Review of the metabolism of 2-hydroxy-4-(methylthio) butanoic acid*

J.J. DIBNER

Novus International, Inc., 20 Research Park Drive, Missouri Research Park, St. Charles, MO 63304 USA, e-mail: julia.dibner@novusint.com

This report will cover studies of the metabolism of a supplemental source of L-methionine (L-met), Alimet® feed supplement. The goal of these studies was to gain an understanding of how this methionine precursor is converted to L-methionine and how it is used by the chick, in order to optimise the conditions for its use. The methionine precursor in Alimet is 2-hydroxy-4- (methylthio) butanoic acid (HMB). HMB differs from methionine by having a hydroxyl group on the alpha carbon rather than an amino group. Like synthetic DL-met, HMB has one asymmetrical carbon atom and therefore occurs as a mixture of 50% L-isomer and 50% D-isomer. Because HMB bears a hydroxyl group instead of an amino group, it is an organic acid. Until it is converted to L-met antimicrobial properties of HMB resemble those of organic acids such as lactic acid. Once inside the tissue of the animal, however, HMB is rapidly converted to L-met. Following conversion to L-met, HMB will have the same availability for methyl group transfer or cysteine synthesis as L-met from any other source. This report will cover the bioavailability of HMB polymers, the conversion of HMB to L-met and the role of HMB in normal L-met intermediary metabolism. It will also include a discussion of HMB absorption and incorporation into protein.

Keywords: 2-hydroxy-4- (methylthio) butanoic acid HMB; methionine metabolism

Polymer availability

This feed supplement is a concentrated solution of HMB (88%) in water (12%). Until HMB is converted to L-methionine, it is an α-hydroxy mono-carboxylic acid similar to lactic acid. Its chemistry and biological activity resemble those of other organic acids in the gut in that it reduces digesta pH and shows anti-microbial benefits (Dibner and Buttin, 2002); however, once in the tissues it is converted to L-methionine. Before it is mixed onto feed, the product consists of an equilibrium mixture of HMB monomer (65-70%), HMB linear dimer (15-20%) and HMB trimer (3%) (Baudichau *et al.*, 1987). The esterification that results in dimer formation takes place between the carboxyl group of one monomer and the α-hydroxy group of another, with the liberation of water. The equilibrium is

*Alimet® feed supplement is a registered trademark of Novus International Inc. in the United States and other countries.

World's Poultry Science Journal, Vol. 59, March 2003
Received for publication June 1, 2002
Accepted for publication July 16, 2002

dynamic, with formation and hydrolysis of dimers and trimers occurring continuously. These chemical reactions do not require or give off energy but are influenced by the matrix in which HMB is found. For example, the percentage of monomer, dimer and trimer described above is that found in a concentrated solution of the supplement. Once on feed, however, the equilibrium shifts in the direction of monomer (Bruyer and Vanbelle, 1990a), possibly due to more moisture or the availability of free calcium in feed resulting in the formation of the calcium salt of HMB. After ingestion, monomer formation is favoured by the availability of water, simply on a chemical basis. Thus, the amount of dimer and trimer of HMB would be highest in the supplement and would decrease in feed and after ingestion. In addition to these chemical processes, the availability of dimer and trimer would also be affected by normal digestion.

The ester bonds between HMB molecules are the same as those found in other naturally occurring macromolecules like fat, and in fact closely resemble those occurring in lactic acid, which also undergoes homologous intermolecular esterification (Bruyer and Vanbelle, 1990b). The action of digestive enzymes on HMB dimer is of interest because pancreatic and intestinal digestive enzymes readily break down other esters.

Two models were used to study HMB polymer digestion; a solution of pancreatin in phosphate buffer was used to approximate the enzymatic environment of the intestinal lumen, and a short term organ culture of washed intestine segments was used to examine the contribution of intestinal epithelial cells.

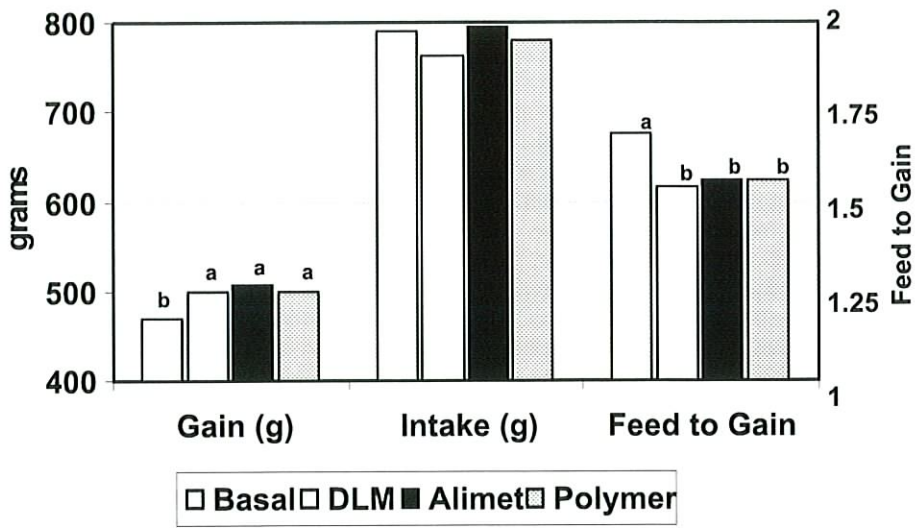
In the first model system, HMB dimer rapidly disappeared from an equilibrium mixture dissolved in the pancreatinc/phosphate buffer solution. The rapid hydrolysis of dimer was shown to be enzyme-mediated by experiments in which heat denaturation resulted in a total loss of hydrolytic activity (Lawson and Ivey, 1986) and in studies of purified pancreatic and intestinal enzymes (Bruyer *et al.*, 1988). Of those tested, pancreatic lipase showed the greatest activity toward the oligomeric forms of HMB. Since lipase hydrolyses ester bonds between fatty acids and glycerol, it is not surprising that the HMB esters would be substrates for this enzyme.

A second model system was used to determine the effect of the intestinal epithelial cells on HMB. A solution of 100% HMB dimer in phosphate buffer was placed in the lumen of intestinal segments and incubated in culture. Following incubation, the monomer and dimer content of the lumen, the intestinal tissue and the external buffer was determined by HPLC as described in Knight and Dibner (1984) and Ontiveros *et al.* (1987). During the two-hour organ culture, 92% of the HMB dimer was hydrolysed to monomer. An important consideration in the interpretation of this rate of hydrolysis is the gut transit time, which is greater than two hours, even in very young chicks (Golian and Polin, 1984). Thus the rate observed using pure dimer *in vitro* was more than adequate to hydrolyse the polymer fraction of the equilibrium mixture during its residence time in the gut. These techniques indicate that there can be more than one source of hydrolytic activity for HMB that would have an additive effect *in vivo*.

Other work has confirmed the equivalence of HMB polymers to DL-met and Alimet (*Figure 1*) in chick performance studies. The production of a polymer fraction for a feeding study is complicated by the fact that upon concentration involving the simple removal of water, the dimer and trimer will form much larger oligomers than are present in the product. In the feeding study of *Figure 1*, the polymer fraction was obtained by extraction. The free hydroxyl groups of the extracted polymer fraction were reacted with a blocking agent to prevent formation of very large oligomers during the concentration process (Bruyer and Vanbelle, 1990a, 1990b). These larger oligomers, which are formed when polymer with free hydroxyl groups is freeze dried or concentrated by solvent evaporation, do not occur in the unaltered Alimet product (Boebel and Baker, 1982; Van Weerden *et al.*, 1992). This can be avoided using the techniques described by Lawson and

Ivey (1986). Polymer fractions that accurately represent those found in the product have been demonstrated to be fully active (*Figure 1*) in chick performance studies (Bruyer and Vanbelle, 1990a; 1990b).

Figure 1 Performance of birds fed the polymer fraction extracted from Alimet.
Hubbard broilers were fed a corn sorghum soy diet containing .31% basal L-met for 7 – 19 days. Performance is equivalent between DL-met, Alimet and Polymers.



Hubbard Males, 7-19 days, Met .31% Corn Sorghum Soy Vanbelle, 1990. Ann. Zootech 39:45-51

Biochemistry of the conversion of HMB to L-methionine

Biochemical conversion of hydroxy acids to amino acids takes place in two steps. The first reaction is an oxidation of the alpha-hydroxyl group yielding a keto intermediate, 2-keto-4-(methylthio) butanoic acid in the case of HMB. A transamination yields the corresponding L-amino acid as the sole product of the second step. Conversion of HMB to L-met by L-hydroxy acid oxidase (L-HAOX, EC 1.1.3.15) has been studied in numerous organisms (Robinson *et al.*, 1962; Gordon and Sizer, 1965; Langer *et al.*, 1971). In all of these, the first conversion step was found to require molecular oxygen and a co-enzyme and to produce hydrogen peroxide as a byproduct. L-HAOX is found in liver, kidney, and other tissues in peroxisomes, a subcellular organelle containing catalase (EC 1.11.1.6), which converts the peroxide byproduct of these reactions to water and molecular oxygen (Dixon and Webb, 1979). Among the many peroxide-producing enzymes found in peroxisomes is one that is responsible for the oxidation of the D isomer of methionine, D-met: D-amino acid oxidase (D-AAOX, EC 1.4.3.3). Since DL-met is also used as a nutritional source of L-met, this enzyme was also studied. Interestingly, the conversion of D-met to L-met has the same intermediate as the HMB pathway, 2-keto-4- (methylthio) butanoic acid, (KMB, *Figure 2*). Therefore, any differences in metabolism between these two sources of L-met should have their biochemical basis before or during the first conversion step. Chick liver peroxisomes have been reported by Scott *et al.* (1969) to contain both L-HAOX and D-AAOX. The

specific activity of L-HAOX was greater than three-fold that of D-AAOX (10.3 vs. 2.7 units) in the two-year-old leghorns that Scott *et al.* studied.

In order to study the oxidase enzymes, chick liver homogenate was centrifuged to yield a fraction enriched in peroxisomes (Dibner and Knight, 1984). The conversion of HMB to KMB by this peroxisomal fraction confirmed that KMB was the intermediate in this pathway. Similar experiments also confirmed the requirements for oxygen and a flavin coenzyme (Dibner and Knight, 1984). Using D-met as the substrate for the peroxisomal oxidases, the presence of D-AAOX in chick liver peroxisomes was confirmed.

Following these initial studies, the activity of L-HAOX toward L-HMB versus D-HMB was tested. Reactions containing D-HMB yielded 1.95 milliunits of activity in comparison to 12.68 milliunits of activity toward L-HMB (Dibner and Knight, 1984). Thus, 87% of the total activity was directed toward L-HMB. This is in agreement with other reports that peroxisomal HAOX is specific for the L-isomer (Masters and Holmes, 1977).

An oxidizing flavo-enzyme for D-hydroxy acids, D-hydroxy acid dehydrogenase (D-HADH, EC 1.1.99.6) has been isolated from rabbit kidney mitochondria (Cammack, 1969; Tubbs and Greville, 1961). Because it cannot use molecular oxygen in the oxidation reaction, this enzyme requires an additional cofactor and is therefore referred to as a dehydrogenase rather than an oxidase.

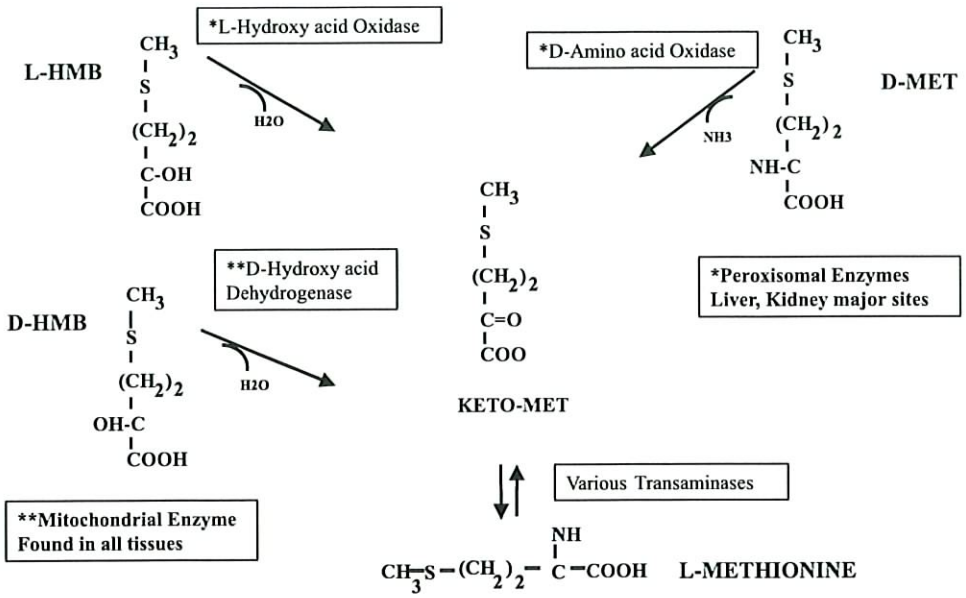
To test for D-HADH, activities of the crude homogenate and the peroxisomal and mitochondrial fractions were compared using either molecular oxygen or another cofactor (phenazine methosulfate, PMS) in the reaction, and the two HMB stereoisomers were tested for activity under these conditions. Little increase in activity accompanied the addition of PMS to any reaction containing L-HMB, while an activity increase of up to ten-fold was seen in reactions containing D-HMB. More oxidase activity was associated with the peroxisomal fractions and more dehydrogenase activity with the mitochondrial fractions (Dibner and Knight, 1984).

A summary of these enzymes and their common intermediate and end product is presented in *Figure 2*. The organelles, in which activity was found, as well as cofactor requirements and isomer specificity, were used to identify activity toward L-HMB as peroxisomal L-HAOX and toward D-HMB as mitochondrial D-HADH. Conversion of HMB and D-met by the three enzymes has also been demonstrated in ruminants (McCollum *et al.*, 2000; Vazquez-Anon *et al.*, 2000) and swine (data not shown).

The conversion enzymes for HMB and D-met were also compared in terms of their tissue distributions. As expected from previous studies of supplemental methionine metabolism and peroxisome tissue distribution, the oxidases for L-HMB and D-met were found in chick liver and kidney. Mitochondria are found in virtually every cell type, however, and therefore many organs were tested for dehydrogenase activity. Conversion of D-HMB was found in every tissue studied (Dibner and Knight, 1984). Activity ranged from a low of 10.89 milliunits in spleen to the high values of 135.71 and 119.54 milliunits in liver and thigh muscle, respectively. The higher D-HADH content of red versus white muscle is consistent with the large number of mitochondria in red fibres (Bowman and Marshall, 1971).

Further investigation of the role of HMB and keto methionine in the liver has demonstrated that HMB is a naturally occurring methionine precursor in the chick (Dibner *et al.*, 1990). The biochemical synthesis of HMB from methionine, methylthioribose-1-phosphate and methylthioadenosine has been reported in rats (Edwards *et al.*, 1973) and various microorganisms (Belasco *et al.*, 1978; Galsworthy and Metzenberg, 1965; Maw and Coyne, 1966). Biochemical synthesis of HMB from radiolabeled methionine was observed by Saroka (1983). Chick liver cytosolic enzymes were used to describe the role of HMB as an intermediate in the synthesis of methionine from methylthioadenosine (Dibner *et al.*, 1990).

Figure 2 Schematic of the conversion of D-HMB, L-HMB and D-methionine to L-met. All the isomers have the same general conversion pathway with an initial oxidation step to keto-methionine followed by transamination to L-met. The major difference among the sources is that D-met yields ammonia as a byproduct of oxidation while HMB yields water.



Absorption of HMB

The mechanism of HMB absorption from the intestinal lumen is also of interest, in part because conflicting rates of absorption have been reported in the literature (Lerner and Kratzer, 1969; Saroka and Combs, 1983; Knight and Dibner, 1984; Maenz and Engele-Schaan, 1996). As might be expected, absorption of HMB resembles that of other short chain organic acids (Mroz, 2000), particularly lactic acid. At low pH, HMB is lipophilic and absorbed by diffusion. This is the predominant mechanism of transport in the upper gut. In fact, *in vitro* tissue preparations of oesophagus and crop have been used to demonstrate transfer of HMB across the wall of the crop (data not shown). This process is responsive to pH and confirms the importance of diffusion in HMB absorption.

At higher pH, such as in the ileal lumen, HMB is ionised and requires a carrier-mediated transport system. The presence of such a system has been described by Brachet and Puigserver (1987), who demonstrated that transport of HMB can be mediated by an energy-independent, proton-dependent carrier system associated with L-lactate. As shown in *Figure 3*, the predominant mode of transport for HMB is by passive diffusion with a lesser role for energy-independent carrier mediated transport, very much like lactic acid. This transport system, while of biochemical interest (Brachet and Puigserver, 1987; Maenz and Engele-Schaan, 1996; McCollum *et al.*, 2000; Pan *et al.*, 2002) will be a minor component of HMB uptake under normal physiological circumstances, in which rapid uptake will occur in the acid environments of the upper gut, where HMB will tend to be undissociated and therefore capable of diffusing across cell membranes (Knight and Dibner, 1984; McCollum *et al.*, 2000). Even in the lower gut, diffusion could be the predominant mechanism because of the presence of an acidic microenvironment in the unstirred layer covering enterocytes.