at −20° C. A new ampule of radioactive HMB was used in each experiment. Nonradioactive HMB-Ca (pharmaceutical grade, Degussa AG, Hanau, Germany) was stored in a desiccator at room temperature. L- and DL-MET (Sigma) were stored at room temperature.

The radiochemical purities of L-[1-^{14}C]-MET and DL-[1-^{14}C]-MET (94% for both sources) were determined by ion exchange chromatography using the amino acid analyzer. The radiochemical purity of DL-[1-^{14}C]HMB was determined by the ion exchange chromatography method of Saunderson,[15] and by the high performance liquid chromatography method of Lawson and Ivey.[23] A radiochemical impurity and some minor non-radioactive impurities were detected (*Figure 2*).

Results and discussion

Experiment 1 The results of this experiment are shown in *Figure 1*. The statistical analysis indicates that the bioefficacy of DL-HMB-Ca was 73% that of DL-MET (95% confidence interval, 55–92%) based on weight gain, and 72% based on feed conversion (95% confidence interval, 57–87%). The bioefficacy of L-MET was 102% that of DL-MET (95% confidence interval, 81–124%) based on weight gain and 106% (95% confidence interval, 78–134%) based on feed conversion. No significant differences ($P > 0.05$) in carcass fat or water content were detected.

The results of the analyses carried out to confirm the dietary levels of supplements were as follows (in g/kg of diet): 0.34 ± 0.06, 0.94 ± 0.13, 1.48 ± 0.11 and 1.91 ± 0.17 L-MET, 0.39 ± 0.10, 1.08 ± 0.01, 1.21 ± 0.19 and 1.96 ± 0.02 DL-MET, and 0.69 ± 0.25, 1.05 ± 0.28, 1.62 ± 0.34 and 2.86 ± 0.01 DL-HMB-Ca, respectively, for each dietary series.

Experiment 2 The recovery of radioactivity in luminal contents of intestinal segment 1 represented 8.2 ± 2.0, 9.6 ± 3.3, and $22.4 \pm 4.4\%$ of dietary radioactivity for L-MET, DL-MET, and DL-HMB, respectively. This decreased to 3.0 ± 0.8, 4.4 ± 0.5, and $20.9 \pm 4.0\%$, respectively, in segment 5. The statistical analysis revealed no significant interactions between segment and methionine source ($P > 0.10$). Across methionine sources there was a significant difference between segment 1, and the average of the remaining segments ($P < 0.05$), but no significant difference between segment 2 and the average of 3, 4, and 5, or between 3 and the average of 4 and 5 ($P > 0.05$). Segment 4 was lower than the average of 5 and 6, and segment 5 was lower than 6 ($P < 0.05$).

When this experiment was repeated using samples from four intestinal segments, the results were similar to the first experiment. The radioactivity in luminal contents in segment 1 represented 4.8 ± 0.6, 6.4 ± 1.4, and $20.4 \pm 3.0\%$ of initial dietary radioactivities for L-MET, DL-MET, and DL-HMB, respectively. The radioactivities decreased to 1.8 ± 0.2, 2.7 ± 0.4, and $11.8 \pm 2.0\%$ of initial dietary radioactivities, respectively, in the fourth segment. There was no significant interaction between diet and segments ($P > 0.10$). Across all sources, significant disappearance of radioactivity occurred between segment 1 and the average of the remaining segments, and between segment 2 and the average of segments 3 and 4 ($P < 0.05$): radioactivities of the luminal contents of segments 3 and 4 were not different ($P > 0.10$).

In related experiments, the level of methionine source, adaptation time, and type of chicken or diet did not affect the amount of radioactivity from any of the sources that was recovered in the last 5 cm of the small intestine (results not shown).

To determine whether the radioactivity in the contents of the most caudal sample of the contents of the small intestine represented actual methionine or DL-HMB, samples of extract were chromatographed by the two methods that were used to check the purities of the radioactive sources.[15,23] The radioactive methionine recovered in the digesta extract of chicks administered DL-[1-^{14}C] methionine was slightly above background and represented less than 20% of the total radioactivity present in the extract. Treatment with L-amino acid oxidase essentially removed all the radioactivity present in the methionine fraction, indicating that no D-MET was present. The recovery of radioactivity from DL-MET in the last 5 cm of small intestine averaged 3.9% (average of five experiments), and 2.5% from L-MET (average of three experiments), and that less than 20% of this fraction was methionine. This indicates that when fed in free form, the amount of DL-MET recovered from the intestine is less than 1% of that provided in the diet.

When the radiochemical purity of the DL-[1-^{14}C]HMB source was determined by the methods of Saunderson[15] and Lawson and Ivey,[23] carbon-14 appeared in two fractions, while carbon-14 from intestinal contents appeared only in one of the two fractions—the one identified as impurity (e.g., fraction D in *Figure 2*). No radioactivity was detected in the fraction corresponding to the retention time of pharmaceutical grade DL-HMB.

Experiment 3 The chromatographic column utilized in experiment 3 was new and this caused the HMB fraction to shift in retention time to 6.2 min. It can be seen in *Figure 3* that a fraction of similar size with a retention time of 6.2 min was detected in extracts of intestinal samples of chicks receiving the basal diet and the diet supplemented with 1% DL-HMB-Ca. Considering that the amount of sample injected and the size of the fractions were equivalent for both dietary treatments, this means that the fraction observed was not unabsorbed HMB. A sample of extract from the basal group was spiked with HMB in the concentration that would be expected if the birds had received 1% DL-HMB in the diet and 10% was unabsorbed. It can be seen that the size of the peak of the sample spiked with DL-HMB was far larger than that of the other two samples.

The retention time of the impurity present in the radioactive stock of HMB that had been recovered in the intestine in previous experiments was 10 min for the column used in this experiment. All the samples appear very similar in this region of the chromatogram

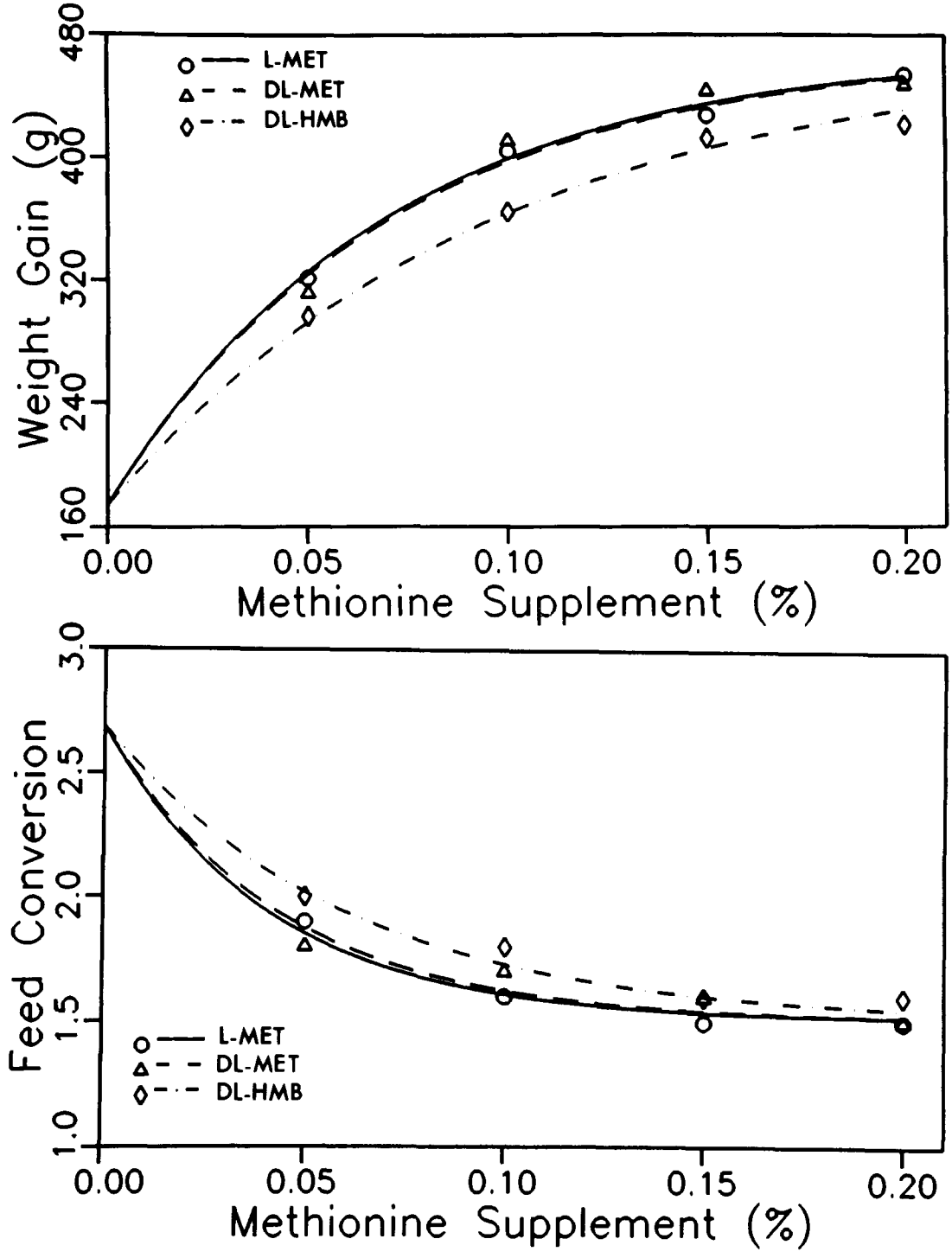

Figure 1 Weight gain and feed efficiency of chicks fed different methionine sources (experiment 1). The equations of the curves are: weight gain = 174.0 + 296.3 $(1-e^{-14.0\times1-14.2\times2-10.2\times3})$ and feed conversion = 2.7 − 1.2 $(1-e^{-23.3\times1-24.7\times2-16.8\times3})$, where x1 is % DL-MET, x2 is % L-MET, and x3 is % DL-HMB (on a molecular equivalent basis) added.

(*Figure 3*). There is no indication of any fractions in the samples from chicks receiving HMB that would not be present in samples from chicks receiving no HMB. The results of experiments 2 and 3 indicate that DL-HMB, L-MET, and DL-MET are completely absorbed in the digestive tract of the chicken.

Excretion studies

Experiment 4 The renal excretions of methionine during infusion of L-MET and DL-MET are illustrated in *Figures 4 and 5*, respectively. As can be seen, urinary losses were very small for both sources, and

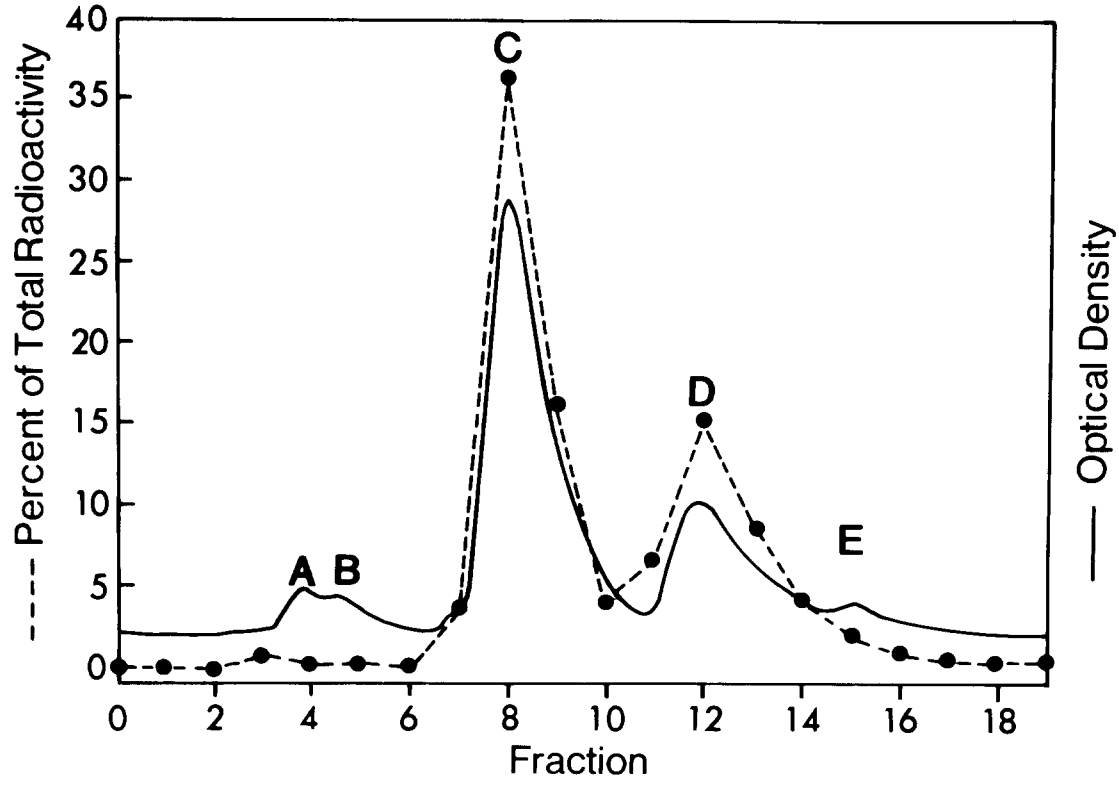

Figure 2 Reverse phase chromatography of DL-[1-^{14}C]HMB in Zorbax NH_2 column. Optical density determined at 215 nm and distribution of radioactivity in the effluent of the column. Each fraction corresponds to a 1-min collection of effluent.

Table 3 Renal excretion of D-methionine (experiment 5)

Final D-methionine plasma concentration = 0.49 ± 0.09[1] mmol/L.
D-methionine in urine as percent of the filtered load = 2.2 ± 0.4%.[1]
Total radioactivity in urine as percent of the filtered load = 3.0 ± 0.8%.[1]
Total radioactivity in the urine as percent of radioactivity infused = 1.9 ± 0.5%.[1]

[1]Mean ± SE of five observations.

did not exceed 3% of the filtered load,* with most of the values around 1% of the filtered load or less. There was no indication that the urinary loss was increased by the plasma concentration over the range of plasma methionine concentrations obtained during these infusions. On the contrary, there was a slight decrease ($P < 0.05$) in excretion of methionine, as a percent of filtered load, during the course of L- and DL-methionine infusion. Treatment of the urine samples of the chicks receiving DL-MET with L-amino acid oxidase resulted in virtual disappearance of methionine, indicating that the losses of D-MET were below the limits of detection.

Experiment 5 Because the amount of radioactivity present in the urine as D-MET was very small and barely above background, only the results corresponding to the last urine collection were calculated. As shown in *Table 3*, the final plasma concentration of D-MET was similar to the final methionine concentrations obtained by infusion of L- and DL-MET in experiment 4. The excretion of D-MET, expressed as percent of the filtered load, was low and similar to the values obtained in the previous experiment for methionine excreted during L- and DL-MET infusion. The excretion of total radioactivity in the urine was low, either when expressed as a fraction of the filtered load or as a fraction of the infused radioactivity. This suggests that the losses of D-MET in the urine are low, even when the presence of radioactivity from D-MET in other compounds is taken into account.

Experiment 6 Because the stock of radioactive DL-HMB was not pure, the precaution taken was to measure only the radioactivity with the same retention time as HMB in the chromatographic procedure of Lawson and Ivey.[23] The assumption was that the composition of radioactive DL-HMB would remain constant throughout the experiment. To test this assumption, infusion solution, plasma, and urine samples were

*The following computation was used in experiments 5 and 6: percent of filtered load = [inulin clearance × concentration of L-MET, D-MET, or D- + L-HMB in plasma] × 100. Units are nmole/min for urinary excretion, mL/min for inulin clearance, and nmole/mL for concentrations of methionine sources in plasma.

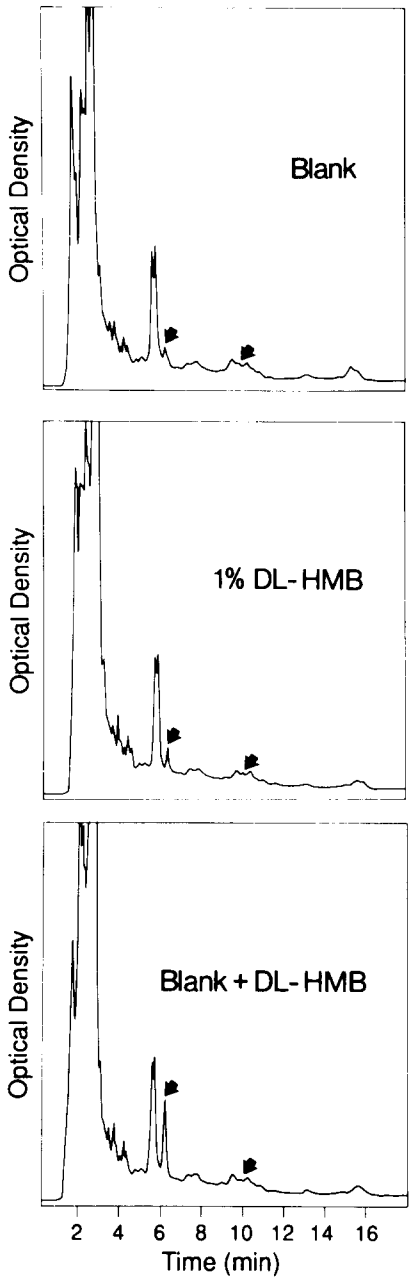

Figure 3 Chromatogaphy of intestinal contents in a Zorbax NH_2 column (experiment 3). Blank = intestinal content of chicks receiving no HMB. 1% DL-HMB = intestinal content of chicks receiving 1% dietary DL-HMB-Ca. Blank + DL-HMB = Blank spiked with the amount of HMB that would be found if the percentage of dietary DL-HMB unabsorbed was 10% in chicks receiving 1% dietary DL-HMB-Ca. Left and right arrows indicate fractions corresponding to the retention times of HMB and impurity, respectively.

mixed with radioactive DL-HMB, and the radioactivity in the HMB fraction was determined before and after the experiment was performed. The proportion of radioactivity in HMB fraction was 61.7±3.6% at the beginning and 60.9±2.7% at the end of the experiment. This indicated that the composition of radioactive HMB remained constant throughout the experiment.

The results of the two groups (i.e., groups receiving infusions of 5 mmol/L or 20 mmol/L HMB) were combined and are shown in *Figure 6*. It can be seen that at low plasma concentrations the excretion of HMB was small, although it appeared to be higher than the excretion of methionine observed in the previous experiments involving the infusions of L-, D-, and DL-MET. The typical values for L-MET excretion in the preceding experiments were in the order of 1% or less of the filtered load throughout the range of plasma methionine concentrations studied: values for DL-MET were slightly higher, and the value for D-MET was approximately 2% of the filtered load at the highest plasma concentrations achieved by infusion. The excretion of HMB at plasma concentrations below 0.1 mmol/L was 6.7±0.7% of the filtered load (mean±SE of 23 observations). At plasma concentrations between 0.1 and 0.3 mmol/L, the excretion of HMB increased gradually to a value of 15–20% of the filtered load. The excretion of HMB rose sharply at higher plasma HMB concentrations, suggesting that there may be a threshold concentration above which HMB would be lost to a very large extent in the urine.

One purpose of the experiment was to determine the influence of dietary level on plasma concentration of HMB. The concentrations of DL-HMB found in plasma were 0.029, 0.042, 0.046, and 0.278 mmol/L, at dietary levels of 0.12, 0.24, 0.36, and 1.00% DL-HMB, respectively. The separation of HMB from other substances in plasma was incomplete. At 0.06% dietary DL-HMB, the optical density corresponding to HMB was masked by a co-eluting substance. Only at 0.12% dietary DL-HMB was the HMB fraction visible. This could explain the apparent lack of proportionality between dietary and plasma concentration. However, at dietary levels between 0.12 and 0.36% DL-HMB, it is apparent that the plasma concentrations of HMB were well below 0.1 mmol/L, a level at which renal excretion is only about 6% of the filtered load. At dietary levels of 1% DL-HMB, plasma concentrations were close to those at which renal excretion of HMB increased sharply.

Radiochemical purity of DL-[1-^{14}C]HMB

The presence of the radioactive impurity in DL-[1-^{14}C]HMB seemed to be caused by the lack of stability of the preparation because the impurity reappeared within days after isolation of the HMB fraction by reverse phase chromatography. No impurity was ever observed during chromatography of solutions of nonradioactive pharmaceutical grade DL-HMB-Ca.

In an experiment conducted during the present study (not shown), the renal clearance of the impurity present in the stock of radioactive DL-HMB was greater than that of inulin (157±23% of the filtered load, based on five chicks). It was calculated that if the impurity represented one-third of the radioactivity, as was found in some vials of stock DL-[1-^{14}C]HMB, the apparent excretion of HMB during constant infusion over a 50 min period would be 11% of the dose. This illustrates the extent of renal clearance needed to achieve large urinary losses. If, in any experiment involving adminis-

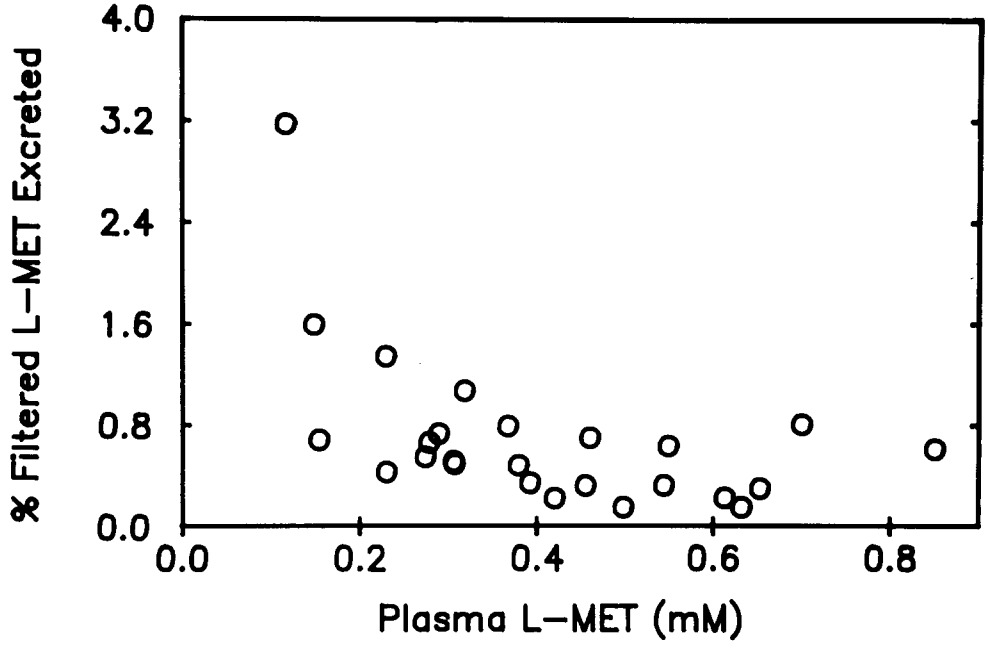

Figure 4 Renal excretion of L-methionine by chicks infused with L-methionine, expressed as percent of the filtered load excreted (experiment 4). Each data point represents one observation.

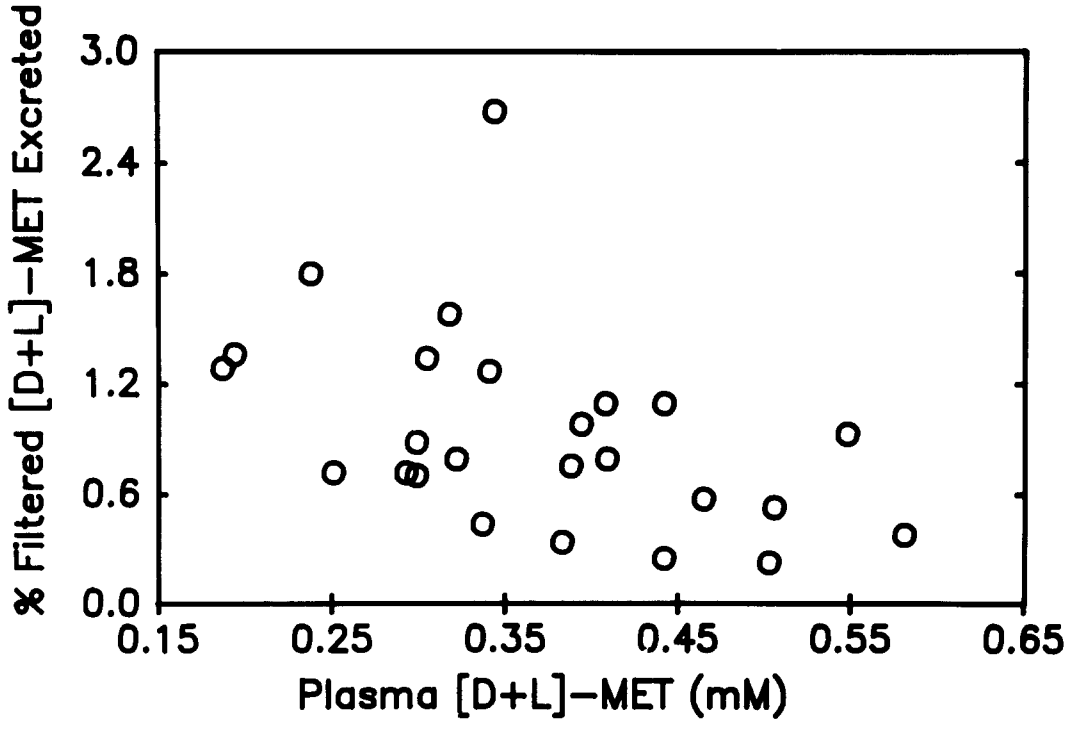

Figure 5 Renal excretion of (D + L)-methionine by chicks infused with DL-methionine, expressed as percent of the filtered load excreted (experiment 4). Each data point represents one observation.

trative radioactive HMB to chickens, there is a substantial portion of radioactive material that could not be absorbed or could be cleared efficiently by the kidney (such as the impurity mentioned above), it could be expected that losses in excreta would be substantial and may appear independent of the route of administration.

Nutritional implications

Experiment 1 simply adds a new observation to the already long list of bioefficacy trials. The results indicate that under the conditions used in this laboratory that there is a difference in the bioefficacies of these

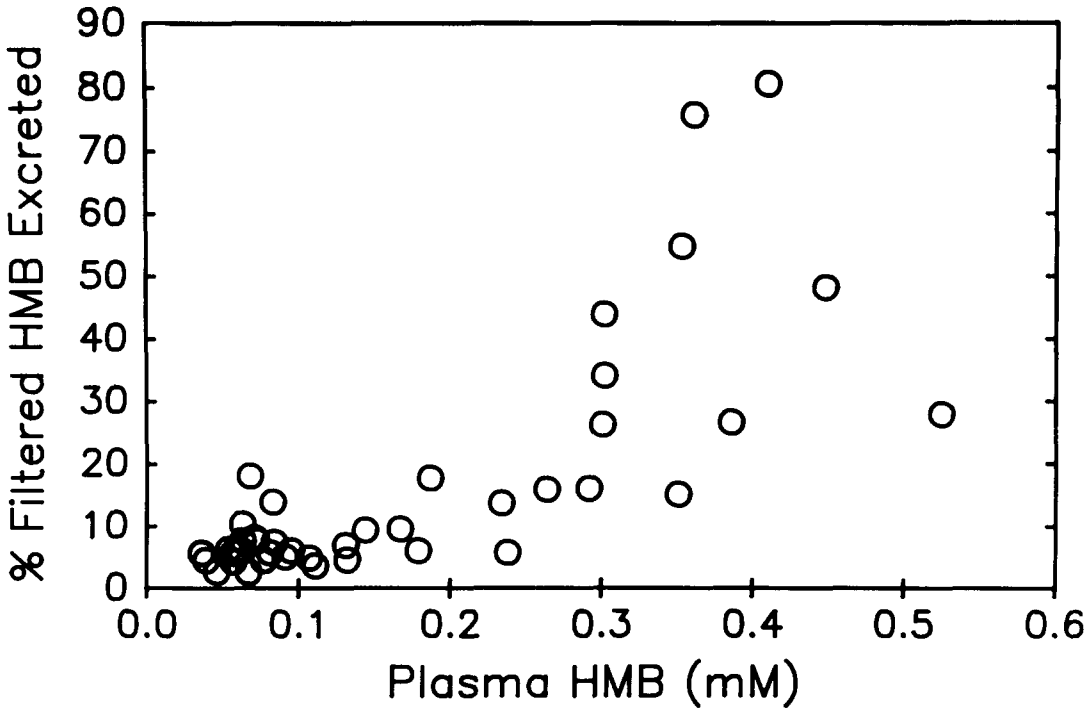

Figure 6 Renal excretion of HMB by chicks infused with DL-HMB-Ca, expressed as percent of the filtered load excreted (experiment 6). Each data point represent one observation.

methionine sources. The purpose of the present studies was to determine whether differences in intestinal absorption or renal excretion could explain the differences of bioefficacy observed in these and other studies.[1-3]

The results of experiments on intestinal absorption indicate that all the methionine sources tested are well absorbed. As was shown in experiment 2, the disappearance of radioactivity from L- and DL-MET was over 90% in a short segment of the small intestine immediately caudal to the duodenum. Considering that what was measured in the chicks receiving DL-HMB was an impurity of the preparation, this means that most of the HMB also had already been absorbed by the time digesta had passed through this segment.

According to Hellier and Holdsworth,[26] L-MET is the most rapidly transported of all free amino acids in the intestine. It was interesting to find that DL-MET also was rapidly absorbed. The studies by Paine et al.[27] and Lerner and Taylor[28] on chicks, by Jervis and Smyth[29] and Aroskar and Berg[30] on rats, and by Lin et al.[31] on hamsters suggest that some D-amino acids, and D-MET among them, can interact with L-amino acid carriers and can be actively absorbed. Recently, Brachet et al.[7] have shown that in the rat there are separate Na^+-dependent carriers for D-MET and L-MET. It is possible that the fast rate of absorption could be explained by the presence of an active transport system for D-MET. Passive diffusion seems also to be an important component of amino acid transport in chicken intestine.[32] Dupuis et al.[33] reported that intestinal mucosal scrapings possess the ability to oxidize D-MET. If the oxidation of D-MET to KMB takes place at a fast rate, the concentration gradient generated between the lumen of the intestine and the mucosal cells may be large enough to facilitate a rapid rate of absorption of D-MET.

Lerner et al.,[34] using Thiry-Vella fistulas, and Knight and Dibner,[11] using intestinal segments, showed that the uptake of DL-HMB was slower than that of L-MET. Brachet and Puigserver[8,9] have shown that HMB can be transported in vesicles prepared from brush border of rat jejunum and chicken intestine by a Na^+-independent carrier that can be inhibited by L-lactate. A diffusional component of transport was also detected, but the total rate of HMB transport was much slower than that of L-MET. The present experiments indicate that the capacity for transport in in vivo is sufficient to permit the complete absorption of DL-HMB in the gastrointestinal tract. Dupuis et al.[33] observed the oxidation of DL-HMB in mucosal scrapings from chick small intestine. The rate of oxidation of DL-HMB was lower than for DL-MET, but could contribute to absorption by lowering intracellular HMB concentrations.

The results of the experiments of renal retention of different methionine sources seem to indicate that urinary losses of these compounds are very small. The low urinary loss of L-MET was expected because the presence of active transport systems for L-amino acids is well established. The excretion values of L-MET obtained under the present conditions may be overestimated because the levels of L-MET measured in urine were only slightly above the limits of detection.

The urinary loss of D-MET was also low. This is consistent with early reports of Crampton and Smyth[13] with cats. The studies of Gordon[14] indicated that D-

MET was not excreted in the urine of chickens as such, in agreement with the present studies. Gordon[14] indicated that infusion of D-MET resulted in urinary losses of KMB, although he was not able to quantitate these losses. The present studies reveal that some radioactivity was excreted in the urine of chicks infused intravenously with radioactive D-MET. However, the amount excreted was small, and even though the plasma concentrations of D-MET were high, it averaged only 1.9% of the radioactivity infused over the collection period.

With regard to the question of whether the urinary losses of HMB observed in the present studies could explain, at least in part, the relative bioefficacy of DL-HMB observed in experiment 1, it is important to consider the fraction of the dietary methionine source that would be lost in the urine. The dietary concentrations of methionine used, even in crystalline amino acid diets, usually do not exceed 0.3% of the diet. At this dietary level the plasma concentrations of HMB observed in the present studies were well below 0.1 mmol/L. The renal excretion of HMB in this range was found to average 6.7% of the filtered load. To put this in proper perspective, a male broiler chicken weighing 300 g consuming 40 g of a diet containing 0.1% DL-HMB would receive 40 mg (268 μmoles) of DL-HMB per day. Assuming a glomerular filtration rate of 2.5 mL/min/kg of plasma, as was observed in these studies, the amount of plasma filtered would be 1080 mL per day. At a plasma concentration of about 30 μmol/L, as observed in experiment 9, 32.4 μmoles of HMB would be filtered per day. Assuming a renal excretion of 6.7% of the filtered load, the amount of HMB lost in the urine would be 2.17 μmoles. This loss would represent 0.8% of the daily amount of DL-HMB consumed. The losses of L- and D-MET would be even smaller. But, clearly, the amount of DL-HMB excreted seems to be very small and does not approach the losses that might be expected to account for the difference of bioefficacy between DL-HMB and L- or DL-MET observed in experiment 1.

Balance studies[4,5] using unlabeled DL-HMB have yielded conflicting results. The amount of HMB excreted was 1–4% of the dietary intake in one study,[5] whereas in a second study,[4] the excreted HMB was equivalent to 14–17% of dietary intake. It is possible that these losses were due to the protocols of the balance trials. Fasted adult roosters were tube fed 30[5] or 40[4] grams of diet containing DL-HMB, and excreta was collected for 56 hr[5] or 48 hr.[4] The excretion of HMB, as a fraction of intake, increased with increasing dietary concentration of HMB.[5] The study[5] yielding the lowest excretion values utilized lower dietary levels of DL-HMB-Ca (0.24% and 0.48%) distributed in two 15 gram intubations of diet over an 8-hr period as compared with a dietary level of 0.56% administered in a single intubation of diet in the other study.[4] In view of the complete intestinal absorption but limited renal reabsorption of DL-HMB observed in the present investigation, the protocols, especially the latter, may have resulted in blood levels of HMB that exceeded the capacity of the kidney for efficient reabsorption of this methionine source.

The results of the present investigation do not explain the differences of bioefficacy between methionine sources. These differences probably are due to metabolic processes involved in the conversion of D-MET and DL-HMB to L-methionine, as suggested by the recent report of Dupuis et al.[33]

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