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Review Article

# A systematic review of metabolism of methionine sources in animals: One parameter does not convey a comprehensive story

Philippe Becquet [a, *], Mercedes Vazquez-Anon [b], Yves Mercier [c], Karen Wedekind [b], Tahir Mahmood [c], Dolores I. Batonon-Alavo [c], Frances Yan [b]

[a] International Methionine Analogue Association, Regus Brussels City Centre, Stéphanie Square, Avenue Louise, 65, B-1050 Brussels, Belgium
[b] Novus International Inc., 20 Research Park Drive, Saint Charles, Missouri 63304, USA
[c] Adisseo France SAS, Antony Parc II, 10 Place Du Général de Gaulle, Antony 92160, France

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ABSTRACT

The goal of this review article, based on a systematic literature search, is to critically assess the state of knowledge and experimental methodologies used to delineate the conversion and metabolism of the 2 methionine (Met) sources DL-methionine (DL-Met) and DL-2-hydroxy-4-(methylthio) butanoic acid (HMTBa). The difference in the chemical structures of HMTBa and DL-Met indicates that these molecules are absorbed and metabolized differently in animals. This review explores the methodologies used to describe the 2-step enzymatic conversion of the 3 enantiomers (D-HMTBa, L-HMTBa and D-Met) to L-Met, as well as the site of conversion at the organ and tissue levels. Extensive work was published documenting the conversion of HMTBa and D-Met into L-Met and, consequently, the incorporation into protein using a variety of in vitro techniques, such as tissue homogenates, cell lines, primary cell lines, and everted gut sacs of individual tissues. These studies illustrated the role of the liver, kidney, and intestine in the conversion of Met precursors into L-Met. A combination of in vivo studies using stable isotopes and infusions provided evidence of the wide conversion of HMTBa to L-Met by all tissues and how some tissues are net users of HMTBa, whereas others are net secreters of L-Met derived from HMTBa. Conversion of D-Met to L-Met in organs other than the liver and kidney is poorly documented. The methodology cited in the literature to determine conversion efficiency ranged from measurements of urinary, fecal, and respiratory excretion to plasma concentration and tissue incorporation of isotopes after intraperitoneal and oral infusions. Differences observed between these methodologies reflect differences in the metabolism of Met sources rather than differences in conversion efficiency. The factors affecting conversion efficiency are explored in this paper and are mostly associated with extreme dietary conditions, such as noncommercial crystalline diets that are very deficient in total sulfur amino acids with respect to requirements. Implications in the diversion of the 2 Met sources toward transsulfuration over transmethylation pathways are discussed. The strengths and weaknesses of some methodologies used are discussed in this review. From this review, it can be concluded that due to the inherent differences in conversion and metabolism of the 2 Met sources, the experimental methodologies (e.g., selecting different organs at different time points or using diets severely deficient in Met and cysteine) can impact the conclusions of the study and may explain the apparent divergences of conclusion found in the literature. It is recommended when conducting studies or reviewing the literature to properly select the experimental models that allow for differences in how the 2 Met precursors are converted to L-Met and metabolized by the animal to enable a proper comparison of their bioefficacy.


* Corresponding author.
*E-mail address:* philippe.becquet@imaa-asbl.org (P. Becquet).
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## 1. Introduction

Numerous studies have been published in recent decades to compare the bioefficacy of different sources of methionine (Met), such as 2-hydroxy-4-(methylthio) butanoic acid (HMTBa) and its calcium salt (HMTBa-Ca) and DL-methionine (DL-Met). However,

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these studies yielded conflicting results. The objective of this article is to extensively and critically analyze publications related to the conversion of the 2 sources of Met. This critical review allows the understanding of the cause for diverging results and provides some insights into how further evaluation should be conducted when the evaluation of the bioefficacy of the 2 molecules is discussed.

## 2. Materials and methods

The review aims to address the following question regarding the relative efficacy of Met sources: Is there any scientific evidence regarding the enzymatic conversion steps of L-Met precursors (i.e., D-Met, HMTBa, keto methyl-thio butanoic acid [KMB]) that can explain differences in the relative efficacy between the sources?

Literature searches of the Web of Science, Scopus, CAB Abstracts and Google Scholar were conducted using combinations of terms covering the different names of precursors, including their CAS numbers (e.g., HMTBa, MHA, Met, DL-Met) and the different species, with a focus on monogastric animals (e.g., poultry, layers, quails). Additionally, no time limit was imposed, allowing the coverage of a period from 1937 to 2019. In addition, specific keywords related to conversion were included in the search strategy. The detailed key words are provided in the supplementary information (Table S1). The literature searches were combined with a search on transport/absorption.

The search resulted in 1417 scientific articles, from which 457 duplicates were removed. All remaining 960 articles were evaluated by 3 independent readers and classified into 1 of the 3 following groups: within scope, excluded, or complementary (for articles not covering the scope of the review but providing complementary elements to be used in the critical assessment).

From the 79 articles included in the scope subjects, 7 were removed, either due to full text language issues or unavailability of the full article.

The 72 remaining articles were further critically assessed for their quality and split into 2 categories relevant to the conversion (40 articles) or to the transport/absorption (32 articles) of the different precursors. The details of the sequence are provided in the supplementary information (Fig. S1). Nine additional articles were evaluated for their quality and reintroduced in the list of articles used for the critical review of the conversion. Therefore, 49 articles were included in this review.

## 3. Chemical structure of methionine sources

Methionine, also known as 2-amino-4-(methylthio) butanoic acid, is an amino acid, as it has one basic amino group and one acidic carboxyl group and no additional acidic or basic group on the side chain. Methionine is considered a neutral amino acid and exists in its zwitterion form as the amino group captures the hydrogen ion from the carboxyl group in the crystalline state and in aqueous solution (Meister, 1957; Wu, 2013). With the amino group on the α-carbon in Met replaced by a hydroxyl group, HMTBa is an organic acid. Without an amino group neutralizing the carboxyl group, the carboxyl group easily donates its hydrogen ion in solution; therefore, it is acidic. Like most organic acids, HMTBa possesses antimicrobial properties, as it is lipophilic in its undissociated form and can cross the bacterial cell membrane, modifying hydrogen ions and their associated anion concentrations in the cytoplasm (Doerr et al., 1995; Enthoven et al., 2002).

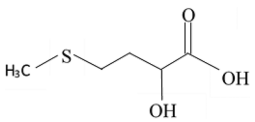
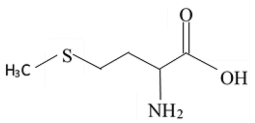
The physical and chemical properties of HMTBa and DL-Met are summarized in Table 1. The Met and HMTBa forms available for practical use are racemic mixtures of D- and L-enantiomers. L-methionine is the only enantiomer used directly by animals for protein synthesis and other metabolic functions. The 50% D-Met in DL-Met and both enantiomers in HMTBa, 50% D-HMTBa and 50% L-HMTBa, need to be converted to L-Met before being used by animals as a Met source. HMTBa has been detected in the liver, plasma, excreta, or urine of chickens and rats that never received HMTBa, indicating that it is a naturally occurring Met precursor in animals (Dibner et al., 1990; Saroka and Combs, 1986; Trackman and Abeles, 1981). The difference in the chemical structure and physical and chemical properties of HMTBa and DL-Met determines that these molecules are absorbed and metabolized differently in animals.

## 4. Conversion of Met precursors into L-Met

### 4.1. Different steps of conversion: location of the enzymes

The biochemical conversion of Met precursors to L-Met is summarized in Fig. 1. It occurs in two-step reactions that have been studied by several authors in vitro (Dibner and Knight, 1984;

**Table 1**
Comparison of the physical and chemical properties of HMTBa and DL-Met.

| Met source | HMTBa | DL-Met |
|---|---|---|
| Name | 2-hydroxy-4-(methylthio) butanoic acid | 2-amino-4-(methylthio) butanoic acid |
| Chemical structure |  |  |
| Molecular formula | $C_5H_{10}O_3S$ | $C_5H_{11}NO_2S$ |
| Molecular weight | 150.12 g/mol | 149.21 g/mol |
| Chemical property | Organic acid | Amino acid |
| pKa | 3.53 | 2.28 (carboxy); 9.21 (amino) |
| pH | Acidic | Neutral |
| Antimicrobial | Yes | No |
| Enantiomer | 50% D-HMTBa; 50% L-HMTBa | 50% D-Met; 50% L-Met |
| L-Met precursor | 100% precursor | 50% L-Met; 50% precursor |
| Commercial product purity | 88% DL-HMTBa | 99% DL-Met |
| Appearance | Light-brown liquid | White crystalline powder |

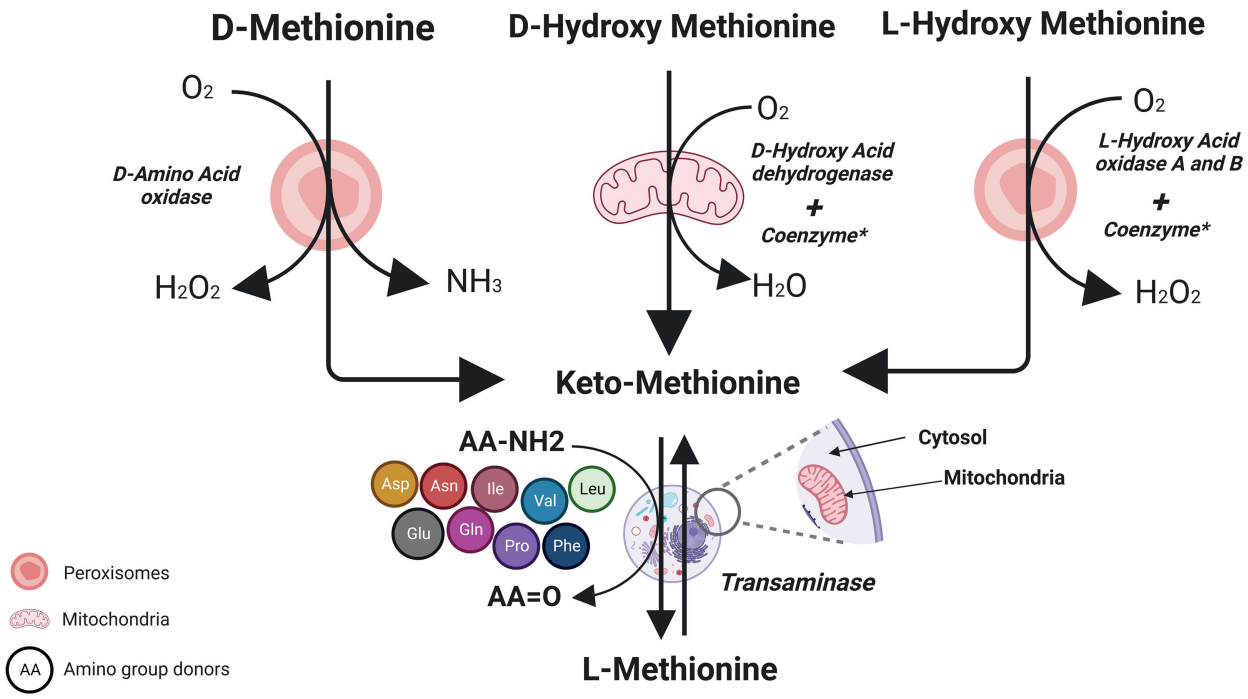



**Fig. 1.** Biochemical conversion pathways of methionine precursors.

Dupuis et al., 1989; Martin-Venegas et al., 2011). Each enantiomer must be converted before being used by the animal. The first step was found to be stereospecific with different enzymes for the D- and L-enantiomers of HMTBa and D-Met. In the case of L-HMTBa, the first reaction is an oxidation of the hydroxyl group to form KMB performed by L-hydroxy acid oxidase, whereas it is a dehydrogenation in the case of D-HMTBa, consisting of the removal of a hydrogen to form KMB by the enzyme D-hydroxy acid dehydrogenase (Dibner and Knight, 1984). The conversion of D-Met into KMB is also an oxidation and is performed via the enzyme D-amino acid oxidase (Gordon, 1965). Once KMB is obtained from the different precursors, it is then transaminated into L-Met through transaminases and amino acid donors (Fig. 1). The following sections describe the different enzymes responsible for the conversion of the 3 precursors D-Met, D-HMTBa and L-HMTBa into L-Met and their intracellular location.

#### *4.1.1. Oxidation of D-Met through D-α-amino acid oxidase (D-AAOX)*

D-α-amino acid oxidase is a flavoprotein catalyzing the oxidative deamination of D-amino acids with its prosthetic group of flavin-adenine dinucleotide (FAD) amino acids (Masters and Holmes, 1977). D-α-amino acid oxidase is present in many vertebrate species and is found predominantly in the kidney and, to a lesser extent, in the liver, except in the mouse liver (Masters and Holmes, 1977). D-α-amino acid oxidase has been reviewed by several authors for its conversion of unnatural D-enantiomorphs of amino acids and detoxification of endogenous D-amino acids accumulated in the organism during racemization (Khoronenkova and Tishkov, 2008; Momoi et al., 1988; Pilone, 2000). In animals, only a few authors (Bauriedel, 1963; Fang et al., 2010b; Gordon, 1965) have studied its role in converting D-Met into KMB. The distribution of mRNA abundance and the specific activity of D-AAOX across different tissues in multicatheterized piglets were measured, and it was found that diamine oxidase 1 mRNA was present in higher levels in the kidney, duodenum, jejunum, and ileum compared with the liver and muscle (Fang et al., 2010b), whereas Bauriedel (1963) demonstrated that there was no difference in D-AAOX activity in the livers and kidneys of chicks fed either L-Met or D-Met. Supplementation with D-Met did not increase or decrease the amount of D-AAOX in the chicken liver or kidney (Bauriedel, 1963).

The enzyme is present in the peroxisomal fractions of these tissues (Beaufay et al., 1964; Thiskov and Khoronenkova, 2005). During the biochemical conversion of D-Met into KMB, hydrogen peroxide ($H_2O_2$) is produced. Peroxisomes are organelles containing predominantly oxidases that use various substrates, such as L-α-hydroxy acids and D-amino acids. They also contain catalase decomposing the toxic oxidase product $H_2O_2$ (Duley and Holmes, 1976). Thus, such localization of D-AAOX provides the efficient removal of the cell toxicant $H_2O_2$ (Momoi et al., 1988). Peroxisomes consist of 4 compartments (Hayashi et al., 1973). When subjected to differential and density gradient centrifugation, the peroxisomes were identified by their contents of catalase, α-hydroxy acid oxidase, D-AAOX, and urate oxidase (Scott et al., 1969). D-α-amino acid oxidase is contained in a biochemical "compartment" of the peroxisome that is resistant to solubilization and similar to urate oxidase (Masters and Holmes, 1977). According to Scott et al. (1969), D-AAOX and urate oxidase behaved similarly and were measured in the peroximal-lysosomal fraction of rats or in the peroxisomal fraction of birds, with variable proportions appearing in the soluble fraction.

#### *4.1.2. Oxidation of L-HMTBa using L-α-hydroxy acid oxidase (L-HAOX)*

L-α-hydroxy acid oxidase, a flavin-containing protein, catalyzes the oxidation of L-α-hydroxy acids to produce the corresponding α-ketoacids and $H_2O_2$ (Masters and Holmes, 1977). This enzyme is mainly found in the peroxisomes of the liver and kidney (Dibner

and Knight, 1984; Fang et al., 2010b). For this first conversion step, a molecule of oxygen and a coenzyme are required and result in KMB and $H_2O_2$ as a byproduct. The coenzyme was identified as FAD in rat liver (Langer, 1965), flavin mononucleotide (FMN) in hog kidney (Robinson et al., 1962) and nicotinamide-adenine dinucleotide in Ascaris (Langer et al., 1971).

L-α-hydroxy acid oxidase exists as 2 isozymic forms (isozymes A and B) in mammalian tissues that differentially oxidize a variety of L-α-hydroxy acids (Duley and Holmes, 1976). Isozyme A and isozyme B refer to short- and long-chain hydroxy acid oxidases, respectively (Duley and Holmes, 1976). As described by Masters and Holmes (1977) and Duley and Holmes (1976), mammalian liver isozyme A preferentially oxidizes short-chain aliphatic hydroxy acids and exhibits no activity with aromatic hydroxy acids (Duley and Holmes, 1976). It is sometimes referred to as glycolate oxidase because of its high activity toward this substance. In contrast, isozyme B exhibits no activity with glycolate but has activity for many aliphatic and aromatic acids, with carbon chains longer than hydroxy butyrate (Gordon, 1965). This isozyme also catalyzes the oxidative deamination of different L-amino acids (Robinson et al., 1962). This latter study from Robinson et al. (1962) on the hog renal cortex suggested that L-HMTBa may be a better substrate for Isozyme B than for Isozyme A. However, other studies have shown that L-HAOX purified from hog kidney consists of a single form able to oxidize glycolate and, to a lesser extent, L-HMTBa (Langer et al., 1971). Additionally, isozyme A activity was detected in chicken liver and kidney peroxisomes (Scott et al., 1969). Martin-Venegas et al. (2011) were not able to precisely identify which isozyme was responsible for the conversion of L-HMTBa, but it was shown that both isozymes were present in Caco-2 cells. They observed the highest activity on glycolic acid and HMTBa in the control cells, thus suggesting that HMTBa was preferentially oxidized by isozyme A unless isozyme B has a higher affinity for HMTBa than for L-2-hydroxy-isocaproic acid. However, in HMTBa-supplemented conditions, there were no differences between the substrates, indicating a similar increase in glycolic acid and HMTBa and a more pronounced increase in L-2-hydroxyisocaproic acid, thus suggesting that isozyme B is more sensitive to HMTBa availability. Altogether, these results thus confirmed the role of L-HAOX in the conversion of L-HMTBa into KMB, as demonstrated by different authors. The 2 L-HAOX isozymes are differentially localized between the highly compartmentalized environment of the peroxisome and the cell sap (Duley and Holmes, 1976). However, as specified by Duley and Holmes (1976), the 2 isozymes are 2 structurally homologous and evolutionarily related isozymes and not 2 separate enzymes.

Dibner and Knight (1984) obtained a peroxisomal fraction by differential centrifugation of chick liver homogenate based on methods described by deDuve and Baudhuin (1966) and showed that the peroxisomal fraction catalyzed the oxidation of HMTBa to KMB, thus confirming the presence of L-HAOX in liver peroxisomes. Similarly, Dibner and Ivey (1992) measured the total chick liver capacity to convert L-HMTBa into KMB. They showed that when each enantiomer of HMTBa was tested individually, some KMB formation was obtained. The reaction was examined in the presence of a cofactor, phenazine methosulfate (Dibner and Ivey, 1992), which was not used in the study of Dupuis et al. (1990). Phenazine methosulfate was added in some studies as an artificial electron acceptor. This factor is only required for the conversion of D-HMTBa and not for the oxidative conversion of L-HMTBa or the use of the racemic mixture of both D- and L-HMTBa (Dibner and Ivey, 1992).

#### *4.1.3. Dehydrogenation of D-α-hydroxy acid dehydrogenase (D-HADH)*

The enzyme for D-HMTBa oxidation into KMB was identified as mitochondrial D-HADH. It was shown to oxidize D-lactate and several long-chain D-α-hydroxy acids, D-malate, D-glycerate and meso-tartrate, to the corresponding ketoacids (Camack, 1969; Tubbs and Greville, 1961). D-α-hydroxy acid dehydrogenase is present in many species, including birds. D-HADH is found in every tissue tested in piglets (Fang et al., 2010b).

The conversion of D-HMTBa was evaluated by different authors in both poultry and swine (Dibner and Knight, 1984; Dibner and Ivey, 1992; Dupuis et al., 1989; Martin-Venegas et al., 2011; Schreiner and Jones, 1988). All these studies agreed that D-HMTBa was oxidized into KMB by D-HADH and found that this enzyme was mainly present in the liver and kidney. Schreiner and Jones (1988) established that D-HADH and L-HAOX were both present in porcine kidney fibroblasts and that the 2 enzymes displayed similar conversion rates for their respective enantiomers of HMTBa. Additionally, in the study of Dibner and Knight (1984), the crude homogenate, peroxisomal, and mitochondrial fractions were assayed by using oxygen and phenazine methosulfate as the electron acceptors. An increase in D-HADH activity up to 10-fold that of L-HAOX was obtained with phenazine methosulfate addition. D-α-hydroxy acid dehydrogenase activity was measured in the mitochondrial fraction. A high D-HADH content was also observed in red versus white muscle in relation to the large number of mitochondria in red fibers. However, the activity of this enzyme was initially difficult to assay because of the cofactor required and because it was active over a limited pH range (Dibner and Knight, 1984). Using D-lactate as a substrate, Tubbs and Greville (1961) reported an optimal pH of 8.2 to 8.5 with an abrupt loss of activity between pH 8.5 and pH 9.0, whereas Dibner and Knight (1984) found a strong response of D-HADH to pH, with an optimal pH of 8.24. This is additional evidence that D-HMTBa oxidation was catalyzed by mitochondrial D-HADH.

One of the limitations that could be raised regarding the study of Schreiner and Jones (1987) is the lack of cofactors important for the metabolism of Met precursors. As mentioned earlier, the success of the D-HMTBa assay is very much related to the use of a cofactor. Dibner and Ivey (1992) used phenazine methosulfate, which was found to be the most efficient for the conversion of HMTBa into KMB. The crude enzyme mixture has a very limited capacity for KMB synthesis from D-HMTBa in the absence of this cofactor (Dibner and Ivey, 1992). Phenazine methosulfate is not naturally occurring; however, it has been used as an artificial electron acceptor in biochemical studies of purified D-HADH from several animals (Cammack, 1969). Naturally occurring acceptors, such as FAD, FMN, nicotinamide-adenine dinucleotide, menadione, coenzyme Q, and oxygen, were tested in chick liver homogenates (Dibner and Knight, 1984). The activity obtained with these possible cofactors was higher than that with no cofactor but was still below the activity seen with phenazine methosulfate (Dibner and Knight, 1984). As explained by Dibner and Ivey (1992), the activity of L-HAOX could be detected because the cofactor FAD was included in the reaction mixture. A synthetic electron acceptor is only required for D-HMTBa conversion because the naturally occurring cofactor for this enzyme has not been identified (Dibner and Ivey, 1992). When DL-HMTBa is used, oxidase activity toward L-HMTBa obscures the lack of activity toward D-HMTBa. This could mean that the enzyme activities toward DL-HMTBa in the absence of the cofactor for D-HADH are due to the action of L-HAOX only. However, this contradicts the results of Schreiner and Jones (1987), who observed no growth of porcine kidney fibroblasts in the presence of L-HMTBa but observed the same protein and DNA content per cell between D-HMTBa, DL-HMTBa, or L-Met. They concluded that cultured porcine kidney fibroblasts can metabolize the D-isomer, whereas the L-isomer was inert. Despite the lack of consistency, these results could either be related to the nature of the cells used (porcine kidney fibroblast requirements may have been poorly

documented), the lack of any naturally occurring cofactor and the culture conditions of the cells or the lack of transporters (Schreiner and Jones, 1987).

#### 4.1.4. Transamination

Following the formation of KMB, the second step of conversion of Met precursors is its transamination into L-Met, which is ubiquitous (Table 1). For amino acids, the transamination reaction consists of a transfer of an amino group from an amino acid to an α-ketoacid, in this case KMB. The reaction is catalyzed by pyridoxal phosphate-dependent transaminases, also known as amino transferases. Amino transferases are present in the cytosol, mitochondria, and peroxisomes (Sakuraba et al., 1991) and play an important role in integrating various metabolic pathways. Amino transferases are present in the cytosol and mitochondria, but their activity is much higher in the cytosol (Bhagavan and Ha, 2011).

Transamination of KMB to L-Met was documented in Ascaris (Langer et al., 1971), chick liver (Gordon, 1965), rat liver (Langer, 1965), and Caco-2 cells (Martin-Venegas et al., 2011) using various donor amino acids. Glutamine serves as an amino donor in the formation of Met from the alpha keto acid in rat liver (Gordon, 1965). Similarly, glutamine and asparagine transaminases convert KMB into L-Met (Langer, 1965). In the latter study, the replacement of glutamine by glutamate, aspartate or ammonium sulfate did not restore activity, but asparagine could partially replace glutamine (Langer, 1965). Nevertheless, while most of the studies concluded that glutamine is the amino donor used to convert KMB, Martin-Venegas et al. (2011) found that transamination was not linked to a specific amino group donor since all the amino acids tested promoted the formation of L-Met, although leucine was the preferred amino group donor. In this sense, Rangel-Lugo and Austic (1998) observed that there is a larger capacity for KMB to be transaminated in the liver, kidney, intestine, and muscle of chickens. All amino acids (L-isoleucine, L-glutamate, L-leucine, L-valine, L-aspartate, L-alanine, L-glutamine, L-asparagine and L-phenylalanine) investigated as nitrogen donors promoted the conversion of KMB in all 4 tissues. A higher affinity was observed for some amino acids compared with others depending on the tissues, e.g., in cytosols, branched chain amino acids and glutamine were the most preferred. In the intestinal mucosa, asparagine was also effective. The preferred substrates in mitochondria were glutamate in the liver, isoleucine and alanine in the kidney, and branched chain amino acids and glutamate in skeletal muscle. In the chick system, branched chain amino acids were found to serve as amino group donors, whereas glutamine was found to be ineffective (Gordon, 1965).

In the different studies mentioned above, different models and amino donors were used, which could explain the difference in the findings. In the study of Rangel-Lugo and Austic (1998), tissue samples were obtained from chickens fed a balanced diet supplemented with DL-Met, whereas Caco-2 cell models were used by Martin Venegas et al. (2011). While the first model is representative of in vivo conditions, the second model is a well-characterized intestinal in vitro model enabling evaluation of the ability of chemicals to cross the intestinal barrier and to study their transport mechanisms and metabolism (DeAngelis and Turco, 2011; Sun et al., 2008). The Caco-2 cell monolayer is a human colon adenocarcinoma cell line in which most intestinal transporters and enzymes are expressed, including the enzymes responsible for converting Met precursors. Despite their colorectal origin, differentiated Caco-2 cells express many of the morpho-functional features of mature small intestine enterocytes (Picariello et al., 2016), e.g., columnar polarization, apical microvilli, and tight junctions. Caco-2 cells have the disadvantage of lacking the mucous layer found in the intestinal wall (Gamboa and Leong, 2013); however, this does not preclude the accuracy of the Caco-2 cell method for evaluating the conversion of Met precursors (Martin-Venegas et al., 2011). Therefore, the Caco-2 cell monolayer remains to be prevalently used for the assessment of drug permeability, transport, and metabolism because the system is enterocyte-like, well characterized, and easily maintained and managed (Sun et al., 2008). Consequently, the findings of Martin-Venegas et al. (2011) are completely valid in establishing the amino donors for the transamination of KMB into L-Met, whether KMB is produced from D-Met or DL-HMTBa oxidation. It should be noted that the conclusions of Gordon (1965) and Langer et al. (1971) are only valid for the amino acids tested as amino donors (glutamine and leucine (Gordon, 1965) and glutamine, glutamate, and asparagine (Langer et al., 1971)) and cannot be extended to others.

Finally, when any one of the 3 acids (alpha hydroxy, alpha keto, or alpha amino) is added to the homogenates, the other two are formed very quickly, regardless of which one was used as a substrate, indicating extensive metabolic interconversion (Gordon, 1965). All amino acids except alanine supported Met synthesis from KMB in the mitochondria of the intestinal mucosa. From these studies, it can be concluded that a wide variety of amino acids can serve as substrates for the transamination of KMB in chickens and that the availability of nitrogen donors is unlikely to be a limiting factor in the conversion of KMB to Met.

### 4.2. Where does the conversion occur?

Sites of conversion of Met precursors to L-Met have been studied both in vitro and in vivo. For in vitro studies, tissue homogenates, cell lines, primary cell cultures, and everted gut sacs have been evaluated for their ability to synthesize L-Met and/or KMB. While in vitro experiments provide insights into whether certain biochemical reactions can occur in vivo, they may not be directly applicable because the conditions are different. Hence, extrapolation of the degree of metabolism in various tissues based on in vitro experiments needs to be further confirmed with in vivo trials.

#### 4.2.1. Liver

As an organ that plays a central role in metabolic processes in the body, the liver has long been recognized for its ability to convert HMTBa and D-Met to KMB and L-Met in multiple animal species. Using the supernatant fraction of tissue homogenates, the activity of the liver to convert HMTBa-Ca to L-Met was higher than that of the small intestine, brain, or muscle but lower than that of the kidney on a per gram tissue basis, and most of the activity within the liver cells was present in the supernatant fraction (Langer, 1965). However, based on its much greater weight, Langer (1965) hypothesized that the liver must play a predominant role in converting HMTBa-Ca to L-Met in intact rats. The crude homogenates of chick or rat liver readily converted HMTBa to L-Met (Gordon, 1965). The mitochondria preparation mainly converted HMTBa to KMB, and the supernatant fraction (without mitochondria) converted KMB to both L-Met and HMTBa. Minimal D- or L-Met was oxidized by the mitochondrial preparations. The fact that the supernatant (obtained by differential centrifugation of disrupted liver cells) of rat liver homogenates converted HMTBa-Ca to L-Met (Langer, 1965) and the supernatant of liver homogenates (obtained by the removal of mitochondria from the supernatant) only converted KMB to L-Met (Gordon, 1965) is most likely due to the different methods used to prepare the supernatant fraction. Gordon (1965) specifically mentioned that their supernatant fraction was obtained by differential centrifugation of disrupted liver cells. Using primary cultures of chick hepatocytes combined with radiolabeling techniques, chick liver was capable of converting HMTBa to L-Met and incorporating L-Met into protein

(Dibner, 1983), and when HMTBa was compared with DL-Met, equimolar quantities of the radioactivity from the 2 sources were detected in liver protein at all time points measured (15 min, 2 h, 4 h, 8 h, 24 h, and 48 h) (Dibner, 1983). Equivalent incorporation of HMTBa and DL-Met into the culture medium as early as 15 min after their introduction was an indication of rapid conversion to L-Met. This result demonstrated that at the cellular level in the liver, both HMTBa and DL-Met are biochemically equivalent sources of Met for protein synthesis (Dibner, 1983). Among all chicken tissues tested, including the liver, kidney, gastrointestinal tract, brain, spleen, and muscle, liver homogenates had the highest D-HADH activity per gram of tissue (Dibner and Knight, 1990). Dupuis et al. (1989) studied the specific activity of various chicken tissues to convert HMTBa, D-Met, and L-Met to KMB using 1-$^{14}$C-labeled tracers and found that the specific activity of the liver on a mg protein basis was lower than that of the kidney but higher than that of the intestinal mucosa and breast muscle. Taking into consideration organ mass, they concluded that at physiological concentrations, the liver contributed principally to the whole-body oxidation (conversion to KMB) of both HMTBa and D-Met in chickens. The authors also observed that the rate of conversion to KMB was greater for DL-Met compared with HMTBa in various tissues, including the liver (Dupuis et al., 1989). It was pointed out by Dibner and Ivey (1992) that an essential cofactor for D-HADH was missing in the work of Dupuis et al. (1989), therefore underestimating the conversion rate of HMTBa to KMB. The transamination activity from KMB to L-Met in chicken liver homogenates was similar to or slightly higher than that in intestinal mucosa and skeletal muscle but lower than that in kidney tissue (Rangel-Lugo and Austic, 1998). The activity of liver homogenates to convert D-Met, D-HMTBa, and L-HMTBa to KMB was reported to be lower than that in the kidney but higher than that in the intestines in piglets (Fang et al., 2010b). Based on in vitro studies from different laboratories, one would expect that the liver should be the major site of conversion for both D-Met and HMTBa in chickens and rats.

In contrast to in vitro studies, the limited in vivo work in the literature does not seem to support the idea that the liver is the major organ for conversion to L-Met. When anesthetized broilers were infused with HMTBa in the hepatic portal vein, the liver removed 86% of HMTBa entering from both the hepatic portal vein and hepatic artery; therefore, the authors concluded that the results indicated that the liver was a major site of removal and further metabolism of HMTBa in chickens (Wang et al., 2001). However, the fate of HMTBa extracted by the liver was not evaluated in the study, and it was unclear whether it was primarily converted to L-Met or catabolized. In addition to being anesthetized, the birds in the study had no access to feed or water for 6 h before blood sample collection, and the doses of HMTBa infused during the 10-min infusion periods, 2.2 to 22 mg/min per kg body weight, were 10- to 100-fold those of the normal Met intake from feed for 24 h. The results, therefore, most likely reflect the liver's ability to remove and catabolize extra HMTBa, not its role in converting HMTBa to L-Met under normal conditions in broilers (Wang et al., 2001). Using similar techniques, researchers from the same laboratory infused hepatic veins of Single Comb White Leghorn males with either HMTBa or DL-Met at either 0.22 or 0.88 mg/min per kg body weight and investigated liver extraction of Met along with other amino acids (Song et al., 2001). At both doses of HMTBa and a low dose of DL-Met, the liver removed 32% of the Met entering the liver; however, a high dose of DL-Met, 4-fold the normal Met intake, exceeded the liver's capacity, and the Met removal rate became negative. It is worth noting that the 4-fold infusion of DL-Met resulted in negative liver extraction in the study of Song et al. (2001), whereas chicken liver removed 86% of up to 100-fold HMTBa in the former study (Duley and Holmes, 1976; Enthoven et al., 2002). Considering that the 2 studies were from the same laboratory and used the same technique, it appeared that chicken liver has a greater ability to extract HMTBa than DL-Met. Although the liver's role in converting HMTBa to L-Met was not the objective of their investigation, it was demonstrated that part of HMTBa flowing out of the liver was converted to L-Met by extrahepatic tissues and secreted into the systemic circulation (Song et al., 2001). Additionally, the fact that the Met concentration in the hepatic vein was lower than that in the portal vein was probably an indication that the liver was not the primary organ that converted HMTBa to L-Met and that HMTBa is present in the systemic circulation for other tissues to use in chickens.

Using stable isotope dilution techniques in lambs, 87% HMTBa infused in the abomasum was recovered in the portal vein, and 37% of this liver was extracted, leaving the majority for posthepatic metabolism (Lobley et al., 2006). A significant amount of L-Met was synthesized by the liver, equivalent to 22.3% Met entry into cells; however, only small quantities of L-Met synthesized from HMTBa by the liver were exported to the circulation, and substantial and significant input was from posthepatic tissues in conscious lambs (Lobley et al., 2006). Using the same stable isotope dilution techniques in lambs, the liver was found to extract 25% of HMTBa infused into the mesenteric vein, which did not increase Met release from the liver, again indicating that the liver was not a major site of Met synthesis and secretion (Wester et al., 2006). Comparing their findings with the results of Wang et al. (2001) in chickens, the authors concluded that there appeared to be a genuine difference between chicken and lamb in the liver's role in HMTBa metabolism, which could represent avian vs. mammalian contrast or a nonruminant vs. ruminant difference (Wester et al., 2006). However, it is possible that the different results observed are due to different conditions used, such as doses of HMTBa infused (physiological vs. pharmaceutical), feeding status (fed vs. fasting), and conscious state of the animals (conscious vs. anesthetized). In summary, from the literature, it is unequivocal that the liver converts a significant amount of HMTBa and D-Met to L-Met for further metabolism, but whether it is a major conversion site across species remains a question. The role of the liver in the secretion of L-Met from these Met precursors for other tissues to use is also a question. The liver may convert Met precursors to L-Met for its own use and not secrete it into the bloodstream. It has been demonstrated in ruminants that the liver is not a major site for the conversion of HMTBa to L-Met and secretion into the bloodstream, and it is highly likely that this could also be the case in nonruminant animals. Further work is warranted to clarify the role of the liver in HMTBa and D-Met conversion to L-Met in nonruminant animals.

#### *4.2.2. Kidney*

Not as much work has been done on the kidney compared with the liver. Based on in vitro studies using tissue homogenates, among the various tissues tested, the kidney was found to possess the highest activity to convert HMTBa-Ca to L-Met (Langer, 1965) or to convert HMTBa, DL-Met, and L-Met to KMB (Dupuis et al., 1989). Despite this higher activity in the kidney, these authors speculated that the liver should be the major organ to convert Met precursors to L-Met in rats and chickens in vivo due to its higher mass. Chicken kidney homogenates readily converted D-Met to KMB; however, minimal conversion of HMTBa to KMB was observed (Gordon and Sizer, 1965). The activity of D-HADH in crude kidney homogenates was lower than that in liver and muscle but similar to that in the small intestine (Dibner and Knight, 1984).

The lack of activity related to the conversion of HMTBa to KMB (Gordon and Sizer, 1965) and low D-HADH activity in the kidney are not in agreement with other findings (Dupuis et al., 1989; Langer,

1965), where the highest conversion activity was observed. Chicken kidney homogenates had the highest transamination activity to form L-Met from KMB compared with the liver, intestinal mucosa, and skeletal muscle (Rangel-Lugo and Austic, 1998). Pig kidney homogenates were found to contain the highest activity to convert D-Met, D-HMTBa, and L-HMTBa to KMB among various tissues tested (Fang et al., 2010b). Without detailed information on preparations of the kidney homogenates and reaction conditions, it is difficult to delineate the reasons for this discrepancy. As the kidney is an organ with a complex structure and different regions serve specific but distinct functions, regions selected for testing could have contributed to the difference in results observed. There was no mention of whether the whole kidney or specific regions of the kidney were used to prepare homogenates in any of the publications. Metabolism of HMTBa was also studied in porcine kidney fibroblast cell lines, and the fibroblasts were found to be able to utilize the sodium salt of D-HMTBa as the sole Met source, but a higher concentration was needed compared with that of Met (Schreiner and Jones, 1987). Additional studies from these authors demonstrated that the inability of porcine kidney fibroblasts to utilize L-HMTBa was not due to a lack of enzyme, as L-HAOX activity was detected, but rather due to unavailability of substrate as the stereospecific transporter was only found for D-isomer, not for L-isomer (Schreiner and Jones, 1988). Although cell lines provide an important tool for biochemistry and cell biology research, the metabolism of immortalized cell lines may differ from that of normal cells. In addition, fibroblast cells are not the principal functional cells in the kidney but the cells that provide a structural framework; therefore, they may not represent the normal functions of the kidney in animals.

In vivo studies related to the conversion of DL-Met and/or HMTBa to L-Met in the kidney are limited. Conversion of HMTBa to L-Met was studied in lambs in vivo using a stable isotope technique, and the kidney was found to convert the greatest amount of HMTBa to L-Met, equivalent to 23.6% Met entry into cells, and to be the only organ that showed isotope-labeled Met enrichment higher than plasma, suggesting that it must be a primary source of plasma Met from HMTBa (Lobley et al., 2006). They demonstrated that while both the liver and kidney converted a significant amount of HMTBa to L-Met, the liver retained the synthesized L-Met, but the kidney secreted it into the systemic circulation, making it available for other Met-requiring tissues. This is consistent with the role of the kidney in reabsorption of amino acids in urine. Additionally, with stable isotope methodology, but a different approach, the kidney was also found to be the principal organ responsible for converting D-Met to L-Met in rats in vivo (Hasegawa et al., 2011). The authors performed nephrectomy on rats to remove five sixth of the kidney mass, which resulted in a reduction in clearance of intravenously administered D-Met, and the metabolic clearance value associated with converting D-Met to L-Met was reduced by five sixth compared with that in sham-operated rats. The highest specific conversion activity of rat and chicken kidney homogenates observed in most in vitro studies and the critical role of the kidney observed in trials in vivo with lambs and rats suggest that the kidney is a major site of conversion for D-Met and HMTBa in animals, which needs to be confirmed by in vivo studies in species other than lambs and rats.

#### 4.2.3. Intestine

With the kidney and liver serving as the main organs for HMTBa and D-Met conversion, other organs have also been reported to convert HMTBa or D-Met to L-Met. In vitro studies with tissue homogenates demonstrated that the intestine possesses the third highest activity among the organs to convert HMTBa-Ca to L-Met in rats (Langer, 1965), HMTBa and D-Met to KMB in chickens (Dupuis et al., 1989), and KMB to L-Met in chickens (Rangel-Lugo and Austic, 1998), followed by the kidney and liver. Methionine appeared in the serosal compartment (serosal pH 7.4) of chicken duodenal, jejunal, and ileal everted sacs incubated with HMTBa, confirming the capacity of the intestine to convert HMTBa to L-Met, and the Met appearance was higher in the presence of an $H^+$ gradient (mucosal pH 5.5 vs. mucosal pH 7.4) (Martin-Venegas et al., 2006). The capacity of piglet stomach, duodenum, jejunum, and ileum homogenates to convert D-Met to KMB was much lower than that of kidney or liver; however, their capacity to convert D-HMTBa or L-HMTBa was only slightly lower than that of the kidney or liver (Fang et al., 2010b). It was demonstrated that differentiated intestinal Caco-2 cells had the capacity to convert HMTBa to L-Met, and the conversion was upregulated by nutritional HMTBa supplementation (Martin-Venegas et al., 2011).

Apart from in vitro work, in vivo studies also demonstrated that intestines play a role in converting HMTBa to L-Met. The increase in portal vein levels of Met and the increase in portal-drained viscera appearance for Met following HMTBa infusions into the portal vein of anesthetized chickens indicate that the intestines were synthesizing and secreting Met into the vasculature (Song et al., 2001). When lambs were infused with stable isotope [1-$^{13}$C] HMTBa into the abomasum, synthesis of Met from HMTBa by gut tissues, including rumen, omasum, abomasum, duodenum, jejunum, ileum, and ceca, was equivalent to 5% to 8% Met entry into cells, lower than that in the liver and kidney but higher than that in the muscle, skin, brain, and lung (Lobley et al., 2006). Across species, both in vitro and in vivo studies supported the intestine as an important site of HMTBa conversion along with the kidney and liver, but its role in converting D-Met to L-Met is less documented.

The fraction of HMTBa and DL-Met that can pass through the gastrointestinal tract to circulation to be further metabolized in the body has been the subject of several studies (Fang et al., 2010a, 2010b; Zuo et al., 2019). When diets containing HMTBa or DL-Met supplied at a level to provide 0.12% Met activity (based on 99% Met activity for DL-Met and 88% Met activity for HMTBa) were fed to barrows at hourly intervals, the fractional portal Met balance, expressed as the fraction of ingested directly available L-Met, was higher in pigs fed HMTBa diets, suggesting a potential difference in the first-pass metabolism of Met by the intestine (Fang et al., 2010a). Note that the denominator in the calculation of the fractional portal Met balance was dietary, directly available L-Met, which excluded D-Met and HMTBa. In a companion paper on the same study, the fractional net portal balance was calculated with ingested total Met (L-Met from feed ingredients plus supplemental DL-Met or HMTBa) as the denominator, and no significant difference was observed between DL-Met and HMTBa diets (Fang et al., 2010b), indicating that the fractional portal Met difference was mainly due to a lower input of directly available L-Met in HMTBa-supplemented diets. Researchers from the same laboratory conducted further research using the intestinal cell line IPEC-J2 and concluded that HMTBa was more likely to bypass first-pass intestinal metabolism than DL-Met (Zuo et al., 2019). Further examination of the data revealed that these IPEC-J2 cells did not convert HMTBa to L-Met as the intracellular concentrations of Met and its metabolites, such as S-adenosylmethionine, S-adenosylhomocysteine, homocysteine, and cystathionine, of the HMTBa-treated cells were completely different from those of the Met-treated cells but very similar to those of the untreated control cells. Based on tissue homogenates, everted sacs, and the in vivo studies discussed above, intestinal cells possess the ability to convert HMTBa to L-Met, so it is questionable whether the IPEC-J2 cell line is an appropriate model for such studies. Taken together, there is no adequate evidence in the literature to suggest a difference in intestinal first-pass metabolism between HMTBa and DL-Met in animals.

*4.2.4. Organs other than liver, kidney, and intestine*

In addition to the liver, kidney, and intestine, conversion of Met precursors to L-Met has been reported to occur in other organs. The conversion in other organs appears to support the needs of that organ, with L-Met being used for protein synthesis in that location. The specific activity of muscle homogenates on a mg protein basis was similar to or lower than that of the intestine to convert HMTBa-Ca to L-Met in rats (Langer, 1965), HMTBa and D-Met to KMB in chickens (Dupuis et al., 1989), and KMB to L-Met in chickens (Sun et al., 2008). However, due to its greater mass, muscle could play a greater role than intestine in L-Met synthesis from Met precursors in vivo. Factoring in mass, Dupuis et al. (1989) calculated the total activity of different organs to form KMB and concluded that muscle should be the main site for HMTBa oxidation when it is present at concentrations higher than physiological concentrations and for L-Met oxidation regardless of concentration. The D-HADH activity of crude muscle homogenates was found to be lower than that in the liver but higher than that in the kidney, intestine, brain, and spleen, with the activity in muscle with oxidative metabolism being 2.2-fold that in muscle with glycolytic metabolism in chickens (Dibner and Knight, 1984). Muscle homogenates contained the lowest D-AAOX activity among all tissue tested, i.e., liver, kidney, intestine, and muscle; however, the activity of D-HADH was equivalent to that in the kidney and higher than that in all other tissues, and the activity of L-HAOX was only lower than that in the kidney and higher or equivalent to that in other tissues (Fang et al., 2010b). When lamb abomasum was infused with the stable isotope [1-$^{13}$C] HMTBa, muscle was found to synthesize L-Met from HMTBa; however, the amount only represented the 3% Met that entered from the plasma, and a further 8% of Met that entered the muscle had been synthesized from HMTBa elsewhere and transported to muscle via the plasma, both together meeting the Met requirement needed to support muscle growth in these lambs (Lobley et al., 2006).

Skin, brain, and lung synthesized L-Met from HMTBa, but the conversion in these organs was lower than that in the kidney, liver, and intestinal tissues (Lobley et al., 2006). Brain tissue was evaluated along with other tissues, and its activity regarding conversion of HMTBa-Ca to L-Met was lower than that in the small intestine but higher than that in the pectoralis muscle (Langer, 1965), whereas the activity of brain homogenates to convert D-HMTBa to KMB was reported to be similar to that of the kidney and small intestine but much lower than that of the liver and muscle (Dibner and Knight, 1984). Conversion of HMTBa to L-Met in vivo was found to occur in the mammary gland, which accounted for 15% of L-Met synthesized from HMTBa and used for milk protein secretion, and the remaining 85% was synthesized in splanchnic and peripheral tissues and transported to the mammary gland (Lapierre et al., 2011), whereas only L-Met, but not D-Met, was extracted by the mammary gland, which indicated that the enzymes responsible for converting D-Met into L-Met were not present (Lapierre et al., 2012). Based on in vitro and in vivo research, conversion of HMTBa to L-Met occurs in all tissues tested, which provides the biological basis for the highly efficient conversion of HMTBa to L-Met in animals, whereas conversion of D-Met to L-Met in organs other than the liver and kidney is poorly documented.

### 4.3. Conversion efficiency: methodologies and affecting factors

The efficiency of conversion of D-Met and HMTBa to L-Met in vivo was estimated by determining urine/feces and/or respiratory excretion, plasma concentration or tissue incorporation of radioactivity from the labeled test articles, L-Met, and/or its precursors at certain time points after test article administration.

*4.3.1. Urinary, fecal, and respiratory excretion*

The amount of urinary excretion of a substance is mainly determined by whether it is actively reabsorbed or excreted by the kidney. In general, amino acids are actively reabsorbed. Urinary excretion of D-Met has been studied in various animal species by quantification of D- and L-Met. Except for humans and monkeys, all animals tested, including pigs, rats, rabbits, and dogs, excreted minimal D-Met in urine (Cho and Steginк, 1979; Cho et al., 1980; Steginк et al., 1971, 1980). Miniature pigs, adult rabbits, and adult dogs excreted less than 1%, 1.3%, and 0.4% D-Met, respectively, when it was provided via different routes, namely, intravenously infused for miniature pigs, in food for rabbits, and in drinking water for dogs (Cho et al., 1980). Rats that were intravenously infused for a 24-h period excreted in urine less than 1% D- or L-Met. Despite minimal excretion, urinary excretion was greater for DL-Met, and more than 90% of urinary Met was in D-form, suggesting loss due to inefficient kidney reabsorption of D-Met (Cho and Steginк, 1979).

The minimal excretion of D-Met in pigs, rats, rabbits, and dogs does not extend to humans and monkeys. For young infants fed formula containing DL-Met, urinary Met excretion was excessive, and 88% to 93% of excreted Met had the D configuration (Steginк et al., 1971). It has been reported that 26.8% of ingested D-Met is excreted in the urine of monkeys during the 7-d test period, indicating that they absorb and metabolize D-Met like humans rather than like rats, chickens, pigs, or rabbits (Steginк et al., 1980). While excessive excretion of D-Met in humans and monkeys certainly reflects inefficient conversion to L-Met, minimal excretion of D-Met in all other species may indicate efficient conversion and utilization. However, D-Met could also be modified and excreted in forms other than D-Met. This was explored in only one study (Cho and Steginк, 1979). In addition to D- and L-Met, they also measured urinary Met sulfoxide and KMB, and only trace amounts were detected in rats receiving parenteral solutions containing either L-Met or DL-Met.

Urinary excretion of radioactivity was evaluated in chickens fed $^{35}$S radiolabeled HMTBa or L-Met (Saroka and Combs, 1986). Athens–Canadian Random Bred roosters (14 months old) were fasted for 20 h and administered 0.5 mL of $^{35}$S radiolabeled 10 mmol/L L-Met or 10 mmol/L HMTBa solution by crop intubation, and urine samples were collected at 15, 30, 45, 60, 75, and 90 min after dosing for radioactivity measurement. The amount of radioactivity excreted in the urine increased at 30 min and stabilized after that with a larger amount being excreted for L-Met compared with HMTBa at each time point and during the entire 90 min, 24.15% and 10.22% of the dosed activity for L-Met and HMTBa, respectively (Saroka and Combs, 1986). The high excretion of L-Met in chickens observed in this trial was not in good agreement with the trials in species other than humans and monkeys, where 1% or less of D-Met or DL-Met was excreted in urine. Protein synthesis requires the presence of all amino acids at the same time. Therefore, the high amount of excretion of L-Met and HMTBa was likely due to the catabolic state after the fasting conditions used in the trial and, therefore, did not represent the efficiency of L-Met and HMTBa under normal conditions. The percentage of radioactivity from L-Met, HMTBa, and KMB in the urine was also measured in the trial, and the results showed that very similar proportions of the 3 compounds were observed except at the first time point. At all other time points, excretion of L-Met was greater than that of HMTBa and KMB combined, regardless of the Met source administered. This result suggests that α-keto Met, HMTBa and L-Met were interconverted in vivo. It is interesting that under fasting conditions, where L-Met could not be used to synthesize protein efficiently, HMTBa excretion was only 42% of L-Met excretion. The data indicated complex catabolic changes following fasting when

Met sources were administered in excess. Glucogenic amino acids from protein catabolism and the carbon skeleton of HMTBa may be used as an energy source under these conditions, contributing to excess free Met in the bloodstream. Saroka and Combs (1986) concluded that HMTBa is not actively excreted by avian kidneys based on their results.

Excretion of HMTBa has been compared with L-Met, DL-Met or both in chickens using $^{35}S$ or $^{14}C$ radiolabeling techniques (Gordon and Sizer, 1965; Lingens and Molnar, 1996; Saunderson, 1985, 1987). Twenty-four hours after oral feeding of 4- to 6-wk-old New Hampshire cockerels a dose of 10 μCi $^{35}S$ mixed into their feed, 8.6%, 19.3%, and 8.4% of the dosed $^{35}S$ were recovered in the excreta when it was derived from labeled L-Met, DL-Met, and HMTBa, respectively (Gordon and Sizer, 1965). Broiler chicks at 14 to 32 d old were injected intraperitoneally with 5 μCi/kg body weight of 1-$^{14}C$ labeled L-Met, DL-Met, or HMTBa, and excreta were collected over 6 h after administration; 1.88%, 9.59%, and 20.99% radioactivity was recovered for radiolabeled L-Met, DL-Met and HMTBa, respectively (Saunderson, 1985). Another publication from the same author demonstrated that the treatment difference in $^{14}C$ radioactivity excretion among L-Met, DL-Met, and HMTBa was similar when birds were fasted overnight before the trial (feed was offered during the 6-h test) or fed a Met-deficient diet (Saunderson, 1987). Male 16-d-old broilers received 1 mCi radiolabeled $^{14}C$ from either DL-Met or HMTBa-Ca in 0.5 mL of aqueous solution by crop intubation, and within 72 h after administration, 4.40% and 16.99% were recovered from the DL-Met and HMTBa-Ca groups, respectively (Lingens and Molnar, 1996). It is apparent that the findings from these studies are not in good agreement among themselves. As excretion could be affected by many factors, such as dosage and route of administration, feeding status, nutrient profile of diets, and sampling time, the relevant information regarding these radiolabeling trials was summarized in Table 2 to investigate whether any of the discrepancies could be explained by different conditions employed in these trials. Although being excreted at higher amounts in the urine (Saroka and Combs, 1986), L-Met in the excreta was low compared with DL-Met (Gordon and Sizer, 1965) or with DL-Met and HMTBa (Saunderson, 1985, 1987). DL-Methionine excretion was greater than HMTBa excretion in one trial (Gordon and Sizer, 1965) but less than HMTBa excretion in another trial (Saunderson, 1985). In the Gordon and Sizer (1965) trial, 10 μCi $^{35}S$ radiolabeled L-Met, DL-Met, and HMTBa were mixed with the diet and fed to chickens, representing the practical intake route. However, no specific activity or purity was provided, and amounts of Met sources fed to animals could not be calculated; therefore, it is unclear whether the difference in excretion could be influenced by the administered doses. In Saunderson (1985), 5 μCi/kg body weight $^{35}S$-radiolabeled L-Met, DL-Met, and HMTBa were injected intraperitoneally at a dose equivalent to 6.32 μg of L-Met, 7.12 μg of DL-Met, and 71.2 μg of HMTBa based on specific activities of 401, 338–364, and 35.1 μCi/mg for L-Met, DL-Met, and HMTBa, respectively. With much lower specific activity for HMTBa, to achieve similar radioactivity, the injected HMTBa amount had to be approximately 10 times the amount of L-Met or DL-Met, which could have contributed to its higher excretion. HMTBa-Ca excretion was compared with DL-Met in one trial (Lingens and Molnar, 1996), and it was found to be excreted more than DL-Met. Based on the specific activity, 2.15 and 4.22 Bq/mmol (equivalent to $1.8 \times 10^{-3}$ and $8.0 \times 10^{-4}$ μCi/mg) for DL-Met and HMTBa-Ca, 258 g of DL-Met and 297 g of HMTBa-Ca would have been needed to provide 1 mCi radioactivity. Compared with the Saunderson (1987) study, which also used 1-$^{14}C$-labeled Met sources, the specific activity in this trial was much lower ($8.0 \times 10^{-4}$ to $1.8 \times 10^{-3}$ μCi/mg compared with 35.1 to 401 μCi/mg in the Saunderson (1985) trial, but the dose administered was much higher (1 mCi/390–500 g body weight

**Table 2**
Summary of information on urinary/fecal excretion in trials with radioactive labeling.

| References | Test article form | Labeled chemial element | Specific activity & purity | Animals | Dose | Route | Feed or fasting | *N* | Time points | Excreta or urine | Form in excreta/urine | Results (% of the dose) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gordon and Sizer (1965) | L-Met; DL-Met; HMTBa | $^{35}S$ | Not provided | 4–6 wk old New Hampshire cockerels | 10 μCi/bird | Encapsulated and mixed in a standard chicken ration | Feed (a standard chicken ration) | 4 | 24 h | Excreta | Not measured | L-Met 8.6%, DL-Met 19.3%; HMTBa 8.4% |
| Saroka and Combs (1986) | L-Met; HMTBa | $^{35}S$ | Not provided | 14-month-old Athens–Canadian Random Bred roosters | 0.5 mL of a 10 mmol/L solution per 100 g BW (approximately 35 mg, i.e., 5% daily requirement) | Crop intubation | 20-h fasting | Not provided | 15, 30, 45, 60, 75, 90 min | Urine | L-Met; HMTBa; and KMB measured | L-Met 24.15%; HMTBa 10.22% |
| Saunderson (1985) | L-Met; DL-Met; HMTBa | 1-$^{14}C$ | 401, 338–364, 35.1 μCi/mg for L-Met, DL-Met, HMTBa; Purity 98% for L- and DL-Met; 90% for HMTBa | 14–32 d broiler chicks | 5 μCi/kg BW (6.23, 7.12, 71.2 μg for L-Met, DL-Met, and HMTBa; 0.0016%, 0.0019% and 0.0187% daily requirement) | Intraperitoneal injection | Feed (chick starter ration) | 8 | 6 h | Excreta | Pattern examined; suspect HMTBa was excreted as is | L-Met 1.88%; DL-Met 9.59%; HMTBa 20.99% |
| Lingens and Molnar (1996) | DL-Met; HMTBa-Ca | 1-$^{14}C$ | 2.15, 4.22 Bq/mmol ($1.8 \times 10^{-3}$, $8.0 \times 10^{-4}$ μCi/mg) DL-Met and HMTBa-Ca | 16-d broiler chicks (390–500 g BW) | 1 mCi in 0.5 mL of solution per bird (258 g L-Met; 297 g HMTBa-Ca; approximately 650 × daily requirement) | Crop intubation | Feed containing 0.365% Met activity | 5 | 24, 48, 72 h | Excreta | Not measured | DL-Met 4.40%; HMTBa-Ca 16.99% |

compared with 0.5 μCi/kg body weight in the Saunderson (1985) trial). Either the specific activity or the dosage provided in the paper from Lingens and Molnar (1996) must be inaccurate because it would have been impossible to dissolve 258 g of DL-Met or 297 g of HMTBa-Ca in 0.5 mL of solution, and these amounts would have been toxic to chickens (Benevenga, 1974). Additionally, the chickens in this trial were fed diets containing 0.37% Met activity and 0.35% cysteine for a total of 0.72% total sulfur-containing amino acids (TSAA), which was not very deficient considering the age and growth rate of these chickens. Additional administered Met at high doses would probably need to be catabolized and excreted. Therefore, the conditions of the trial may have tested the chickens' ability to eliminate excess Met sources rather than the conversion efficiency of Met precursors. It is surprising that all the studies investigating excretion (Gordon and Sizer, 1965; Lingens and Molnar, 1996; Saunderson, 1985, 1987) provided little or no justification on the radioactive doses applied, the equivalent dose on a weight basis, the Met amount provided in the diets, and the percentage of radioactive doses relative to the daily requirement. Without this information, it is difficult to know whether the trials were testing the chickens' ability to convert Met precursors to L-Met for utilization or their ability to remove excess Met sources. Furthermore, unless similar specific activity and purity of radiolabeled Met sources can be achieved, different amounts of compounds need to be administered to deliver the same radioactivity, which could be a confounding factor in deriving conclusions intrinsic to the test compound. Another problem is the lack of radiochemical analysis of excreted metabolites. It is impossible to discern whether the excreted label represents the source material or its metabolites. In summary, based on available published data, no clear difference was demonstrated in the conversion efficiency of D-Met and HMTBa based on excretion of radioactivity in urine or excreta of chickens. Future research in this area should take into consideration all these affecting factors, provide adequate information, and discuss potential implications of the conditions used.

In addition to the urinary and fecal routes, Met sources could also be excreted via respiration by the lungs. Limited work on this topic suggests that different Met sources are oxidized and exhaled to a similar degree. The production of $^{14}CO_2$ from L-Met, DL-Met, and HMTBa over 6 h after intraperitoneal injection was found to be similar, namely, 4.27%, 5.52%, and 5.04% of the given dose for L-Met, DL-Met, and HMTBa, respectively, in broiler chicks (Saunderson, 1985). Similarly, within 24 h post-crop intubation, no significant difference in $^{14}CO_2$ production was observed between DL-Met (15.8% of the given dose) and HMTBa-Ca (11.4% of the given dose) in broiler chicks (Lingens and Molnar, 1996). The higher percentage of expiration observed in the study of Lingens and Molnar (1996) is probably related to its higher doses administered compared with Saunderson's work (1985), as discussed above.

#### 4.3.2. Plasma concentration of L-Met and precursors

The plasma concentration of a nutrient is the net result of intestinal absorption, tissue utilization, and renal excretion. As slow tissue uptake and utilization will result in high plasma concentrations at certain time points, it is not difficult to understand that it does not necessarily serve as a good indicator of the bioavailability of a nutrient (Cho et al., 1980; Cho and Stegink, 1979; Lapierre et al., 2012; Stegink et al., 1971). Furthermore, when evaluating a nutrient precursor, the plasma concentration of the nutrient does not necessarily reflect conversion efficiency, as the precursor can be transported in plasma as it is taken in, converted to the nutrient, and utilized by various tissues locally. In vivo studies have evaluated the plasma concentration of Met or radioactivity from radiolabeled test articles at various time points after administration across different animal species.

The efficiency of D-Met conversion to L-Met in vivo was estimated by evaluating the kinetics of plasma appearance and clearance of the test compound and L-Met using a stable isotope technique. Over 90% of intravenously administered D-[$^2H_3$]Met is converted to L-[$^2H_3$]Met in vivo in rats (Hasegawa et al., 2011). Lapierre et al. (2012) reported that a minimum of 75% D-[1-$^{13}$C]Met was transformed to L-[1-$^{13}$C]Met considering only the L-Met synthesized intracellularly and released to the plasma, while L-Met synthesized and utilized within the cell was not accounted for. The kinetics approach offers the advantage of direct detection of the conversion of Met precursors to L-Met, and the conversion efficiency is estimated based on the plasma test compound and L-Met concentration kinetics over a period of 6 to 8 h. Therefore, this helps avoid the biases associated with specific time points; however, it underestimates the conversion efficiency, as only L-Met released into plasma is measured and modeled, and those synthesized and utilized in local tissues are not accounted for. As HMTBa has been demonstrated to be converted to L-Met and utilized in all tissues tested in vivo, the underestimation of the conversion efficiency for HMTBa is expected to be greater than that for D-Met with the plasma kinetics stable isotope technique.

The plasma Met concentration after the administration of DL-Met versus L-Met has been investigated in several studies. For young human infants fed formula containing DL-Met, 35% and 25% of the circulating Met had the D configuration at 2 and 3–4 h postprandially, respectively, indicating substantial concentrations of D-Met circulating in the plasma at all times (Stegink et al., 1971). When rats were intravenously infused with 8.5% amino acid solution containing DL-Met for 24 h, elevated plasma Met levels were observed (20.6 μmol/100 mL) compared with those infused with amino acid solution containing L-Met (11.2 μmol/100 mL). For DL-Met, 45% of Met in plasma was in the form of D-Met (Cho and Stegink, 1979). Similarly, for miniature pigs ingesting a protein-free diet and intravenously infused with 8.5% amino acid solutions differing only in the Met isomer for a 2- or 3-d period in a crossover design, the plasma Met level was higher when an amino acid solution containing DL-Met compared with L-Met was infused and 25% of plasma Met was in the form of D-Met (Cho et al., 1980). After dairy cows received portal vein infusions of DL-Met for 2 d, the fractional removal of D-Met was 6 to 7 times lower than that of L-Met, with a mean half-life of 52 min for D-Met compared with 8 min for L-Met. After 5 d of abomasal infusion of either DL-Met or L-Met, the arterial Met concentration was higher for DL-Met than for L-Met (37.4 μmol/L for DL-Met and 25.4 μmol/L for L-Met), with 37.1% for D-Met (Lapierre et al., 2012). These studies across different mammalian species demonstrated that feeding DL-Met can increase plasma Met concentration; however, it is not an indication of higher conversion efficiency. This is due to a slower fractional removal from plasma and a slower utilization of D-Met by various tissues, as suggested by Lapierre et al. (2012).

The plasma Met concentration of feeding HMTBa was evaluated in swine, poultry, and ruminants by several authors. For barrows infused intragastrically and constantly with 1-$^{13}$C Met at 7.0 μmol/kg body weight per hour, when they were fed a diet containing 0.12% added DL-Met or HMTBa at equimolar concentrations, arterial and portal blood samples taken at hourly intervals until 6 h after the first meal were found to have Met concentrations that were not significantly different from each other, although DL-Met-fed pigs had numerically higher Met concentrations at 2 and 4 h after the first meal (Fang et al., 2010a). For this study, plasma Met came from a basal diet containing 0.22% Met, supplemental Met sources, and infused L-Met. The possible explanation for the lack of difference in blood Met concentration between DL-Met- and HMTBa-fed pigs is that the basal and infused L-Met could dilute the effect of the supplemental Met source on plasma Met levels,

rendering it less sensitive to dietary supplementation with DL-Met or HMTBa, especially when the supplementation level was low compared with the sum of the basal and infused L-Met contents. The plasma concentration of Met after oral intake of HMTBa is usually lower than that for equimolar intake of DL-Met (Dibner, 2003; Gonzales-Esquerra et al., 2007; Vázquez-Añón et al., 2003). Interpretation of this difference is complicated by differences in the metabolism, sites, and mechanisms of absorption of the 2 molecules. In addition, the plasma concentration of DL-Met-fed animals is complicated by the accumulation of D-isomers due to a slower removal rate (Cho et al., 1980; Cho and Stegink, 1979; Lapierre et al., 2012). For HMTBa, the plasma concentration of Met from HMTBa-fed animals will depend on the sites of its conversion to L-Met and the molecular form being transported in the plasma. Wester et al. (2006) demonstrated that all HMTBa infused in the mesenteric vein of conscious lambs was recovered at the portal vein with 25% extracted by the liver. The portal appearance of Met was unaltered by HMTBa infusion, but arterial Met concentration and Met irreversible loss increased, equivalent to 40% of the HMTBa re-entering the plasma after conversion to L-Met. Using an infusion of 1-$^{13}$C labeled HMTBa into the abomasum of conscious lambs, 87% was recovered at the portal vein, 37% of which was extracted by the liver and 63% of which bypassed the liver. All tissues synthesized L-Met from HMTBa, but the synthesized L-Met was retained and utilized locally. The exception was the kidney, which was identified as the primary source of plasma L-Met derived from HMTBa (Lobley et al., 2006). HMTBa is transported to tissues by blood as unmodified HMTBa. It is converted to L-Met by tissues, and except for the kidney, the majority of the synthesized L-Met is used and retained by the tissue that converts it. Therefore, it is erroneous to conclude that the low plasma L-Met concentration of HMTBa-fed animals is an indication of poor conversion of HMTBa to L-Met.

Some studies evaluated plasma radioactivity at different time points after administering radiolabeled $^{35}$S-HMTBa, DL-Met or L-Met in poultry. Gordon and Sizer (1965) found that the accumulation of $^{35}$S in blood at 0.5 and 2 h after oral feeding of 4–6 wk old New Hampshire cockerels a dose of 10 μCi $^{35}$S derived from labeled L-Met, DL-Met, and HMTBa mixed in the ration was found to be similar between HMTBa and L-Met but lower for DL-Met. In a study reported by Larbier and Perrot (1984), chicks (28 d old) were injected intraperitoneally with $^{14}$C-labeled L-Met, DL-Met, or HMTBa-Ca at 1 μCi/kg body weight. The accumulation of $^{14}$C in the free amino acid form and incorporation into plasma proteins was measured at 1, 2, 4, and 24 h post-injection. $^{14}$C accumulation was found to be both pool- and time dependent. The $^{14}$C accumulation in the free amino acid pool and plasma proteins at 1 and 2 h after injection was similar for HMTBa-Ca and DL-Met but lower for L-Met. The accumulation of $^{14}$C in the free plasma amino acid pool from HMTBa-Ca was similar to that in L-Met but lower than that in DL-Met at 4 h post-injection, whereas at 24 h post-injection, it was lower than that in L-Met or DL-Met, with no difference between L-Met and DL-Met. The $^{14}$C incorporation into plasma proteins at 4 and 24 h post-injection was the highest for DL-Met, the lowest for L-Met, and HMTBa-Ca was in between (Larbier and Perrot, 1984). In a similar experiment, Saunderson (1985) reported the distribution of $^{14}$C in deproteinized plasma following intraperitoneal injection of 2.5 μCi/kg radiolabeled L-Met and HMTBa. Samples were measured at 10, 30, 60, 90, and 120 min after injection in chicks, and $^{14}$C appeared quickly in blood and decreased over time for both Met sources. The $^{14}$C accumulation was found to be greater for HMTBa across sample time points, but the magnitude of difference with L-Met decreased dramatically with sampling time (Saunderson, 1985). When radioactivity is measured in blood, it captures the labeled element regardless of form. Based on the studies of Larbier and Perrot (1984) and Saunderson (1985), the removal rate of HMTBa in plasma is slower than that of L-Met but similar to or faster than that of DL-Met. The results of the Gordon and Sizer (1965) study on lower plasma radioactivity for DL-Met compared with L-Met or HMTBa were not in agreement with those of other studies (Cho et al., 1980; Cho and Stegink, 1979; Lapierre et al., 2012; Larbier and Perrot, 1984). From these radiolabeling studies, plasma radioactivity measurements were very sensitive to L-Met and its precursors. However, interpretation of the data is difficult due to the complicated interactions among the Met source, measurement pool, and sampling time. The observed difference reflects the difference in the metabolism of Met sources rather than their conversion efficiency.

*4.3.3. Tissue incorporation*

As tissue deposition is typically a good measurement of the bioavailability of a nutrient from different sources, incorporation of radioactivity was measured in various tissues for some studies comparing Met sources (Gordon and Sizer, 1965; Larbier and Perrot, 1984; Lingens and Molnar, 1996; Saunderson, 1985). Liver, muscle, and kidney were the primary tissues studied, and other tissues, including intestine, brain, heart, lung, skin, spleen, and pancreas, were also evaluated.

Two hours after intravenous injection of $^{35}$S-labeled L-Met, DL-Met, and HMTBa, $^{35}$S incorporation into liver protein as Met was found to be similar between L-Met (4.39 CPM/mg isolated Met) and HMTBa (4.28 CPM/mg isolated Met), and both were higher than DL-Met (3.02 CPM/mg isolated Met) in chickens (Gordon and Sizer, 1965). Larbier and Perrot (1984) reported that radioactivity accumulation in chicken liver after intraperitoneal injection of 1-$^{14}$C labeled Met sources was affected by sampling time, measurement method, and Met source. In this report, accumulation of 1-$^{14}$C in the free amino acid pool was similar between DL-Met and HMTBa-Ca, both higher than L-Met, at 1 h post-injection; the accumulation was highest for DL-Met and similar between L-Met and HMTBa-Ca at 2 h post-injection; and it was similar among L-Met, DL-Met and HMTBa-Ca at 4 and 24 h post-injection. The accumulation of 1-$^{14}$C in liver protein was the highest for HMTBa-Ca, and no difference was found between L-Met and DL-Met at 1 and 2 h post-injection, whereas at 4 and 24 h post-injection, there was no difference between D-Met and HMTBa-Ca, and both were higher than that of L-Met.

The $^{14}$C incorporation into liver proteins was found to be equivalent between L-Met, DL-Met, and HMTBa at 6 h after intraperitoneal administration in chickens (Saunderson, 1985). Similarly, no significant difference was detected for $^{14}$C incorporation into the liver at 24, 48, and 72 h after crop intubation of 1-$^{14}$C-labeled DL-Met and HMTBa-Ca in chickens (Lingens and Molnar, 1996). It is unclear why some experiments showed higher incorporation of L-Met and HMTBa over DL-Met (Gordon and Sizer, 1965), some showed higher HMTBa-Ca and DL-Met incorporation over L-Met (Larbier and Perrot, 1984), and others found no difference among Met sources (Lingens and Molnar, 1996; Saunderson, 1985). No consistent difference was demonstrated in the incorporation of radiolabeled Met sources into liver protein for L-Met, DL-Met, and HMTBa in chickens. The kidney was identified as the organ that had the highest $^{14}$C incorporation for DL-Met compared with L-Met or HMTBa at 6 h post-intraperitoneal administration (Saunderson, 1985) and compared with HMTBa-Ca at 24, 48, and 72 h after crop incubation (Lingens and Molnar, 1996). The high accumulation of DL-Met in chicken kidney is in agreement with the primary role that kidney plays in converting D-Met to L-Met as discussed above but is not an indication of higher or lower conversion efficiency.

Incorporation of $^{14}$C into the muscle free amino acid pool was found to be the highest for DL-Met, and there was no difference between L-Met and HMTBa-Ca at 1, 2, 4, and 24 h after

intraperitoneal injection in chickens; however, $^{14}C$ incorporation into muscle protein was higher for L-Met compared with DL-Met and HMTBa-Ca at 1 and 2 h post-injection and compared with HMTBa-Ca at 4 and 24 h post-injection, and DL-Met-injected birds had higher $^{14}C$ muscle protein incorporation than HMTBa-Ca-injected birds at 2, 4, and 24 h after administration (Larbier and Perrot, 1984). The deposition of $^{14}C$ in breast and leg muscle protein was higher for L-Met compared with DL-Met or HMTBa, which were similar to each other 6 h after intraperitoneal injection (Saunderson, 1985). The rate of incorporation of $^{14}C$ into the breast and leg muscle was found to be higher after administration of 1-$^{14}C$ DL-Met compared with 1-$^{14}C$ HMTBa-Ca at 24, 48, and 72 h after crop intubation (Lingens and Molnar, 1996). As L-Met is directly available for incorporation into muscle after absorption, D-Met and HMTBa need to be converted to L-Met first before incorporation; it is not surprising that more L-Met was deposited in muscle compared with DL-Met and HMTBa during the early sampling time points.

The findings of Larbier and Perrot (1984) and Lingens and Molnar (1996) regarding the greater incorporation of DL-Met than HMTBa into muscle protein were not in agreement with the finding of a similar incorporation between the two from the study of Saunderson (1985). However, the conclusion that HMTBa was less available for muscle deposition based on its lower incorporation into muscle in these 2 studies could be questioned regarding its relevance to practical situations, as HMTBa-Ca injected intraperitoneally (Larbier and Perrot, 1984) does not represent the form being absorbed, i.e., HMTBa, when it is fed to animals. In addition, the high dose administered (Lingens and Molnar, 1996) may have tested the chickens' ability to eliminate excess Met sources rather than to utilize them for maintenance and growth, as discussed previously.

Similar to breast and leg muscle, the heart, skin, and brain showed lower $^{14}C$ incorporation from DL-Met and HMTBa at 6 h after intraperitoneal administration compared with L-Met, and no difference was observed between DL-Met and HMTBa (Saunderson, 1985). The incorporation of $^{14}C$ into the digestive tract, lung, heart, spleen, and pancreas was found to be higher after administration of $^{14}C$-DL-Met than after administration of 1-$^{14}C$-HMTBa-Ca (Lingens and Molnar, 1996), which again was most likely complicated by the high dose administered in this study. As D-HMTBa, L-HMTBa, D-Met, and L-Met were metabolized by different metabolic pathways with different timeframes at various sites in animals, as discussed in Section 4.1 and 4.2, deposition in a specific tissue at a specific time point may not reflect the efficiency of conversion from D-Met or HMTBa to L-Met of the whole body. Therefore, deposition in a specific tissue at a single time point does not serve as a good indication of the bioavailability of Met sources but reflects differences in the metabolism of these molecules.

### 4.4. Factors affecting conversion efficiency

As the conversion of D-Met or HMTBa to L-Met involves several biochemical reactions, which are known to be affected by various conditions, it has been reported that the conversion efficacy is affected by multiple factors. All species tested (human, monkey, chicken, dog, lamb, cows, rabbit, and Ascaris sum) can convert D-Met and HMTBa to L-Met, but the conversion efficiency varies. Humans and monkeys were found to utilize D-Met poorly, whereas nearly complete utilization was observed for all other species tested (Langer et al., 1971; Steging et al., 1971).

#### 4.4.1. Can enzymes be rate limiting for the conversion of methionine precursors?

Enzymes responsible for the conversion of HMTBa and D-Met and their locations in tissues are now well known and described. However, the question arises regarding whether enzymes are available in sufficient quantities in animals to allow the full and efficient conversion of these precursors into L-Met. In the studies reported by Dibner and Ivey (1992), the total liver enzyme capacity was studied in broiler chicks fed HMTBa or DL-Met, whereas Dupuis et al. (1989) demonstrated that at low substrate concentrations, the liver contributes principally to the whole-body oxidation of both HMTBa and DL-Met. At concentrations greater than physiological concentrations, HMTBa is oxidized principally in skeletal muscle. According to Dibner and Ivey (1992), the total enzyme activity for converting synthetic Met sources into L-Met was not different between the sources tested. Using optimum reaction conditions and saturating substrate concentrations, Dibner and Ivey (1992) calculated that the total liver enzyme conversion activity was essentially equal for the 2 sources, i.e., 564 μmol/h for HMTBa and 529 μmol/h for DL-Met at a substrate concentration of 20 mmol/L. At high commercial supplementation levels of Met sources, calculations indicated that the liver of a 21-d-old broiler could convert approximately 500 μmol/h of either HMTBa or DL-Met if these substrates were present at saturating concentrations (Dibner and Ivey, 1992). The data presented by Dibner and Ivey (1992) also showed that no accumulation of HMTBa (no isomeric configuration indicated) occurred in the livers of chicks fed with HMTBa because 92% of the liver content was measured as Met.

Broilers have biochemical capacity in excess to convert practical supplemental levels of HMTBa or DL-Met to L-Met. The relative excess of enzyme capacity to conversion requirement increases with the age of the animal, as justified by the following 3 points (Dibner and Ivey, 1992). First, the sulfur amino acid requirements and, thus, the required supplemental Met levels decrease with age in a commercial diet, as observed in many nutrient recommendation tables (Dibner, 2003; National Research Council, 2012). Second, the liver continues to grow in growing animals; therefore, the total hepatic enzyme capacity would increase. Third, the hepatic activities of the enzymes L-HAOX (Scott et al., 1969) and D-HADH increase with age.

A calculation of the enzyme capacity of broilers was made by Dibner and Ivey (1992) on a 3-wk-old bird. Assuming that broilers were consuming a diet supplemented with 0.25% HMTBa or DL-Met, the amount of Met activity that would have to be converted in the liver, based on 100 g of feed intake/day, would be approximately 1,600 μmol/d, or approximately 70 μmol/h. Based on the same idea, considering early 7-d-old birds fed a corn–soybean meal diet with 0.38% supplemental HMTBa or DL-Met, with a daily feed intake of 30 g (Aviagen, 2019), the estimated amount of Met activity that would need to be converted would be 752 μmol/d, i.e., 31 μmol/h.[1] Compared with the total liver enzyme capacity determined above (an average of 546 μmol/h) by Dibner and Ivey (1992), these calculations suggest that the liver alone can convert the amount of Met precursors for 3-wk-old animals 7 times an hour, whereas this capacity rises up to 18 times for 7-d-old birds. Nevertheless, it was demonstrated by several authors that the enzymes responsible for the conversion of Met precursors are available in different organs: small intestine, kidney, and muscle (Martin-Venegas et al., 2006; Marret et al., 1964). Therefore, the additional enzyme present in renal peroxisomes and in the mitochondria of virtually every organ indicates that supplemental Met levels would not overwhelm or exceed the animal conversion capacity at commercial levels or even at high levels (Bauriedel, 1963; Dibner and Ivey, 1992). Supplementation with 7.70% HMTBa (77 kg/t) would be required to overwhelm the liver enzymatic capacity of a

[1] Met activity was calculated according to the following equation [Daily feed intake, g/d × Methionine supplemental level, %/(Molar mass of DL-HMTBa, 150.2 g/mol × 100)] × $10^6$.

7-d-old bird eating 30 g of feed/d. This is unlikely to occur in commercial diets.

*4.4.2. Other factors*

Based on growth performance in chicks, Marrett et al. (1964) demonstrated that when a basal-amino acid diet containing 15% D-amino acids (3.8% of total ration) was used and graded D-Met and L-Met were added, L-Met was utilized better than D-Met; when a basal L-amino acid mixture was used, L-Met and D-Met were utilized equally well. This indicated that the utilization of D-Met was affected by the total dietary D-amino acid level. In a separate study, D-Met utilization in chicks was found to be affected not only by the total amount of D-amino acids present in the diet but also by specific D-amino acids, which are more sensitive to D-valine than to D-threonine or D-tryptophan (Marret and Sunde, 1965).

Based on the tissue protein-bound $^{14}C$ to free $^{14}C$ ratio at 6 h after intraperitoneal injection, Saunderson (1987) concluded that brain and probably liver tissues from fasting and Met-deficient birds showed an improved rate of conversion from D-Met and HMTBa to L-Met. The author used the protein-bound $^{14}C$ to free $^{14}C$ ratio as a measurement of D-Met and HMTBa conversion efficiency, with higher values indicating better conversion. This conclusion was based on a significant interaction between Met source and nutritional status for the brain protein-bound $^{14}C$ to free $^{14}C$ ratio. However, examination of the data revealed that the interaction was driven by the fact that the protein-bound $^{14}C$ to free $^{14}C$ ratio was reduced by fasting and Met deficiency only for L-Met but not for DL-Met or HMTBa. Therefore, the data did not support an improved conversion rate from D-Met or HMTBa to L-Met but suggested altered L-Met metabolism by fasting and Met deficiency in the brain. Therefore, it is questionable whether this protein-bound $^{14}C$ to free $^{14}C$ ratio is a reliable indication of conversion efficiency for D-Met and HMTBa.

It is well known that diet type is a major contributing factor for the reported conflicting bioefficacy values between HMTBa and DL-Met. In diets designed to be very deficient in Met, i.e., crystalline amino acid diets or semipurified diets, the 2 sources have different dose response characteristics based on different dietary intake. In these diets, low levels of supplementation favor DL-Met, and high levels of supplementation favor HMTBa. Nevertheless, no consistent difference between sources is observed for practical diets (Elkin and Hester, 1983; Tipton et al., 1966). Some dietary factors have been identified to affect the utilization of HMTBa in chickens. Supplementation of HMTBa-Ca alone in a purified diet did not support growth performance equal to L-Met, in contrast to a mixture of HMTBa-Ca and L-Met containing approximately 50%–60% L-Met. This indicates that the utilization of HMTBa-Ca was affected by dietary L-Met levels in chicks (Marret and Sunde, 1965).

Similarly, the efficacy of HMTBa-Ca in broilers fed diets based on a crystalline amino acid mixture was found to depend on its level in the diet and on the levels of Met and cystine. HMTBa was less effective when fed without Met, had an intermediate value when fed with Met only and was most efficacious when fed with a mixture of Met and cystine (Marret and Sunde, 1965). Compared with DL-Met, HMTBa-fed chicks had lower body weight gain under Met deficiency but higher body weight gain under Met excess; however, when pair-fed to equalize feed intake, no difference in body weight gain was observed between HMTBa and DL-Met, indicating that the differences in body weight gain below and above the peak response were the result of the difference in feed intake (Knight et al., 2006). With a series of 5 trials, Dilger and Baker (2008) reported similar growth performance for HMTBa-Ca and DL-Met in the absence of excess dietary cyst(e)ine but lower performance for HMTBa-Ca in the presence of excess cyst(e)ine, indicating that excess dietary cyst(e)ine affected HMTBa-Ca utilization in chicks. These trials used either purified crystalline amino acid-based diets or a corn peanut meal–based diet. An additional 0.15% to 0.35% L-cystine was added to the purified diet, and an additional 0.10% L-cystine was added to the corn peanut meal–based diet to create cyst(e)ine excess. Typical practical poultry diets use soybean meal as the primary protein source. They are deficient in TSAA and are almost universally supplemented with Met. In formulating diets, minima are usually expressed for Met and TSAA. Often, when the minimum TSAA is met, the dietary Met level exceeds the minimum Met, resulting in an excess of Met. The excessive Met is used to meet the requirement of cysteine, indicating that there is no excess of dietary cysteine in the practical diets without L-cystine supplementation.

## 5. Is there any alternative pathway(s) for the conversion of methionine precursors?

L-Methionine has been qualified by many authors as the most toxic of the natural amino acids (Benevenga, 1974; Daniel and Waisman, 1968). The attempts to explain this toxicity led to an intense investigation of Met metabolism and to the suggestion of an alternate pathway that may not involve the activation of Met to S-adenosyl-L-Met (Case et al., 1976; Mitchell and Benevenga, 1978). It was concluded that the metabolic basis for Met toxicity cannot be attributed to catabolism via the transsulfuration pathway and is related to the metabolism of the methyl portion of the molecule and that formaldehyde and formate were 2 intermediates in this pathway (Casey et al., 1976; Perry, 1967).

Transamination is known to be the first step in the degradation of natural amino acids, including Met. Although it was thought that transamination was a primary means for the conversion of KMB, issued from Met precursors (D-Met and HMTBa), several authors have shown that the synthesis of KMB from L-Met by transamination is the first step in its degradation (Jones and Yeaman, 1986; Livesey and Lund, 1980). As shown by Ikeda et al. (1976), Met is a better substrate than leucine for leucine transaminase purified to apparent homogeneity from rat liver mitochondria, and sulfur amino acids are effective amino donors in glutamine transaminase (Copper and Meister, 1972) and histidine-pyruvate aminotransferase (Noguchi et al., 1976). Using rat and monkey liver homogenates, Mitchell and Benevenga (1978) and Steele and Benevenga (1978) demonstrated that a substantial proportion of Met oxidation into carbon dioxide occurred via transamination of Met to KMB, followed by decarboxylation of the α-ketoacid to 3-methylthiopropionate (3-MTP). Similarly, in a study aiming to investigate the biochemical conversion of L-HMTBa by L-HAOX-A, Ferjancic-Biagini et al. (1995) identified 3-MTP as a decarboxylation product derived from KMB.

Nevertheless, there are specific conditions in which this decarboxylation occurs. In the study reported by Ferjancic-Biagini et al. (1995), they incubated in vitro (reaction mixture of the 2 products in a 10 mmol/L Tris–HCl buffer) L-HMTBa with L-HAOX-A, one of the isozymes responsible for converting HMTBa into KMB. KMB was measured first and then oxidized into 3-MTP, as confirmed by the time courses of formation of the 2 products. In this study (Ferjancic-Bioagini et al., 1995), in addition to the use of only one isozyme, there was also no mention of including any of the amino donors mentioned in the previous sections, which could prevent the transamination of KMB into L-Met. The lack of any other pathway for KMB utilization in these experimental in vitro conditions may have led to the formation of 3-MTP. Additionally, this decarboxylation may also occur when the Met concentrations are higher than standard physiological conditions or when the capabilities of transsulfuration have been exceeded or impaired

(Mitchell and Benevenga, 1978; Steele and Benevenga, 1978). Therefore, this is an escape pathway allowing the animal to dispose of the excess Met, similar to the case of an infant described by Perry (1967). This baby, who died of 'hypermethioninemia', had elevated plasma concentrations of Met and tyrosine and excreted large amounts of Met, KMB, tyrosine, p-hydroxyphenylpyruvic acid (keto-tyrosine), and p-hydroxyphenyllactic acid in the urine. A normal amount of cystathionine was excreted, and brain cystathionine concentration was normal on autopsy, thus indicating an apparent normal transsulfuration pathway. This infant may have suffered from an impairment in its ability to catabolize KMB (Perry, 1967). Likewise, rats fed a basal diet supplemented with a high level of 3-MTP (2.57%) for 2 wk have exhibited toxicity symptoms identical to those seen in Met toxicity when supplemented at equimolar levels (Steele et al., 1979). A pronounced depression in growth and feed intake and markedly darkened spleens were observed in the 3-MTP group. While supplemental glycine or serine alleviated the toxicity of dietary Met, similar levels added to the 3-MTP-fed group were ineffective (Steele et al., 1979). Consequently, this result confirms that the transamination pathway of Met catabolism may be important with respect to the toxicity of Met, and this is not related to the single oxidation of HMTBa, as suggested by Ferjancic-Biagini et al. (1995). This phenomenon is unlikely to occur in practical feeding conditions since adequate doses of Met sources are supplied to meet animals' requirements in sulfur amino acids.

Furthermore, only a few studies exist on the subsequent metabolism of 3-MTP and were produced in the 1960s to 1980s. To the best of our knowledge, Steele and Benevenga (1979) produced the first report of 3-MTP oxidation in a mammalian system. They showed that once formed, 3-MTP is catabolized into carbon dioxide and sulfate in rat liver homogenates. Intermediates for this degradation are identified as methanethiol, formaldehyde and hydrogen sulfide (Steele and Benevenga, 1978, 1979). However, there is an additional product formed resulting from the dethiomethylation of 3-MTP. It has not been clearly identified, but it is likely to be acrylate or propionate since 3-methylthioacrylate and 3-MTP were identified as Met metabolites in a strain of *Streptomyces* bacteria incubated in a medium containing DL-Met (Arima et al., 1970). They suggested that 3-methylthioacrylate was formed from 3-MTP. Another product, 3-methylthiopropionamide, has been identified as the product of pyridoxal phosphate-dependent decarboxylation of Met by horseradish peroxidase (Mazelis and Ingraham, 1962).

From these results, the alternate pathway in Met metabolism has been summarized in Fig. 2. Taken together, it can be concluded that under normal practical conditions, there is only one pathway through which Met precursors are metabolized (D-Met and HMTBa), which is bioconversion into L-Met. The other alternate pathway described here is a survival process to expel the excess Met or KMB generated from transamination of L-Met.

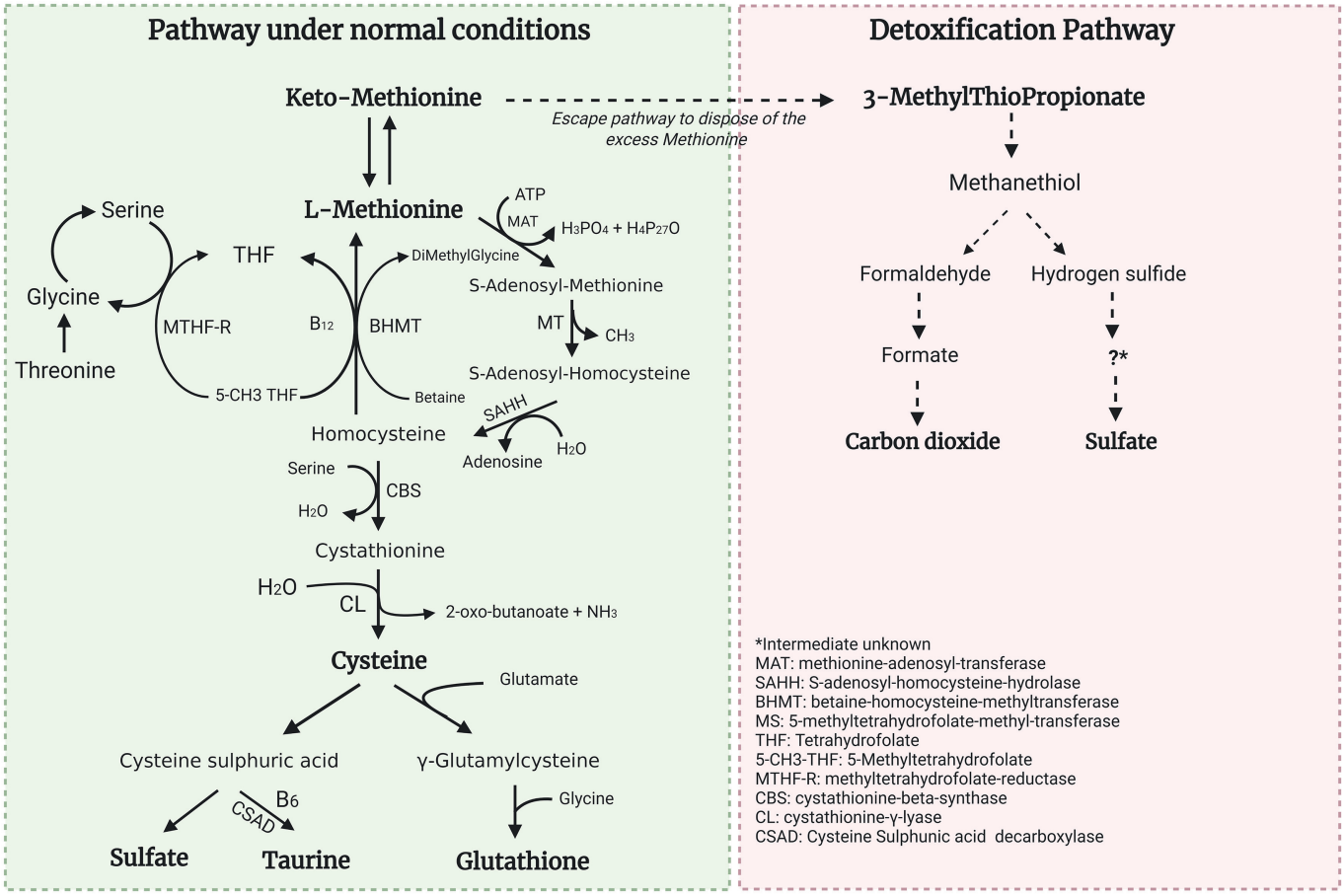



**Fig. 2.** Metabolism of methionine under normal and toxic conditions (detoxification).

## 6. The use of radioisotopes for studying the metabolism of methionine precursors: what are the limitations and impacts on the outputs?

Radioisotopes have shown to be an indispensable tool in investigating the absorption, distribution, metabolism, and excretion properties of a given molecule in biomedical research (Dalvie, 2000). According to Dalvie (2000), radioisotopes have been mainly used because of the ease of detection and the achievement of high sensitivity, especially in the case of compounds with high specific activity. The results from radiolabeled studies help to understand the behavior of an exogenous molecule (Penner et al., 2012). In animals, they have also been used by several authors to study the absorption (Esteve-Garcia and Austic, 1993; Malik et al., 2009), conversion pathway or efficacy of the conversion of Met precursors (Dibner and Ivey, 1992; Lingens and Molnar, 1996; Saunderson, 1985, 1987; Schreiner and Jones, 1987). In the following section, only the impacts on conversion and metabolism are covered.

The most common radionuclide used is $^{14}C$ because of its multiple properties, such as synthetic versatility, relative safety, and favorable nuclear properties dictating the optimal half-life and specific activity (McCarthy, 2000). Several other isotopes, such as $^{35}S$, $^{32}P$, $^{125}I$, $^{131}I$, and $^{59}Fe$, have been applied in studies on the metabolic disposition of compounds (Dalvie, 2000). However, in some studies, $^{3}H$ was used as a replacement (or in addition to) $^{14}C$ to obtain in vitro (Guroff et al., 1967; Linnet, 2004) and in vivo (Ehlhardt et al., 1998; Prakash et al., 1997) biotransformation data. As described by Shaffer et al. (2006), the risk associated with using $^{3}H$-labeled compounds in biological systems is the potential chemical and metabolic instability of the $^{3}H$ atom itself at its specific site within the test molecule. Due to the intrinsic properties of the hydrogen atom, $^{3}H$ exchange within a molecule may occur readily in aqueous physiological environments of varying pH by chemical or metabolic means (Lewis et al., 1988). $^{3}H$-labeling of Met was also found to have a different metabolic fate, as cells exhibited a differential preference for the same molecule when the labeling was made on either $^{3}H$ or $^{14}C$ atoms. According to Kuang et al. (2014), the uptake of L-[methyl-$^{3}H$]-Met was higher than that of L-[1-$^{14}C$]-Met in rat hepatocytes and woodchuck HCC cell lines. In addition, it was found that L-[methyl-$^{3}H$] is predominantly degraded through lipid synthesis (phosphatidylcholine).

Conversely, radiolabeling a compound at the $^{14}C$ atom may lead to the separation of this atom from the substructure retained by most metabolites because of its incorporation at a metabolically labile site (Chasseaud et al., 1974; Larsson and Lund, 1981). The study reported by Saunderson (1985) investigated the metabolism of Met sources in broilers using $^{14}C$-labeled tracers in vivo. They found that HMTBa, D-Met and L-Met are metabolized differently and are excreted in differing proportions. There was also a difference in the ability of each Met source to act as a precursor for protein synthesis in tissues other than the liver. These results were in disagreement with the study of Gordon and Sizer (1965), who used $^{35}S$-labeled molecules. In addition to the nonequivalent amounts of $^{14}C$-labeled Met sources injected (HMTBa dose was 10 times higher than the dose of the other Met source), the labeling on the first carbon could have also limited the estimation of the whole metabolism of Met (Saunderson, 1985). Indeed, Met's whole carbon chain is replaced by serine's in the transsulfuration pathway, thus allowing the conversion of homocysteine into cysteine. Only the sulfur atom is maintained in this biological process (Sbodio et al., 2019). Therefore, the conditions of the Saunderson (1985) and Saunderson (1987) trials may have only allowed us to understand one aspect of Met metabolism. Sulfur-labeled molecules may have been better candidates than $^{14}C$-labeled molecules to study Met source metabolism. In the study of Gordon and Sizer (1965), $^{35}S$ was used at doses representing practical feeding conditions. However, the lack of details on the specific activity or purity of the products rendered it unclear whether the difference in excretion could be influenced by the administered doses. Consequently, further work may be required to clarify the metabolism of Met sources using sulfur-labeled molecules.

## 7. Differences in methionine metabolism between different methionine precursors

Methionine metabolism is described in Fig. 2. Once formed, L-Met is converted into S-adenosyl-Met and S-adenosyl-homocysteine. S-adenosyl-Met is a metabolic product of Met generated by Met-adenosyltransferase, which catalyzes the reaction at the expense of the complete dephosphorylation of one molecule of adenosine triphosphate in the presence of potassium and magnesium ions. S-adenosyl-Met is then utilized as a donor of its methyl group to methylate many substrates to fulfill diverse biological functions, while S-adenosyl-homocysteine is produced in this process and then hydrolyzed to homocysteine (Gao et al., 2018). This pathway is described as the transmethylation pathway. Homocysteine can either be remethylated to generate Met or be irreversibly converted to cysteine (transsulfuration). Homocysteine is converted to cystathionine by cystathionine β-synthase, which is acted on by cystathionine γ-lyase to generate cysteine. The transsulfuration pathway plays a central role in sulfur metabolism and redox regulation in cells. It consists of the transfer of sulfur from homocysteine to cysteine and is the only route for biosynthesis of cysteine (Sbodio et al., 2019).

Methionine metabolism from different Met precursors has been studied by several authors (Martin-Venegas et al., 2006, 2011; Rasch et al., 2019; Zuo et al., 2019). Using everted sacs from the chicken small intestine, Martin-Venegas et al. (2006) measured the serosal Met appearance after HMTBa incubation and compared the serosal appearance of cysteine and taurine after HMTBa and L-Met incubation. They found a significantly higher serosal appearance of cysteine in the duodenum, jejunum and ileum after HMTBa incubation than after L-Met incubation. Similarly, following HMTBa incubation, significantly higher values were obtained for taurine. Likewise, other authors have identified an increased amount of taurine following HMTBa consumption by pigs (Fang et al., 2010a) and broiler chickens (Yodseranee and Binchasak, 2012). In contrast, a study performed by Zuo et al. (2019) in an intestinal porcine epithelial cell line (IPEC-J2) showed that the concentrations of intracellular Met, S-adenosyl-Met, and S-adenosyl-homocysteine and the ratio of S-adenosyl-Met to S-adenosyl-homocysteine were significantly lower in the HMTBa group than in the DL-Met and L-Met groups, whereas the opposite was observed for 5-methyltetrahydrofolate. The authors concluded that HMTBa mainly affects the transmethylation and remethylation of Met and that HMTBa can promote trans-sulfur metabolism with no difference from the other Met sources (Zuo et al., 2019). Comparably, Rasch et al. (2019) found in piglets that HMTBa, compared with L-Met and DL-Met supplementation, increases remethylation and reduces the transsulfuration rate to conserve L-Met. The IPEC-J2 cells used by Zuo et al. (2019) are intestinal porcine enterocytes isolated from the jejunum of a neonatal unsuckled piglet (Vergauwen; 2015). They are described as a nontransformed, nontumorigenic small intestinal cell line that conserves its epithelial nature, maintains its differentiated characteristics, and exhibits strong similarities to primary intestinal epithelial cells (Schierack et al., 2006). According to Vergauwen (2015), IPEC-J2 cells were first used to investigate transepithelial ion transport and enterocyte differentiation, and they have been used for the characterization of epithelial cell interactions with

enteric bacteria and viruses, thus providing insight into initial host–pathogen and nonpathogen interactions. The results obtained for Met source metabolism raised some questions that were not clarified by the authors. From the figures presented by Zuo et al. (2019), it is more likely that HMTBa had a limited ability to be converted by IPEC-J2 cells, thus behaving almost similarly to the Met-deprived treatment. HMTBa is known to be transported by both passive diffusion and through active monocarboxylate transporter 1 (MCT1) (Martin-Venegas, 2014). First, there was no measurement of HMTBa within the cells or in the basolateral region to confirm its full absorption. Additionally, lower mRNA levels of D-AAOX, HADH and HAOX were found in IPEC-J2 cells than in primary pig hepatocytes. This could explain the lower Met levels found with HMTBa and question the relevance of this model to study Met source metabolism. Moreover, Zuo et al. (2019) also showed no significant difference in cysteine and reduced glutathione (GSH) between Met sources. Even though the conversion into cysteine was proven in IPEC-J2, its amplitude may have been limited by the lower Met availability, thus demonstrating opposite results to those obtained by Martin-Venegas et al. (2006), Martin-Venegas et al. (2011), and Martin-Venegas et al. (2013). It is well known that HMTBa must be first converted into Met before being converted into cysteine, taurine and GSH. In addition, according to Støy et al. (2013), it is difficult to extrapolate information from IPEC-J2 cells, as they can respond differently to environmental stimuli (e.g., diet). Diet-induced gene expression patterns differ between IPEC-J2 cells and intestinal tissue from preterm and newborn piglets, making interpretation rather difficult (Støy et al., 2013). Therefore, the relevance of this studying the metabolism of Met sources can be challenged, especially with regard to extrapolation to in vivo conditions.

In the study performed by Rasch et al. (2019), the piglets were fed diets either deficient in Met + cysteine (69% of Met + cystine recommendation) or supplemented on an equimolar basis (0.15%) with L-Met, DL-Met and HMTBa. However, it appears that all treatments were deficient in Met + cysteine (87% of the recommendation for supplemented treatments and 69% for the control diet). Additionally, the animals were infused with L-[1-$^{13}$C; methyl-$^{2}H_3$]-Met and L-[3,3-$^{2}H_2$]-cysteine to determine the kinetics and protein synthesis rates. The authors concluded that Met kinetics did not differ between groups during feed deprivation and were approximately 3 times higher in the fed state. Remethylation was 31% and 45% higher in HMTBa than in DL-Met and control-deficient pigs, respectively. The authors further argued that the lower transsulfuration rate in HMTBa may be due either to an inhibitor of cystathione β-synthase, the rate-limiting enzyme of the transsulfuration pathway or the shift of homocysteine to remethylation, resulting in homocysteine not being available for transsulfuration. These arguments are not completely supported by the other results in the same study, as no difference among Met sources was found in the plasma levels of Met, cysteine, and taurine for 54-d-old piglets that were either feed-deprived or fed. Likewise, protein synthesis and growth performance were not different among Met sources. The infusion of L-[3-3-$^{2}H_2$] cysteine could be another debatable point of this study since interactions can occur between HMTBa and L-cysteine supplementation, as shown by Dilger and Baker (2002).

Although studies have shown that the kidney and liver account for most of the total body conversion of HMTBa (Dupuis et al., 1989; Gordon and Sizer, 1965; Langer, 1965), the study of Martin-Venegas et al. (2006) demonstrated that the small intestine, among other tissues, also participates in the conversion of HMTBa. The reasons for this preferential diversion of HMTBa to the transsulfuration pathway remain to be clarified. However, it has been demonstrated that this could be beneficial to protecting the epithelial barrier (Martin-Venegas, 2013) or the animal per se (Swennen et al., 2011; Willemsen et al., 2011) under some specific oxidative stress conditions. Martin-Venegas et al. (2013) showed that HMTBa increased the production of taurine and GSH in an in vitro model of intestinal inflammation in Caco-2 cells treated with $H_2O_2$ or tumor necrosis factor-α. This led to prevention of an increase in paracellular permeability of the epithelial barrier induced by $H_2O_2$ or tumor necrosis factor-α. The results obtained for DL-Met under the same conditions were similar, although the protective role of the latter was less pronounced than that of HMTBa (Martin-Venegas et al., 2013). The potential role of HMTBa in alleviating stress responses following lipopolysaccharide injection has also been demonstrated by Matsushita et al. (2007) and Zhang et al. (2019).

## 8. Nutritional implications

The difference in the molecular structure of HMTBa compared with DL-Met results in major differences in their metabolism. This has nutritional implications in their evaluation as a source of L-Met in nonpractical and experimental conditions. It is difficult to extrapolate experimental results to practical applications under commercial conditions. As HMTBa is transported in blood to tissues where it is converted to L-Met and the majority of the synthesized L-Met is utilized locally without being released back in to circulation, the plasma Met level is lower for HMTBa-fed animals than for animals fed DL-Met (Dibner, 2003; Gonzales-Esquerra et al., 2007; Lobley et al., 2006; Vázquez-Añón et al., 2003). It has been demonstrated that the low plasma Met level of HMTBa-fed broilers is associated with lower feed intake under Met deficiency and higher feed intake under Met excess (Gonzales-Esquerra et al., 2007; Vázquez-Añón et al., 2003). Additional performance trials and further meta-analyses of studies published in the literature demonstrated different dose responses in feed intake and body weight gain between HMTBa and DL-Met in broilers, with HMTBa outperforming DL-Met at commercial and higher levels and DL-Met outperforming HMTBa at deficient levels (Vázquez-Añón et al., 2006a, 2006b). With different dose responses between HMTBa and DL-Met, the relative bioefficacy of HMTBa to DL-Met is not a fixed value but is dose dependent. It is lower at deficient TSAA levels but similar or higher at commercially relevant or higher TSAA levels. As discussed earlier, dietary cysteine levels also have an impact on HMTBa utilization (Christensen and Anderson, 1980; Dilger and Baker, 2008). Therefore, HMTBa bioefficacy values obtained using amino acid mixture-based purified or semipurified diets at severely deficient Met levels are not applicable to commercial practice; bioefficacy comparison of HMTBa compared with DL-Met should be performed with practical diets at Met and cysteine levels relevant to commercial production. At these practical levels, performance trials and meta-analyses of published trials have demonstrated the full relative bioefficacy of HMTBa over DL-Met (Vázquez-Añón et al., 2006a, 2006b; Agostini et al., 2016).

## 9. Conclusions

The thorough assessment of the literature in this review demonstrates that the physico-chemical characteristics of HMTBa and DL-Met are distinct, leading to unique behaviors with respect to their metabolism in animals. Therefore, it is important to select methods that consider these aspects when comparing the bioconversion of HMTBa and DL-Met. Some methods are not appropriate to compare the conversion of the 2 molecules to L-Met, leading to contradictory results. These contradictory results in the literature can be explained by the use of inappropriate methodologies, either for DL-Met or HMTBa.

## Author contributions

**Philippe Becquet**, **Mercedes Vazquez-Anon**, **Yves Mercier**, **Karen Wedekind**, **Tahir Mahmood**, **Dolores Batonon-Alavo**, **Frances Yan**: Writing – Original draft, Review & Editing.

## Declaration of competing interest

The authors are employees of association and corporate companies producing and placing on the market methionine sources, such as DL-methionine, HMTBa and HMTBa-Ca. The corresponding author is the General Manager of the International Methionine Analogue Association (IMAA asbl).

## Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.01.009.

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