

KINETIC CHARACTERIZATION OF METHYL DONOR SUBSTRATES
AND INHIBITORS OF HUMAN, PIG, AND RAT LIVER BETAINE
HOMOCYSTEINE S-METHYLTRANSFERASE (BHMT)

BY

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THESIS

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ABSTRACT

Betaine-homocysteine S-methyltransferase (BHMT) catalyzes the transfer of a methyl group from betaine to homocysteine to form dimethylglycine and methionine, respectively. BHMT is primarily expressed in the liver and kidney of mammals. BHMT catalyzes an ordered bi bi reaction where the first product released, dimethylglycine, can compete for the betaine binding site and inhibit homocysteine utilization. There is considerable interest in the regulation of homocysteine metabolism since even moderate elevations in plasma total homocysteine have been established as an independent risk factor for the development of vascular diseases and thrombosis. In people with homocystinuria, treatment using supplemental betaine showed a significant decrease in total plasma homocysteine but does not lower homocysteine levels within the normal range, and the moderate levels that remain are highly correlated with vascular disease. Therefore, the BHMT catalyzed reaction is a target for the treatment of homocystinuria. Finding alternative methyl donors for the BHMT reaction that following methyl transfer have less potent inhibitory properties than dimethylglycine are desired. Sulfonium analogs of betaine are considered for this research. The main objectives of this research were to determine the inhibitory properties of the demethylated product of betaine and its sulfonium analogs and also to determine the Michaelis constants in order to use them as alternative methyl donors for the BHMT reaction in homocystinuric

patients. The methyl donor substrates used are betaine, dimethylsulfonioacetate, dimethylsulfoniopropionate, and their respective demethylated products are dimethylglycine, methylthioacetate and methylpropionate. Dimethylglycine had the lowest IC_{50} values, ranging from 28 to 35 μ M for all three enzymes. Methylthioacetate had IC_{50} values ranging from 65 to 106 μ M, and values for methylthiopropionate ranged from 400 to 800 μ M. There was no significant difference between the IC_{50} values obtained for the different enzymes when assayed in the presence of DMG or MTA, but for MTP the IC_{50} values were significantly different from one enzyme to another. Kinetic studies for betaine was conducted for all three enzymes. The K_m of betaine varied from 1.8 ± 0.5 mM, 0.7 ± 0.06 , and 0.5 ± 0.06 mM for overexpressed human BHMT, pig and rat liver BHMT, respectively. We are unable to determine the K_m for DMSA and DMSP. Further studies as the catalytic efficient (K_{cat}) for all three substrates are needed in other to determine which one could be a better alternative treatment for homocystinuria.

Be impeccable with your words.

Don't take anything personally.

Don't make assumption

Always do your best.

Don Miguel Ruiz

To God, and to my family for their love, patience and unconditional support.

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ABBREVIATIONS

BHMT	Betaine homocysteine methyltransferase
BSA	Bovine serum albumin
CBS	Cystathionine beta synthase
CH ₂ THF	Methylene tetrahydrofolate reductase
Cys	Cysteine
DMG	Dimethylglycine
DMSA	Dimethylsulfonioacetate
DMSP	Dimethylsulfoniopropionate
EDTA	Ethylenediaminetetraacetic acid
Hcy	Homocysteine
HHcy	Hyper homocysteinemia
IC ₅₀	Half maximal inhibitory
IPTG	Isopropyl β-D-thiogalactopyranoside
MAT	Methionine adenosyltransferase
Met	Methionine
MS	Methionine synthase
MTA	Methylthioacetate
MTHF	Methylene tetrahydrofolate
MTHFR	Methylene tetrahydrofolate Reductase
MTP	Methylthiopropionate
PEMT	Phosphatidylethanolamine- <i>N</i> -methyltransferase
SAH	S-Adenosine homocysteine
SAM	S-Adenosine methionine
TCEP	Tris (2-carboxyethyl) phosphine tHcy
Tyr	Tyrosine

CHAPTER 1

Literature Review

Introduction

Vascular diseases, particularly heart disease and stroke, are the leading causes of death in the United States¹. Atherosclerosis, a process commonly referred to as “hardening of the arteries”, is the most prevalent form of vascular disease. Atherosclerosis can affect the arteries of the heart (cardiovascular disease), the brain (cerebrovascular disease), and the legs (peripheral vascular disease). Due to the prevalence of vascular diseases, efforts to reduce their incidence have increased research in an effort to understand the underlying causes with the overall aim to decrease morbidity and mortality. The primary risk factors for vascular disease include high blood pressure, high blood cholesterol, smoking, and physical inactivity. It is estimated that about half of the adults in the US have at least one of these factors. More recently, elevated levels of plasma total homocysteine (tHcy), or hyperhomocysteinemia (HHcy), has been proposed to be an independent risk factor for cardiovascular diseases. In 1993, Allen and Stabler suggested that even mild increases of tHcy in the range of 16 to 25 $\mu\text{mol/L}$ are highly correlated with all major forms of vascular diseases including coronary artery, cerebral and peripheral vascular². A meta-analysis by Boushey et al. showed that tHcy is a strong, independent, and graded risk factor for atherosclerotic disease in coronary, peripheral, and cerebral vessels³.

Homocysteine and Diseases

Homocysteine (Hcy) is derived from the essential amino acid methionine (Met). It is an intermediate of sulfur amino acid metabolism and is not found in proteins. The level of Hcy in blood and tissues is kept very low. In its reduced form Hcy it has a free thiol group that is susceptible to auto-oxidation at physiological pH to form disulfide bonds. In plasma, only about 1% of Hcy exists in the reduced form, and about 70% is in disulfide linkage with the cysteine (Cys) residues found in plasma proteins, predominantly albumin. The remainder of the Hcy (~30%) is found in disulfide linkage with other low molecular-weight sulfur containing compounds, primarily Cys⁴. Together these forms are called tHcy. In humans, levels of tHcy increase throughout life in both men and women (see table 1)^{5, 6}. As mentioned previously, Hcy is normally kept very low in tissues, and normal levels of fasting tHcy are considered to be between 5 and 15 $\mu\text{mol/L}$ (see table 1)^{4,5}. Elevated levels of tHcy are grouped into three levels; moderate, intermediate or severe, and any level above normal is called HHcy.

Table 1. Plasma total homocysteine (tHcy) levels for men and women, and classification

Age range ⁵	Under 30 years	30-50 years	Over 60 years
tHcy Levels μ M, Men and Women ⁵	4.1- 8.1	4.5- 11.2	5.8 – 11.9
tHcy- Classification ⁵	Moderate	Intermediate	Severe
[tHcy] μ mol/L ^{5,6}	16 – 30	31- 100	>100

The kidney has an important role in the clearance and metabolism of Hcy. Under normal conditions, Hcy is filtered at the glomerulus and is reabsorbed in the tubules and metabolized⁷. Homocystinuria is a pathological condition concomitant with severe HHcy. It is when Hcy accumulates as such high levels that the disulfide form of Hcy, homocystine, accumulates and spills over into the urine. HHcy and homocystinuria result from disturbances in Met metabolism that are typically due to a genetic defect or a vitamin-derived coenzyme deficiency that affect the proteins needed for normal sulfur amino acid metabolism. The most common genetic defects that cause homocystinuria are mutations in the cystathionine beta synthase (CBS) gene, which encodes an enzyme in the transsulfuration pathway required for the degradation of Hcy. More rare genetic causes of homocystinuria are due to mutations in genes encoding methylenetetrahydrofolate reductase (MTHFR) or genes involved in cobalamin metabolism. Deficiencies of folate, vitamin B₁₂ or vitamin B₆ can also cause HHcy because of the role their derived coenzymes have in sulfur amino acid metabolism.

Details of the genes/enzyme and their required coenzymes are discussed in more detail below.

Biochemical and pathological studies in homocystinuric children proposed that elevated tHcy may cause arteriosclerosis⁸ while other clinical studies in humans have suggested that elevated tHcy is an independent risk factor for atherosclerotic vascular diseases, and arterial and venous thromboembolism. Moderate to intermediate HHcy is present in 12-47% of patients with coronary, cerebral, or peripheral arterial occlusive disease. Patients with fasting tHcy ranging from 15 – 50 μ M are at increased risk for vascular disorders⁹. Strong evidence for a causal relationship is accumulating. Researchers observed that individuals with defective CBS or genes involved in cobalamin metabolism had severe premature thrombosis disease, and elevated tHcy was the only common metabolic disturbance¹⁰. Therefore, finding alternative strategies to reduce tHcy levels in homocystinuric patients is key factor to treat the disease. A number of therapeutic trials have used high dietary betaine and/or B-vitamins to reduce tHcy. These trials show that these treatments can drastically reduce tHcy, but not enough to normalize tHcy and the levels that remain are still strongly correlated to vascular disease risk. The research presented in this thesis describes preliminary enzymology using other alternatives to reduce tHcy levels with the hope they can normalize tHcy in individuals with moderate to severe HHcy.

Overview of Sulfur Amino Acid Metabolism and Choline Metabolism

Sulfur Amino Acid Metabolism

Amino acids are the structural units that make up proteins. They form short chain polymers called peptides, or long chain polymers called polypeptides. Amino acids are essential in the human diet because proteins are critical for maintenance and growth of the human body. Proteins contain twenty amino acids and only two of these, Met and Cys, contain the element sulfur. Also, among the twenty, nine cannot be synthesized by the human body and are therefore essential to have in the diet. Not only does Met contain sulfur, but it is also one of the essential amino acids. Besides being needed for protein synthesis, Met contributes substantially to the maintenance and integrity of cellular systems by serving as a source of methyl groups by its conversion to S-adenosylmethionine (SAM), and by its incorporation into glutathione, Met influences cellular redox state and cellular capacity to detoxify toxic compounds, free radicals and reactive oxygen species¹¹.

Methionine Cycle

Besides being important for protein synthesis, Met has important role in methylation reactions following its conversion to S-adenosylmethionine (SAM), which is an intermediate of the so-called Met cycle (Fig 1. 1). The Met cycle begins with its conversion to SAM by the enzyme methionine adenosyltransferase, also known as MAT

(Fig 1. 1 reaction 1). SAM is then used by one of hundreds of methyltransferase enzymes (Fig 1. 1 Reaction 2) whereby the methyl group associated with the Met moiety is transferred to a methyl acceptor to form a methylated product and S-adenosylhomocysteine (SAH). SAM-dependent methylations occur on many different molecules including DNA, RNA, phospholipids and proteins. SAH is then hydrolyzed to form Hcy by a reaction catalyzed by SAH hydrolase (Fig 1. 1 reaction 3).

Hcy is now at a metabolic branch point and has two possible fates; it can be methylated to reform Met (called remethylation) by either betaine-homocysteine S-methyltransferase (BHMT; Fig 1. 1 reaction 10) or methionine synthase (MS; Fig 1. 1 reaction 7), or it can participate in the transsulfuration pathway (Fig 1. reactions 4 and 5) whereby serine and Hcy are joined together by CBS (Fig 1. 1 reaction 4) to form cystathionine, which is then be hydrolyzed by cystathionase (Fig 1.1 reaction 5) to form Cys and α ketobutyrate. If intracellular Hcy production in extra hepatic tissues overwhelms its metabolism through the remethylation and transsulfuration pathways, excess Hcy is released into circulation and metabolized by the liver, and to a lesser extent, kidney. It is important to note that, as depicted in Figure 1, folate and vitamin B₁₂ are needed for the MS-dependent remethylation of Hcy, and that vitamin B₆ is required for both enzymes of the transsulfuration pathway, which explains why deficiencies in these vitamins can result in some degree of HHcy.

As already explained, both BHMT and MS can remethylate Hcy (Fig 1. 1 reactions 10 and 7), however, they use difference sources of methyl groups. The direct source of methyl groups for the MS-catalyzed reaction comes from methylenetetrahydrofolate reductase (MTHFR; Fig 1. 1 reaction 6). This reaction

reduces a methylene group to a methyl group, which is then used by the MS reaction. Ultimately, the methylene group comes from serine and glycine via reactions that are not shown in Fig 1. 1. For the BHMT-catalyzed reaction, the methyl donor is betaine, a compound that is an intermediate of the choline oxidation pathway. The oxidation of choline to betaine aldehyde, and then to betaine (Fig 1. 1 reactions 8 and 9) are discussed in more detail below. However, after betaine (also known as trimethylglycine) serves as a methyl donor for the BHMT reaction, the demethylated product is dimethylglycine (Fig 1. 1 reaction 10).

Transsulfuration Pathway

As stated previously, the transsulfuration pathway catalyzes the conversion of Hcy and serine to Cys and α -ketobutyrate. Cystathionine is an intermediate of the transsulfuration pathway, and Cys is the final sulfur-containing product. The pathway is called transsulfuration because the sulfur of Hcy is transferred to serine, and so the backbone of Cys comes from serine and only the thiol group comes from Hcy. The transsulfuration pathway is irreversible, and the existence of this pathway is why Cys is not an essential amino acid. The transsulfuration pathway is believed to be primarily expressed in the liver, kidney, small intestine and pancreas, and it represents the primarily catabolic pathway for Met. A reduction or lack of CBS activity is the most common cause of homocystinuria because of an inability to degrade Hcy. There have been many mutations described in the CBS gene and there is considerable genetic heterogeneity in the disease due to CBS deficiency. Homocystinuria due to severe

CBS deficiency causes severe growth and mental abnormalities, skeletal abnormalities, dislocation of the optic lens, and the most lethal consequence of having unstable hemodynamics resulting in fatal thrombosis. In severely affected individuals Cys becomes an essential amino acid, and the most common treatment is to supplement B vitamins (B₆, B₁₂ and folate) and betaine in an effort to enhance Hcy remethylation and stimulate any residual CBS activity.

In normal individuals the transsulfuration pathway is important because it provides Cys, which is not only needed for protein synthesis, but is also a component of glutathione, a tripeptide that has a critical role in maintaining thiol redox status in the cell. Glutathione is also involved in the detoxification of some drugs and xenobiotics in liver. In liver the concentration of glutathione is typically 5 to 10 millimolar. Cys is a precursor to taurine, a compound that is important in bile formation (taurocholic acid) and also functions as a renal osmolyte. Cys is also a precursor to sulfates, which are required for glycolipids, polysaccharides and sulpholipids, and hydrogen sulfide production, the latter of which is a gaseous molecule that has recently gained considerable attention since it has been discovered to be a signaling molecule. Hence, the transsulfuration pathway is very important to human health and function and insufficient flux through this pathway probably contributes to the pathologies observed in homocystinurics above and beyond simply having elevated levels of Hcy.

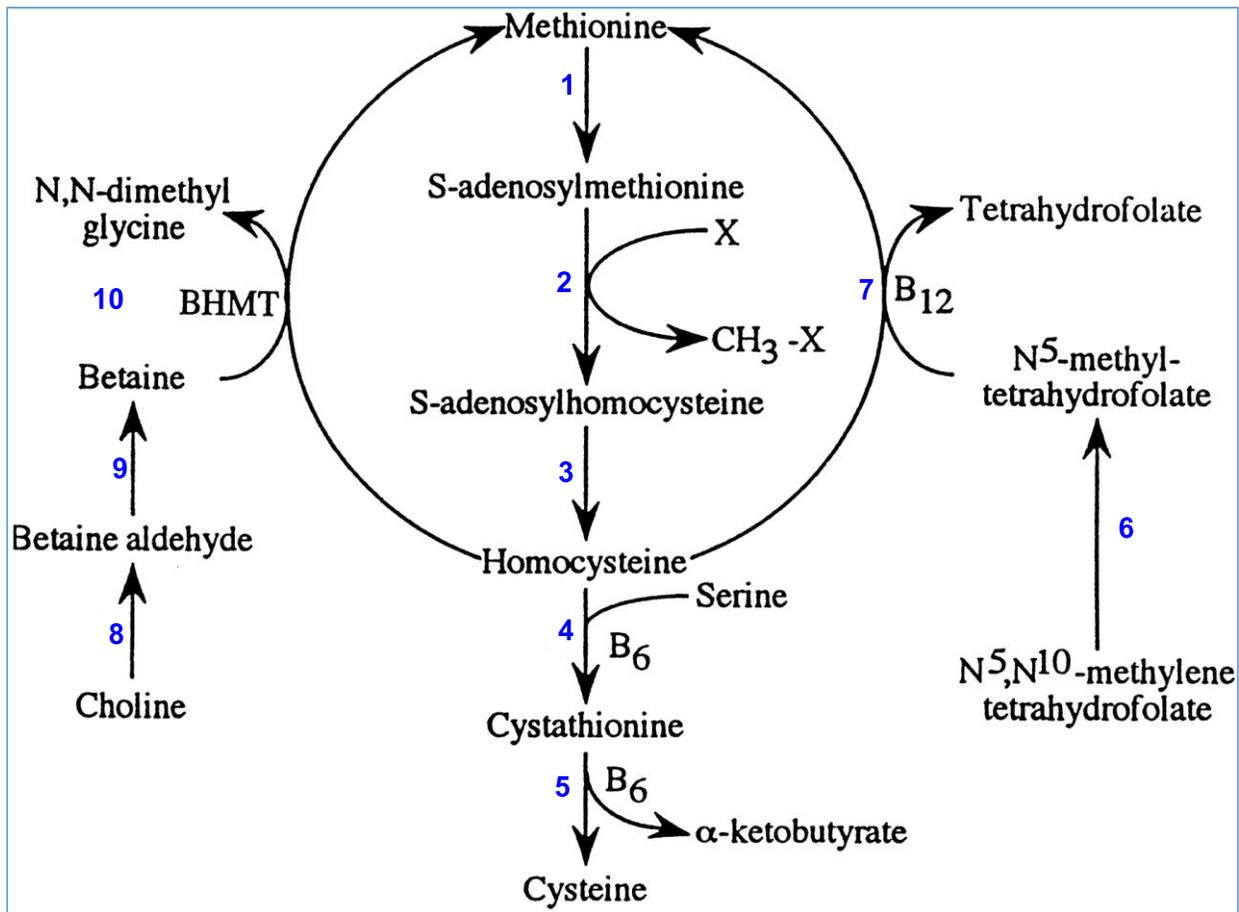


Figure 1. 1 Methionine cycle and transsulfuration pathway: 1. Methionine Adenosyltransferase (MAT). 2. S-Adenosylmethionine methyltransferase 3. S-Adenosylhomocysteine hydrolase 4. CBS 5. Cystathionase 6. MTHFR 7. Methionine Synthase 8. Choline dehydrogenase 9. Betaine aldehyde dehydrogenase 10. BHMT

Choline Metabolism and Oxidation

Choline is a water soluble nutrient that is found in the diet and can also be made from serine and Met in the liver. Although free choline is found in the diet, most dietary choline is associated with the phospholipid, phosphatidylcholine, also known as lecithin. Foods rich in lecithin include egg yolk and meats. For many years choline was not considered to be an essential nutrient as long as the diet contained adequate Met, however, in the late 1990s the Institute of Medicine's declared choline to be an essential nutrient¹². Choline deficiency results in fatty liver, which if not treated can develop into cirrhosis and hepatocellular carcinoma. The fatty liver is the result of a lack of phosphatidylcholine, which is required for lipoprotein (VLDL) synthesis and export of triglycerides out of the liver. The Institute of Medicine set the adequate intake of choline at 550 mg/day for adult men, and 425 mg/day for adult (non-pregnant, non-lactating) females¹².

An overview of choline metabolism is depicted in Figure 1.2 Its biosynthesis requires only one enzyme, phosphatidylethanolamine-*N*-methyltransferase (PEMT; Fig 1.2, reaction 1), which converts phosphatidylethanolamine to phosphatidylcholine using three methyl groups derived from SAM. These methyl groups ultimately come from Met, as described in the section of sulfur amino acid metabolism on the Met Cycle (Fig 1.1). The ethanolamine moiety is derived from serine, which is why the synthesis of choline was previously stated to be possible from serine and Met. PEMT is a liver specific enzyme and accounts for about 30% of the phosphatidylcholine made in hepatocytes¹³.

During normal phospholipid metabolism, the choline head group of phosphatidylcholine (and other choline-containing phospholipids not depicted) can be

removed to generate free choline. Choline is now at a branch point. It can either be re-incorporated into the phospholipid pool (Fig 1.2, reactions 2 to 4), used to generate acetylcholine in nervous tissues (Fig 1.2 reaction 5), or oxidized to carbon dioxide and an ammonium ion (a pathway depicted by step 7). These pathways are briefly discussed in the following paragraphs.

The reincorporation of choline into phospholipids begins with its conversion to phosphocholine, which is catalyzed by choline kinase (Fig 1.2, reaction 2). Phosphocholine is further metabolized to phosphatidylcholine by the reactions catalyzed by CPT: phosphocholine cytidylyltransferase (CT, and CPD-choline: 1, 2-diacylglycerol choline phosphotransferase (or choline phosphotransferase, CPT) (Fig 1. 2 reactions 3 and 4). The regeneration of phosphatidylcholine that begins with the phosphorylation of choline is also known as the Kennedy pathway (Fig 1.2, reactions 2, 3 and 4).

Another fate of free choline is mediated by its conversion to acetylcholine in nerve endings (axon terminals) by a reaction catalyzed by choline acetyltransferase (Fig 1.2, reaction 5). There are many kinds of cholinergic nerve fibers, but perhaps the most familiar are those at the neuromuscular junction that stimulate muscle contraction by stimulating the acetylcholine receptor, which leads to skeletal muscle cell depolarization and contraction. Following release into the synapse, acetylcholine is acted upon by acetylcholine esterase (Fig 1.2, reaction 6), which lowers acetylcholine concentration and stops excitation at the acetylcholine receptor. Then, choline is pumped back into the nerve ending and reused for the synthesis of acetylcholine, stored in vesicles, and re-released into the neuromuscular synapse to initiate another cycle of muscle cell

depolarization and subsequent contraction. Quantitatively, acetylcholine stores represent a small fraction of total body choline.

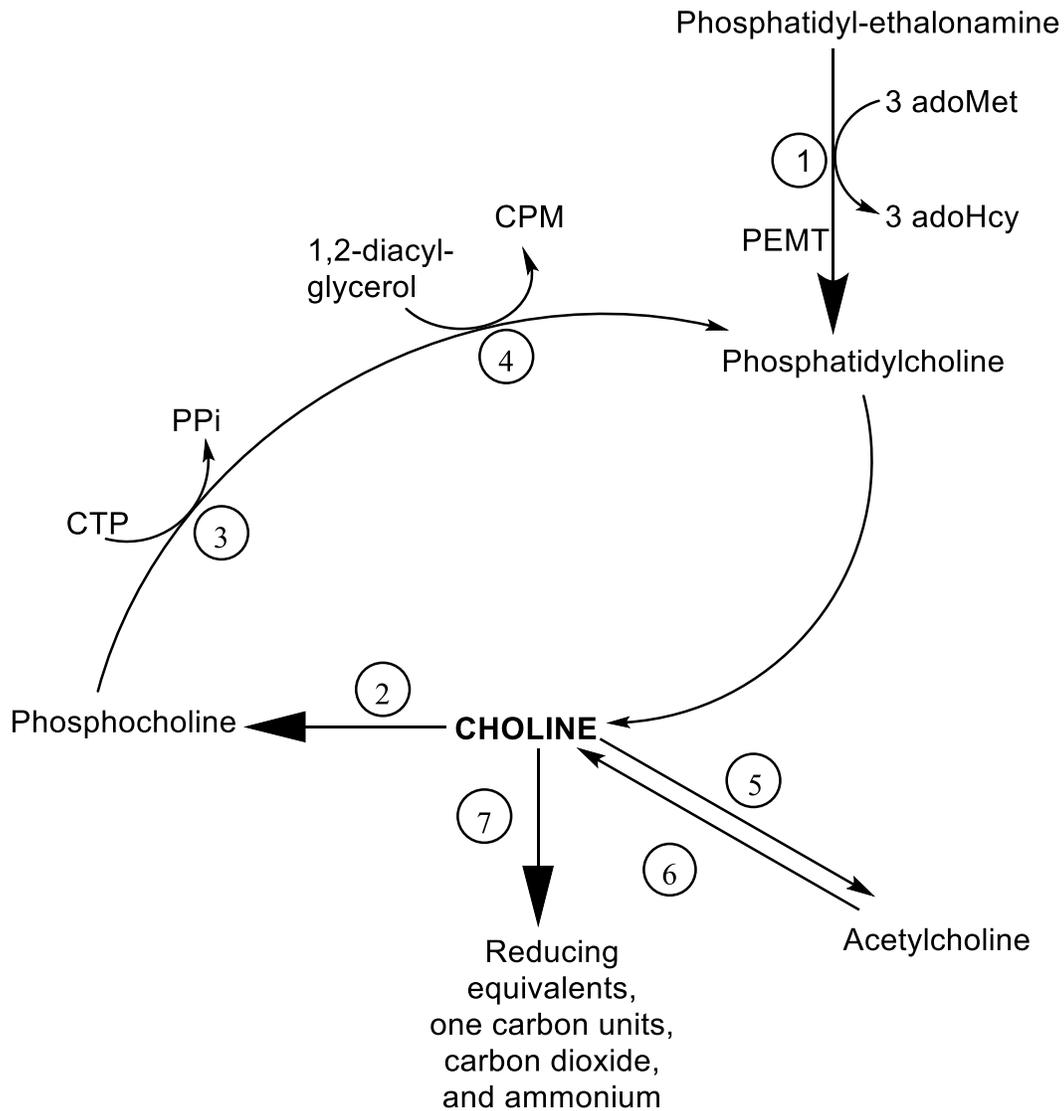


Figure 1. 2 Major biochemical pathways of choline metabolism. 1, biosynthesis of choline by the methylation of phosphatidyl-ethanolamine catalyzed by phosphatidyl-ethanolamine methyltransferase (PEMT). 2, acetylcholine synthesis (choline acetyltransferase). 3, hydrolysis (acetylcholinesterase). 4, choline oxidation. 6, choline kinase. 6, 7 reincorporation of choline into phosphatidylcholine, also known as Kennedy pathway (choline phosphorylation), phosphocholine cytidylyltransferase and cholinephosphotransferase.

The final fate of free choline is that of oxidation. Choline oxidation is a major source of one carbon units and its oxidation occurs by a multistep process that is depicted in Figure 1.3. This process requires enzymes found both in the cytosol and the mitochondria, and these enzymes are very abundant in the liver and kidney. Free choline is transported into the mitochondria from the cytosol where it is oxidized to betaine aldehyde, and then betaine (Fig 1. 3). The enzymes catalyzing these reactions are choline dehydrogenase and betaine aldehyde dehydrogenase, respectively (Fig 1.3 reactions 1 and 2). Betaine is then exported from the mitochondria to the cytosol where it becomes a substrate for the BHMT catalyzed reaction (Fig 1.3 reaction 3). The BHMT reaction transfers a methyl group from betaine to Hcy to form DMG and Met. DMG is transported from the cytosol into the mitochondria where it is metabolized to sarcosine then glycine by DMG dehydrogenase and sarcosine dehydrogenase, respectively (Fig 1.3 reaction 4 and 5). Both reactions are folate- and flavin-dependent. The final reaction of choline oxidation is catalyzed by a protein complex called the glycine cleavage system (Fig 1.3 reaction 6). This enzyme system oxidizes glycine to produce MTHF, carbon dioxide and ammonium ion. The methylene groups associated with folate are then oxidized to formate in the mitochondria (not shown). Formate then leaves the mitochondria into the cytosol, becomes activated back into the folate pool, and can then use for the biosynthesis of purines, serine and Met (via the MTHF and MS; Fig 1.1 reactions 6 and 7). In total, choline oxidation contributes four carbons to the one-carbon pool via the formation of Met by BHMT, and the three MTHF molecules generated from DMG dehydrogenase, sarcosine dehydrogenase, and the glycine cleavage system (Fig1. 3).

The oxidation of choline to betaine is important because betaine is both a renal osmolyte and an important source of one carbon units to support Hcy remethylation and other one carbon needs by its production of MTHF¹⁴. Chiuve et al. demonstrated that the intake of betaine and choline was associated with lower tHcy in a cross-sectional analysis that included 1477 women¹⁵. In BHMT knockout mice, concentration of choline decreased and tHcy concentration increased (8-fold). Similarly, rats fed *S*-(δ -carboxybutyl)-*DL*-homocysteine (CBHcy), a potent and specific inhibitor of BHMT, had decreased plasma choline and an increase in tHcy. These studies showed that BHMT is critical for tissue choline and Hcy homeostasis in rodents. At present, there is no report indicating there are humans that are deficient or lack BHMT activity, but the results from these studies strongly suggest that a mutation in the BHMT gene that results in reduced BHMT activity will have consequences for sulfur amino acid and choline metabolites.

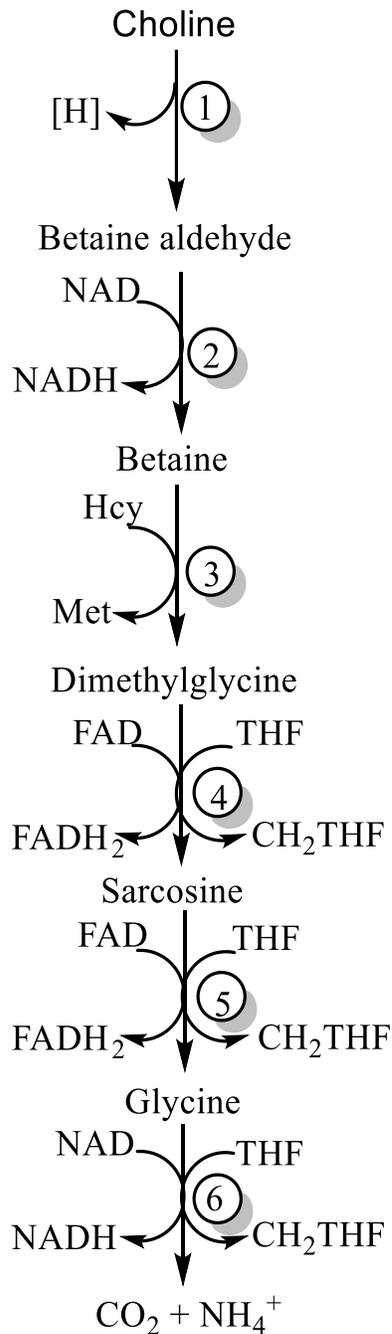


Figure 1. 3 Enzymes in choline oxidation pathway: 1. Choline dehydrogenase 2. Betaine aldehyde dehydrogenase 3. Betaine homocysteine S-methyltransferase 4. Dimethylglycine dehydrogenase 5. Sarcosine dehydrogenase 6. Glycine cleavage. NAD and NADH: oxidized and reduced nicotinamide adenine dinucleotide. FAD and FADH: oxidized and reduced flavin adenine dinucleotide. THF: tetrahydrofolate. CH₂THF: methylene tetrahydrofolate

Key Enzymes in Regulation of Sulfur Amino Acid Metabolism

Methionine Adenosyltransferase (MAT)

Methionine adenosyltransferase (MAT) is the enzyme that catalyzes the formation of SAM by joining together Met and the adenosyl moiety of ATP (Fig 1. 1 reaction 1). In mammalian tissues, there are three distinct forms of MAT; MATI, MATII and MATIII, and these proteins are the products of two different genes, *MAT1A* and *MAT2A*¹⁶. *MAT1A* is expressed only in the liver and encodes MATI (tetramer) and MATIII (dimer). MATIII is the dominant form and often times the formation of SAM in liver is ascribed to MATI/III activity. *MAT2A* is widely expressed in mammalian tissues, including liver, brain, and kidney encodes the MATII enzyme^{17, 18,19}. In liver, MATII only represents about 5% of the total MAT activity.

Although there are catalytic differences between MATI and MATIII, in general, the dominant characteristic of MAT activity in liver is that the activity has a high K_m ($K_m = 200-600 \mu\text{M}$) and displays positive cooperatively (activated allosterically) with respect to SAM concentrations²⁰. Sullivan and Hoffman showed that an increase in Met in the liver can increase SAM levels, which in turn further stimulates its own synthesis by allosterically activating MAT activity. The other form MAT II, primarily expressed in extra hepatic tissues and fetal liver, has a low K_m for L-Met (6-8 μM), and has been shown to be strongly inhibited by SAM allosterically¹⁹.

Methyl Tetrahydrofolate Reductase (MTHFR) and Methionine Synthase (MS)

MTHFR, a rate limiting enzyme for the MS-dependent remethylation of Hcy, is encoded by the *MTHFR* gene. It is a dimer that catalyzes the formation of methyltetrahydrofolate (MTHF) by reducing methylenetetrahydrofolate (Fig 1. 1 reaction 6). This reaction is irreversible under both *in vivo* and *in vitro* conditions, and the enzyme activity is and potently inhibited (allosteric) by SAM²¹. MTHF is required for the MS reaction, one of the enzymes responsible for the *de novo* synthesis of Met. MS also requires vitamin B₁₂ (cobalamin) as a coenzyme²². MS actually catalyzes two methyl transfers, first, the methyl group of MTHF is transferred to cobalamin to form methylcobalamin. Then, methylcobalamin is used to methylate Hcy to form Met. By inhibiting MTHFR, SAM impairs regeneration of Met by decreasing MTHF availability.

Cystathionine Beta Synthase (CBS)

CBS catalyzes the first reaction of the transsulfuration pathway. CBS is a tetrameric protein composed of identical subunits²³ that is dependent on pyridoxal phosphate (PLP) and heme for activity²⁴. This reaction represents one of the two major metabolic avenues to clear Hcy, and therefore its activity is critical to avoid an elevated level of Hcy, which increases risk for vascular diseases. SAM is a required allosteric activator of CBS²⁵, stimulating flux through the transsulfuration pathway.

Betaine Homocysteine S-Methyltransferase (BHMT)

Betaine homocysteine methyltransferase (BHMT) is a zinc metalloenzyme that catalyzes a methyl transfer from betaine to Hcy to form DMG and Met, respectively. This enzyme belongs to a family of enzymes that methylate thiols or selenols, a family that includes MS. BHMT is a tetramer made up of identical monomers. Moreover, BHMT is only expressed in liver and kidney. BHMT catalyzes an ordered bi bi reaction²⁶ whereby Hcy binds first, and then its binding causes a conformational change in the enzyme that creates the betaine binding site. Then, betaine binds and methyl transfer takes place followed by an ordered release of products, first DMG, and then Met.

Betaine is the only methyl donor for the BHMT reaction that is made in the body, but we can also get betaine in the diet. Besides serving as a methyl donor for the BHMT reaction, betaine has an important role as a cellular osmolyte that protects cells from changes in tonicity¹⁰. Therefore, changes in BHMT expression could have the potential to affect betaine levels and cellular volume. It is known that BHMT is strongly inhibited by its end product DMG. This potent inhibition is directly due to its catalytic mechanism as a bi bi reaction; after Hcy binds to the enzyme the second ligand binding site can be occupied by betaine or DMG. If betaine binds, catalysis proceeds, if DMG binds, the enzyme is in an abortive ternary complex that is unable to produce Met. This complex represents a potent form of inhibition for the BHMT reaction²⁶. Therefore, inside a living cell, the flux through this reaction is not only determined by the concentration of substrates, but it is also determined by the concentration of the de-methylated product of the methyl donor and its affinity for the betaine site.

Diet has been shown to affect BHMT activity. BHMT is regulated at the transcriptional level by dietary betaine and Met²⁷. When Met is deficient, dietary betaine or choline induces BHMT activity by increasing the level of its mRNA and subsequent protein levels. Betaine supplementation has been shown to lower tHcy in both hyperhomocysteinemic rats and humans¹⁰, but this supplementation does not usually lower Hcy levels to within the normal range. Allen et al. showed that betaine treatment lowers tHcy to 30-80 μM and eliminates the occurrence of thromboembolism in patients with cobalamin and folate deficiency in patients with severe HHcy². Kinetic studies using purified porcine BHMT indicates that DMG inhibits the enzyme in a competitive way respect to betaine, but in an uncompetitive way with respect to Hcy. Up to 37-fold increase in plasma DMG (250 μM) were observed in individuals receiving betaine for homocystinuria compared with a control population²⁸. These data support the hypothesis that betaine treatment for homocystinuria results in a large increase in DMG, which in turn inhibits BHMT and probably limits the effectiveness of betaine as Hcy-lowering treatment in homocystinurics. Therefore, we propose that a strategy to reduce tHcy further in individuals with homocystinuria is to find alternative methyl donor that is good substrate for BHMT, but whose demethylated product either doesn't accumulate or less potently inhibits the BHMT reaction. In theory, these substrates could enhance the conversion of Hcy to Met and lower Hcy to a greater extent that can be achieved by betaine treatment. It is known that sulfonium analogs of betaine, such as dimethylsulfonioacetate (DMSA) and dimethylsulfoniopropionate (DMSP) can function as methyl donors for the BHMT reaction. However, how well these compounds serve as methyl donors have not been kinetically characterized. DMSA is a synthetic compound

while DMSP is a naturally occurring compound found in plants. The work presented in this thesis characterizes the kinetic properties of substrates and inhibitors of human, pig, and rat liver BHMT as well as finding alternative methyl donor for the BHMT reaction that may improve the treatment for homocystinuria.

Integration of Sulfur Amino Acid Metabolism in Liver

Control of sulfur amino acid metabolism in liver is acutely regulated by SAM concentrations. When the diet contains high levels of Met, the level of SAM increases due to the fact that the flux through MAT increases as a result of its high K_m for Met. Hepatic MAT activity is further enhanced by the resulting increase in SAM, which activates (positive allosteric) the enzyme. High SAM inhibits the remethylation of Hcy by MS because SAM is an allosteric inhibitor of MTHFR, which reduces MTHF production. Moreover, the high level of liver SAM caused by high dietary Met activates Hcy degradation by stimulating the activity of CBS. In short, a high dietary level of Met increases liver SAM, which inhibits the reformation of Met from Hcy, and stimulates the degradation of Hcy via transsulfuration. Conversely, if Met levels are less than adequate, SAM levels are decreased which lifts the inhibition of MTHFR and reduces CBS activation, pushing Hcy toward remethylation.

CHAPTER 2

DIMETHYLGLYCINE, METHYLTHIOACETATE AND METHYLTHIOPROPIONATE AS INHIBITORS OF BETAINE HOMOCYSTEINE METHYL TRANSFERASE (BHMT)

Introduction

Homocysteine (Hcy) is produced from methionine (Met) by a multi-step process beginning with the formation of S-adenosylmethionine (SAM). SAM is the substrate for many methyltransferases, all of which produce S-adenosylhomocysteine (SAH), which is then hydrolyzed to form adenosine and Hcy. There are several genetic diseases that interfere with the use of Hcy. The most common is a cystathionine- β -synthase (CBS) deficiency, a condition that slows or abolishes the condensation of Hcy and serine to form cystathionine. Less common genetic diseases result in a reduction in the conversion of Hcy to Met by the folate- and cobalamin-dependent reaction catalyzed by methionine synthase (MS)²⁹. For each of these diseases, Hcy accumulates abnormally high in blood and spills into the urine, a condition known as homocystinuria. Homocystinuria, a condition caused by severe hyperhomocysteinemia (HHcy), has been associated with thrombosis and atherosclerosis. The treatment for all forms of homocystinuria include supplemental betaine. The treatment focuses on reducing plasma total Hcy (tHcy) by enhancing its methylation by BHMT to form Met. This treatment significantly reduces tHcy but rarely normalizes it, and although the clinical prognosis is greatly improved, for example the most severe complication (thrombosis) is

significantly reduced, the levels of tHcy that remain are still highly correlated to the development of vascular disease.

BHMT makes up 0.5-2% of the soluble protein in liver. It catalyzes a methyl transfer from betaine to Hcy to form dimethylglycine (DMG) and Met, respectively. The reaction mechanism is ordered bi bi, whereby Hcy is the first substrate to bind and Met is the last product off (Fig 2. 1). This reaction mechanism creates the possibility for a potent form of feedback inhibition. In the case of BHMT, once Hcy binds and the second ligand binding site is open for occupancy, either betaine or DMG can bind. If betaine binds, the methyltransferase reaction proceeds. However, if DMG binds to the BHMT-Hcy binary complex, then an abortive ternary complex (BHMT-Hcy-DMG) forms that is inactive. It can be assumed that living cells have steady-state levels of Hcy, betaine and DMG, and therefore if conditions arise that cause an increase in DMG, the flux through the BHMT reaction could be depressed. Since supplemental betaine is a treatment for homocystinuria, any concomitant increase in DMG could limit the Hcy-lowering effect of betaine because of the reduction of active enzyme since some of it is tied up in the BHMT-Hcy-DMG ternary complex.

Does a high level of betaine supplementation cause an accumulation of DMG and therefore limit the Hcy-lowering potential of the BHMT reaction? Previous studies have shown that betaine supplementation significantly reduces tHcy, but does not normalize it, and the level of tHcy that remain are still highly correlated to Hcy-induced pathologies. This has been shown in humans and animal models. For example, human homocystinurics being treated with betaine experienced reductions in tHcy but large increases in plasma DMG². In mice with HHcy due to a lack of CBS activity, high

betaine supplementation also lowered tHcy but the levels of DMG increased greatly¹⁰. In summary, when animals or humans are treated for HHcy with supplemental betaine there is limited success; major reductions in tHcy are observed, but normalization is not achieved because of the concomitant rise in DMG, which inhibits the availability of active enzyme through the formation of the BHMT-Hcy-DMG ternary complex.

Previous work has shown that sulfonium analogs of betaine can function as methyl donor substrates for the BHMT reaction. We have a specific interest in dimethylsulfonioacetate (DMSA) and dimethylsulfoniopropionate (DMSP) (Fig 2.2) as potential alternatives to betaine for the treatment of homocystinuria. DMSA is a synthetic compound. DMSP, a naturally occurring compound found in algae and some terrestrial plants, is thought to be the major precursor of dimethyl sulfide, a gas that has a dominant role in biogenic sulfur emission³¹. It is possible that these sulfonium analogs of betaine might be better supplements for treating homocystinuric patients if their demethylated products inhibit BHMT to a lesser extent than DMG.

The hypothesis is that betaine treatment lowers tHcy because it initially increases flux of Hcy to Met using BHMT, however, over time DMG accumulates and prevents further tHcy-lowering because it inhibits the BHMT reaction. Alternative methyl donors to lower tHcy are needed, and their de-methylated products need to have lower affinity for the BHMT-Hcy binary complex enzyme. If identified, these products will be less likely to limit the flux through BHMT or compete with BHMT Hcy-binary complex site. Hence, a key goal of this research is to find alternative methyl donors that may improve treatment for homocystinuria. The main objective of this chapter is to characterize the

de-methylated products from DMSA and DMSP, methylthioacetate (MTA) and methylthiopropionate (MTP), respectively, as inhibitors for the BHMT reaction (Fig 2.2).

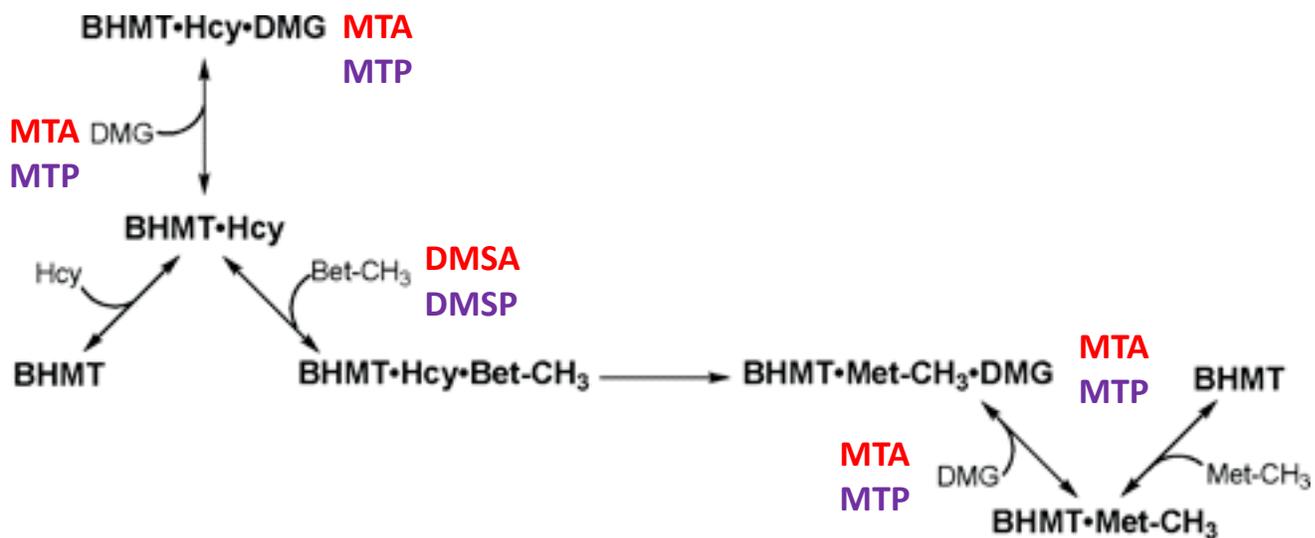
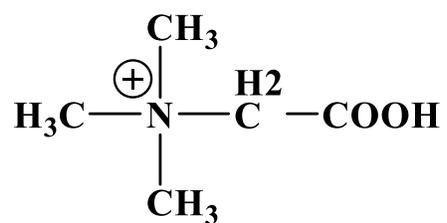
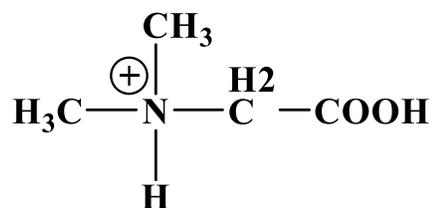


Figure 2. 1 BHMT ordered bi bi reaction with the sulfonium analogs of betaine and their respective demethylated products.

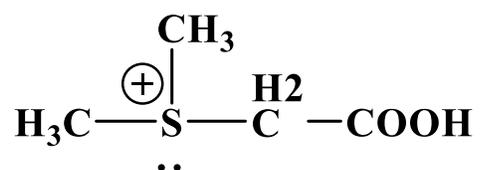
Betaine



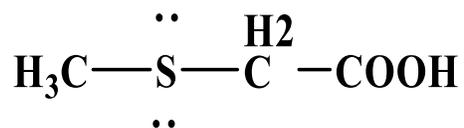
DMG



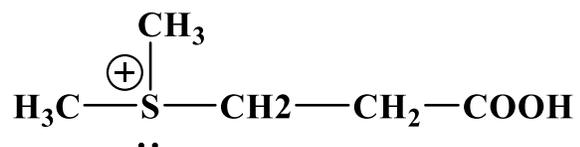
DMSA



MTA



DMSP



MTP

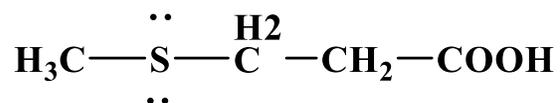


Figure 2. 2 Chemical structure of betaine and the sulfonium analogs of betaine, and their respective demethylated products.

Materials and Methods

Reagents

Ampicillin, zinc chloride (ZnCl_2), isopropyl β -D-thiogalactopyranoside (IPTG), D, L-Hcy thiolactone, Dowex 1x4, dimethylglycine (DMG) and β -mercaptoethanol were obtained from Sigma-Aldrich (St. Louis, MO). Methylthioacetate (MTA) and methylthiopropionate (MTP), ethylenediaminetetraacetic acid (EDTA), protease inhibitors, Tris (2-carboxyethyl) phosphine (TCEP), Tris HCl, Tris base, bovine serum albumin (BSA) were obtained from Thermo Fisher Scientific (Pittsburgh, PA).

Expression and BHMT purification procedure

Vectors containing the wild type BHMT cDNA were transformed into *E. coli*. Cells were added to 50 mL 2XYT media containing 50 μL of ampicillin (100mg/mL), and was incubated overnight in refrigerated shaker set at 20 °C, 250 rpm. When cells reached an optimal OD_{600} of about 0.5 – 0.6, the culture was then centrifuged at 20°C for 30 minutes. The pellet was resuspended in 1 mL of 2XYT broth, and then transferred into a 1 L flask containing 2XYT media, 1 mL of ampicillin (100mg/mL), and 1 mL of 250 μM ZnCl_2 . The resuspended cells were allowed to grow at 37°C, 180 rpm. When the OD_{600} reached 0.7 – 1.0, IPTG was added (0.5mL of 1M IPTG) for the induction of BHMT expression. Induced cells were then incubated overnight at 20 °C, 250 rpm. Following the induction, the cells were collected by centrifugation at 5000g for 90 minutes at 4°C. The cells pellet was resuspended in 20 mL of cold ice buffer. Cells were then lysed

using a French press at 1500 p.s.i, and then sonicated on ice at 1 minute intervals (5 times) at 30% output with a Branson Digital Sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT). The cell lysate was clarified by centrifugation at 15000g for 90 minutes at 4°C. Then the enzyme was purified in the cold room using a chitin affinity column. The clarified lysate was applied (0.5 mL/min) to a chitin affinity column (10 mL) that had been equilibrated with buffer containing 20 mM of Tris buffer pH 8.0, 500 mM NaCl, 1 mM EDTA pH 8.0, and 0.1% of triton. The column was then washed with 500 to 800 mL of the same buffer followed by 300 to 500 mL of buffer containing 20 Mm of Tris buffer pH 8.0, 500 mM NaCl, and 1 mM EDTA pH 8.0. Then 30 mL of elution buffer containing 20 mM of Tris buffer pH 8.0, 50 mM NaCl, 1 mM EDTA pH 8.0, and 30 μ L of β ME was added to the column, which was subsequently was capped and allowed to stand overnight. The BHMT was eluted from the column sitting overnight, and then aliquot and store at -80°C.

Preparation of Crude Extract for Rat Liver and Pig Liver BHMT

Rat liver was purchased from Innovative Research Inc. (Novi, MI) and stored at -80 °C. Five grams of rat liver was partially thawed, minced with scissors, and suspended in 20 mL Tris-phosphate buffer (25 mM phosphate pH 7.0, 25 mM Tris pH 8.0, 0.5 mM EDTA, 5 mM β ME, 200 μ L protease) at 0 °C. Then the mixture was homogenized for 30 seconds using a Power Gen 500 homogenizer (Fisher Scientific, Pittsburgh, PA). Following homogenization, the crude extract was centrifuged at 15000 g for 90 minutes. The supernatant was collected and then dialyzed three times using

two different buffers. The first two dialyses were done in 1 L of 25 mM phosphate pH 7.0, 25 mM Tris pH 8.0, 0.5 mM EDTA, 5 mM β ME. The third dialysis buffer was 2 L containing 25 mM phosphate pH 7.0, 25 mM Tris pH 8.0, 0.5 mM EDTA, 5 mM β ME and 10 % glycerol. After dialysis the crude extract was aliquot and stored at -80 °C. The same preparation was used for the pig liver.

BHMT Assay

BHMT activity was measured as described by Finkelstein and Mudd³² with several modifications. DL-Hcy was prepared by a procedure described by Duerre and Miller³³. DL-Hcy thiolactone hydrochloride (15.4 mg) was dissolved in 400 μ L of 2N sodium hydroxide. The solution was allowed to sit 5 min at room temperature (23°C). The reaction was then neutralized by the addition of 600 μ L of a saturated solution of mono-potassium phosphate and used immediately in the BHMT assay. The standard assay contained 5 mM DL-Hcy, 2 mM betaine (0.1 μ Ci), and 50 mM Tris – HCl (pH 7.5). The final reaction volume was 0.5 mL. Reaction tubes were capped with the rubber stoppers and kept in ice water until the assay was started by transferring the tubes into a 37 °C water bath. Following 1h incubation, samples were chilled in ice water, and 2.5 mL of water was added. The samples were applied to a Dowex 1X4 chloride (-) form (50 – 100 mesh), column (0.6 X 3.75 cm), and the unreacted betaine was washed from the column with water (3 X 5 mL). DMG and Met were eluted into scintillation mixture vials with 3 mL of 1.5 N HCl. Seventeen mL of scintillation mixture (Ecolume, ICN) were added to each vial and counted. Blank vials contained all of the reaction components

except enzyme, and their values were subtracted from the sample values. All samples were assayed in duplicate.

Inhibition Studies and IC₅₀ Determination

The BHMT assay was performed as described above except they contained a lower concentration of betaine (250 μ M) with higher radioactivity (0.5 μ Ci). The concentrations of inhibitors (DMG, MTA, and MTP) were varied as described in the results. An amount of enzyme was used so that no more than 10% of substrate was consumed at the lowest level of inhibitor tested. Inhibitor concentrations tested were consistent with standard protocols³⁴. Final inhibitor concentrations ranged from 1.5 μ M to 15 mM, 10 μ M to 3 mM, and 10 μ M to 50 mM for DMG, MTA, and MTP respectively. All samples were assayed in duplicate, and the blank values were subtracted from all the sample values. The blank contained all of the reaction components except enzyme and inhibitors. Each IC₅₀ determination with a given enzyme and inhibitor was done at least four times to assure repeatability.

Statistical Analysis

For the IC₅₀ determination, all the results were plotted using Graph Pad Prism 6.04 by non-linear progression curve fit. Data are presented as means \pm SEM and means were compared using two way ANOVA. Post hoc differences were compared by Fisher LSD

and p -values of less than 0.05 were considered statistically significant and noted by different superscripts, as presented in Table 2. 1.

Results

Apparent half maximal inhibitory concentration (IC_{50}) of DMG, MTA and MTP for BHMT enzymes – Human BHMT was overexpressed in *E.coli*, purified, and then dialyzed before being used in IC_{50} assays. Pig liver and rat liver were both homogenized and dialyzed, and these crude extracts were used to determine the IC_{50} values. All enzymes were assayed at least four times with the demethylated product; DMG, MTA, and MTP at different concentrations as stated previously in the Methods section. Preliminary assay parameters were established to optimize assay factors and reproducibility. A minimum of 10 concentrations of each inhibitor were used, and each concentration was assayed in replicate for an accurate IC_{50} determination. We used equally spaced concentration ranges (3 fold or half $-\log$ dilutions). We first determined the percent inhibition at a high and a low concentration of each inhibitor. After those results were obtained, we were able to determine an apparent IC_{50} using a more extensive range of concentrations. IC_{50} values were determined for each enzyme-inhibitor pair at least four times, from which a mean IC_{50} was calculated.

Demethylated Product DMG

The results obtained showed that the IC_{50} values for purified human, and pig and rat liver BHMT enzymes in the presence of DMG are low and similar for all three enzymes, ranging between 28 to 35 μ M (Fig 2.3; table 2). The concentration ranges used in these experiments were 1.5 μ M to 15 mM for all three enzymes. The lowest concentration of DMG inhibited only 2% of the BHMT enzymatic activity, while the highest concentration

of DMG inhibited activity more than 98%. Statistically, there was no significant difference between enzymes, which was expected, as we know that mammalian BHMT enzymes share greater than 95% amino acid identity.

Demethylated product MTA

For MTA, the IC_{50} was about threefold greater for all three enzymes compared to DMG, ranging between 65 to 106 μ M (Fig 2.3; table 2). The concentration ranges used in these experiments were from 3 μ M to 10 mM. The lowest concentration of MTA inhibited only 2% of the BHMT enzymatic activity, while the highest concentration of DMG inhibited greater than 98% of the BHMT enzymatic activity. Statistically, there was no significant difference between enzymes using MTA, but there was significant difference between DMG and MTA. DMG so far has the lowest IC_{50} concentration values.

Demethylated product MTP

In contrast, the demethylated product MTP showed significantly greater IC_{50} values compared to the DMG and MTA (Fig 2.3; table 2) for all enzymes. The concentration ranges used in these experiments were from 10 μ M to 50 mM. The lowest concentration of MTP inhibited only 2% of the BHMT enzymatic activity, while the highest concentration of MTP inhibited greater than 96% of the BHMT enzymatic activity. The IC_{50} values for MTP were more than tenfold higher than the IC_{50} values obtained for DMG, and the values were different from one enzyme to another (table 2). For the

recombinant human enzyme, IC₅₀ values were around 700 to 800 μM, for the pig liver enzyme values were around 600 μM, and finally for the rat liver enzyme values were around 400 μM. This variation in IC₅₀ values between enzymes was not expected, but the increase in IC₅₀ compared to DMG and MTA was expected and can be justified. An explanation of this result may be due to slight variations in their molecular structure around their active sites.

Table 2. Apparent half maximal inhibitory concentration (IC₅₀) values for human, rat, and Pig BHMT enzymes with the different demethylated products (DMG, MTA, MTP)

IC ₅₀ values, μM*			
	DMG	MTA	MTP
Human	30 ± 2 ^a	89 ± 8 ^b	436 ± 14 ^c
Rat	33 ± 2 ^a	97 ± 3 ^b	586 ± 26 ^d
Pig	30 ± 2 ^a	95 ± 18 ^b	792 ± 26 ^e

Values are means ±SEM. Means were compared by two-way analysis of variance, and different superscript indicates significant difference (p<0.05)

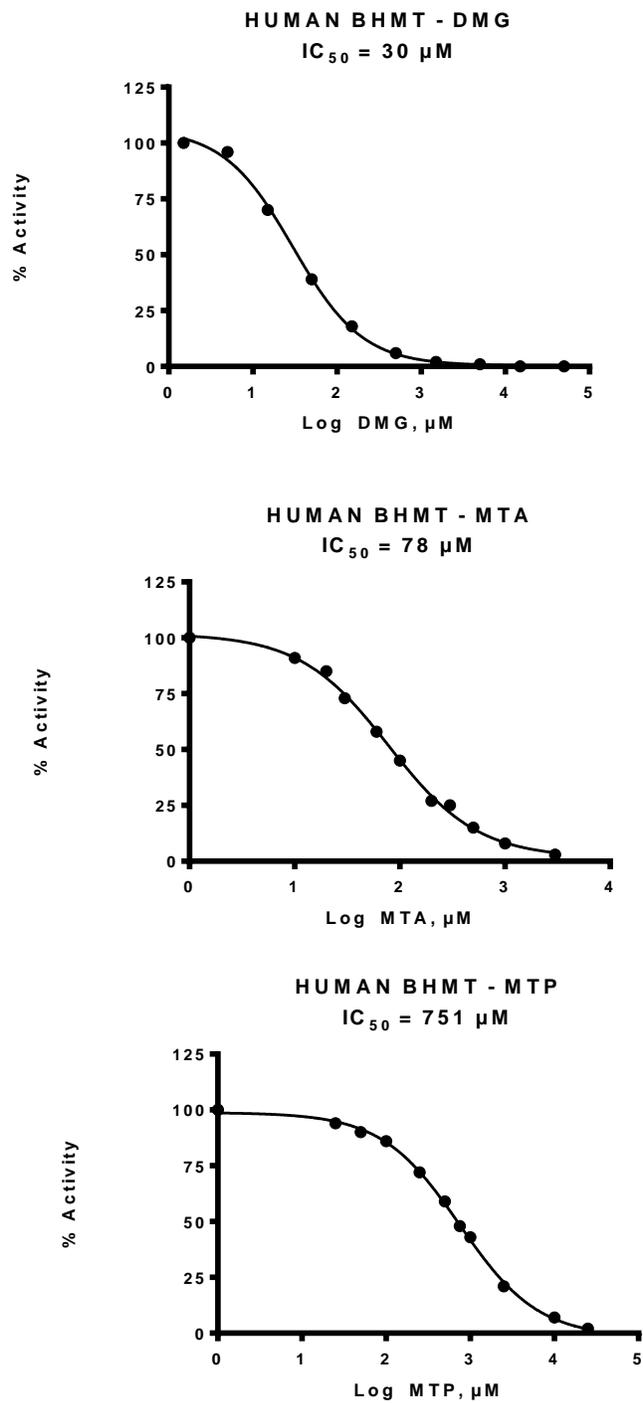


Figure 2. 3 Apparent half maximal inhibitory concentration (IC_{50}) curves for the human BHMT enzyme with DMG, MTA, and MTP

DISCUSSION

Under normal conditions Hcy is either remethylated to produce Met or degraded through the transsulfuration pathway. However, there are genetic conditions that impede the remethylation or degradation of Hcy and result in its accumulation. It has been observed that increased tHcy concentrations in humans correlates with increased risk of coronary, cerebral and peripheral vascular disease³. Since BHMT can use betaine to metabolize Hcy to Met, a high betaine diet has been tried as treatment for homocystinuric people. There are studies that have shown that supplemental betaine can lower but not normalize tHcy in homocystinurics. We hypothesize it is the accumulation of DMG that limits the effectiveness of betaine treatment. Therefore, we want to investigate whether alternative methyl donors, which result in different demethylated products, might be more effective than betaine for the treatment of homocystinuria. We developed an assay to evaluate the inhibitory properties of the three different demethylated products; DMG, MTA, and MTP for the BHMT reaction. DMG has been already reported as a potent inhibitor for the BHMT reaction^{26, 28}. Long term treatment with betaine promotes accumulation of DMG. This accumulation further inhibits the BHMT reaction by binding to the binary complex BHMT-Hcy. When DMG binds to the BHMT-Hcy complex instead of betaine, an abortive ternary complex forms (BHMT-Hcy-DMG), which impedes the BHMT reaction to methylate Hcy. In a study done by Garrow (1996) using an assay that used saturating levels of Hcy and sub-saturating levels of betaine, 50 μM of DMG inhibited about 70% of the BHMT activity³⁵. Reported here, only a small concentration (28 -35 μM) of DMG inhibited BHMT activity

by 50%. The proposed hypothesis that DMG accumulates to further prevent the lowering of Hcy by inhibiting the reaction could be possible according to these data. It is known that the absorption, distribution, and metabolism of betaine into DMG is very fast in healthy people³⁶. When betaine is supplemented in the diet of homocystinurics, DMG quickly accumulates and probably inhibits BHMT and therefore limits the effectiveness of betaine treatment. Why DMG accumulates is not known, but it is probably due to the inability of DMG dehydrogenase and sarcosine dehydrogenase to metabolize it to glycine fast enough to prevent its accumulation.

IC₅₀ values for human, pig and rat BHMT enzymes for MTA were between 60 and 95 μ M. MTA has a relatively low IC₅₀ value and therefore could also inhibit the BHMT reaction if it accumulates, but perhaps in a less potent way than DMG. In a study done by Garrow (1996) using an assay that used saturating levels of Hcy and sub-saturating levels of betaine, 50 μ M of MTA inhibited about 59% of the BHMT activity³⁵, which is less than that reported for DMG and consistent with the results reported here. Comparing the chemical structures of DMG and MTA, DMG has a quaternary ammonium group whereas MTA has a methyl sulfonium group with the same tetrahedral geometry. Therefore, it was expected that the IC₅₀ values for these compounds could be similar. However, since the IC₅₀ of MTA was about threefold higher than DMG, it would be expected to inhibit the BHMT reaction to a lower extent. It is known that DMG could be degraded to sarcosine and glycine by the enzymes DMG dehydrogenase and sarcosine dehydrogenase, respectively, but very little is known about MTA degradation in the body. If MTA could degrade as soon as it is consumed it might not accumulate and compete with betaine for the BHMT-Hcy binary complex.

Besides the conversion of DMSA to MTA, there is not much known about the fate of MTA in animal systems.

After injecting 1.5 mmoles of different solutions of betaine analogs into rats, including DMSA, Slow et al. (2004) found that within 24 h $7.7\% \pm 0.7 \mu\text{M}$ of DMSA were excreted in the urine³⁷. They also found a sulfoxide of MTA, which implied that there was a further metabolism of MTA³⁷. The amount of sulfoxide detected was $138.4 \pm 15.8 \mu\text{M}$ between 0 and 8 h and $33 \pm 3.2 \mu\text{M}$ between 8 and 24 h following the treatment³⁷. This total of excretion is less than 1% of the total of DMSA that was administered to the rats. The small detection of DMSA byproducts and even DMSA in urine excretion makes it challenging and unclear regarding the degradation of this compound. More studies are needed characterized the degradation and toxicity of MTA and any derived metabolites.

In contrast to MTA and DMG, the IC_{50} values of MTP were much higher than DMG and MTA for all three enzymes. Having an additional methylene group might interfere with the capacity of MTP to inhibit the reaction, and compete with betaine for the BHMT-Hcy binary complex. Due to that structural difference, it was anticipated that the IC_{50} values for MTP might be higher than the previous compounds but would not be different from one enzyme to another. Mammalian BHMT enzymes share about 95% amino acid identity and so the variation observed between enzymes were unexpected. MTP should have reacted the same way for all three enzymes because of this particular characteristic, and no significant difference should exist between them in terms of IC_{50} values. In terms of overall number when IC_{50} values are compared for DMG, MTA and MTP, the values for MTP are high compared to the low values obtained for DMG and

MTA. Several studies had reported that DMSP is a substrate for the BHMT reaction. Collinsova et al (2006) showed that DMSP is an effective post-Met load tHcy-lowering agent, and it even decreased the levels of tHcy in mice to a greater extent than betaine³⁸. The catalytic constants of DMSP for BHMT has never been reported, and so it is not known whether the improved tHcy-lowering effect of DMSP was in part due to it being a better substrate, or whether it is entirely due to MTP being a weaker BHMT inhibitor.

In the same study cited previously with MTA, Slow et al. were not able to detect the presence of MTP or its sulfoxide in the urine of rats after injecting DMSP, but approximately two-thirds of the DMSP was excreted in the urine³⁷. Previous studies showed DMSP has good property as a substrate for the BHMT enzymatic reaction and lowering tHcy *in vivo*³⁸, but very little is known about whether its demethylated product MTP is metabolized further *in vivo* or if it is the end product. To some extent it does metabolize but how significant it is needs to be evaluated, and determined if there is no risk of toxicity. Chicks and rats fed with supplemental MTP demonstrated growth depression and splenic hemosiderosis, and they reported levels of toxicity similar to Met toxicity^{39, 40}. There is some evidence that supports the existence of an alternative pathway for Met catabolism called transamination, where MTP serves as an intermediate. During this reaction Met is transaminated to 4-methylthio-2-oxobutyrates, followed by oxidative decarboxylation to 3-methylthiopropionate, and *in vitro* this reaction appears to account for 80% of the Met oxidation when there is high dietary Met. Blom et al showed that patients with hepatic methionine deficiency degraded at least 20% of their dietary Met via the transamination pathway, while in normal subjects

transamination of Met did exist but was quantitatively not important in Met catabolism, not even after Met loading ⁴¹. In contrast, some authors proposed that transamination might be significant in tissues which, unlike liver, do not contain the Met cycle³⁰ and are relevant only at extremely high levels of Met and its metabolites. A better understanding of this pathway and what tissues perform it would help in the understanding of MTP metabolism. As well as for MTA, more understanding is needed for MTP metabolism and the fate of its by-products in the body.

Summary

DMG display greater affinity for the BHMT-Hcy binary complex follows by MTA, and then MTP. The IC_{50} values for DMG and MTA are lower than 100 μ M. MTP was less effective, and a much higher concentration was needed to inhibit the reaction. If DMG accumulation is the main reason for the ineffectiveness of betaine supplementation in lowering tHcy in homocystinurics, these data suggest supplementing with DMSA, and the subsequent production of MTA could behave the same as the betaine-DMG pair. Considering the fact that MTP has less affinity for the BHMT-Hcy binary complex, we can predict that DMSP might be a better alternative. But, further studies are needed to characterize the toxicity of the sulfonium analogs of betaine and how they are degraded in the body. Also, we need to know how well DMSA and DMSP can be used by BHMT, i.e., their catalytic efficiency, before considering their use as alternatives for homocystinuric people. The following chapter of this research characterizes the kinetic properties of betaine and the sulfonium analogs of betaine (DMSA and DMSP) using purified human enzyme.

CHAPTER 3

KINETIC CHARACTERIZATION OF BETAINE AND SULFONIUM ANALOGS OF BETAINE

Introduction

Trimethylglycine, also known as betaine, is found in microorganisms, animals, and plants. In most organisms it is biosynthesized by the oxidation of choline. Interest in betaine metabolism has increased because elevated levels of plasma total homocysteine (tHcy) in blood is associated with certain vascular diseases. Therapies focused on reducing tHcy due to cystathionine β -synthase (CBS) deficiency include high levels of its cofactor pyridoxal phosphate. But approximately 40% of patients with CBS are unresponsive to pyridoxine therapy. For this reason, folic acid and betaine have been used to stimulate the remethylation of homocysteine (Hcy) to methionine (Met) by 5-methyltetrahydrofolate methyltransferase and betaine-homocysteine methyltransferase (BHMT), respectively. Betaine stimulation of BHMT is only partially effective for several reasons such as: DMG accumulation, which further inhibits the BHMT reaction, or Met accumulation, leading to hypermethioninemia.

Searching for other effective alternative treatments that can lower tHcy to a normal range is key. *In vitro* studies have shown that sulfonium analogs of betaine can be used as methyl donor substrates for the BHMT reaction, but it is not known if they would be effective treatments for human homocystinuria. Previous studies showed that sulfonium analogs of betaine, such as dimethylsulfonioacetate (DMSA)³⁵ and

dimethylsulfoniopropionate (DMSP), could be used by the BHMT reaction. Now, we want to evaluate the possibility that they could serve as treatment with homocystinuric patients if they can be shown to be better than betaine as methyl donor substrates for the BHMT enzyme. Our objective is to determine the Michaelis constants (K_m) for betaine and the different sulfonium methyl donors for the BHMT reaction. First, we want to determine the K_m of betaine because there is a discrepancy around its K_m in the literature. A broad range of values have been reported despite the fact that the mammalian BHMT enzymes share greater than 95% amino acid identity. It is possible that this discrepancy is due to experimental error, most likely to an inappropriate over consumption of betaine when assayed at low substrate concentrations (non-initial rate conditions). As depicted in figure 3, the lines represent experimentally determined initial rate velocity rates versus substrate concentration. Because enzyme-catalyzed reactions are saturable, their rates of reaction do not give a linear response to increasing of substrate. The most common error in the determination of K_m values is an over consumption of substrate when tested at very low levels resulting in non-initial rate conditions. Our hypothesis is that some values that have been reported in the literature are low because of this unintentional error (Fig 3. 1; blue curve represents the over consumption of substrate, and the green curve a normal curve at initial rate).

Also, it would be to know how well the enzyme will use the other substrates, DMSA and DMSP. The inhibitory properties of the demethylated products of these sulfonium compounds, MTA and MTP, respectively, are now known. So far, MTP had the highest IC_{50} value, indicating it is not a potent inhibitor. However having a higher IC_{50} values is not the best indicator that the substrate will react with the BHMT reaction

as well as betaine. We want to define the maximal catalytic activity (turn over, K_{cat}) of these methyl donors so we can determine their catalytic efficiencies (K_{cat}/K_m). Due to time limitations and technical difficulties, this research only reported data for the initial velocity rate for betaine using purified human BHMT, rat and pig liver BHMT.

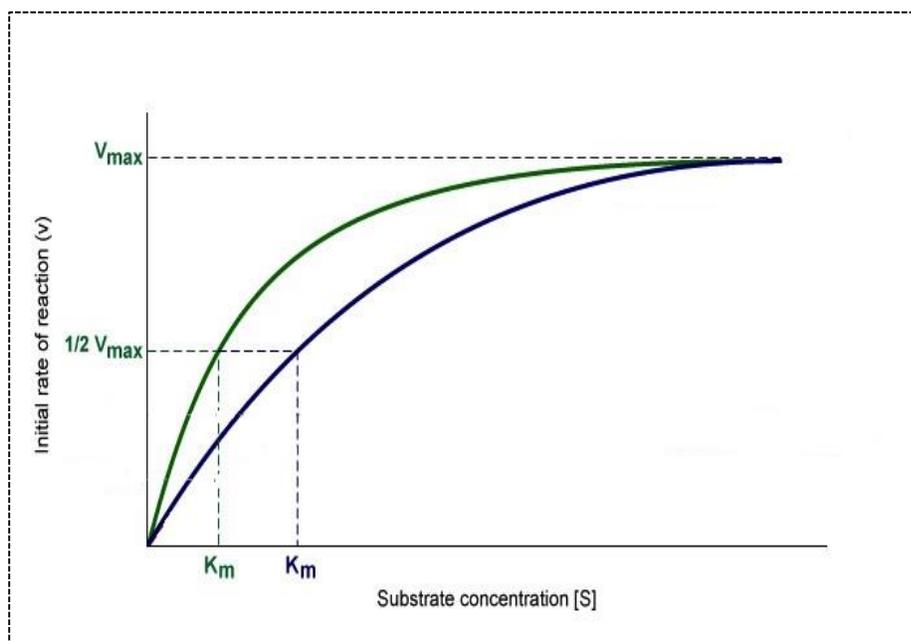


Figure 3. 1 Enzyme initial rate kinetic mechanism

Materials and Methods

Reagents

D, L-homocysteine thiolactone, Dowex 1x4, dimethyl-glycine (DMG) and β -mercaptoethanol were obtained from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) were obtained from Thermo Fisher Scientific (Pittsburgh, PA). Rat liver was obtained from Innovative Research Inc. (Novi, MI). Pig liver was a gift from Dr. L. Rund, Department of Animal Sciences, University of Illinois.

Initial Rate Kinetics of methyl donors

The BHMT standard assay was performed as described in chapter 2 with some modifications. Kinetic assays for all three enzymes (purified human, pig and rat liver) were conducted using the varying levels of betaine. Each assay contained [^{14}C -methyl]-betaine (0.5 μCi) and total betaine was varied from 0.25 to 20 mM. All the experimental data were obtained at fixed Hcy concentration (saturate levels, 100mM) and varying concentrations of betaine. To determine the kinetic constants, the production of nmol of Met/h or the reaction velocity was calculated. All experiments were repeated at least three times and in duplicate for each betaine concentration. The K_m 's were estimated by plotting initial rate data according to the method of Hanes (1932) using a simple linear regression ($r^2 > 0.97$). To compare the results GraphPad Prism 6.04 was also used. The ratio of the initial substrate concentration [S] to the reaction velocity is plotted against [S].

Data Analysis

The data obtained were analyzed using one way analysis of variance to compare experimental to control values. Data are expressed as the mean \pm standard deviation. Comparisons between groups were performed using least significant difference (LSD) test. Differences were considered significant at $p < 0.05$. Results with different letter indicates significant difference (Fig 3.2).

Results

The results of these experiments are showed in figures 3.2 and 3.3. There was no significant difference between the two methods but there was significant difference between the enzymes. Michaelis constants for each BHMT enzyme was 1.83 ± 0.5 mM, 0.71 ± 0.06 mM, and 0.54 ± 0.06 mM for human, pig and rat, respectively. The K_m for the rat liver and pig liver BHMT enzyme showed no significant difference between each other but both were significantly different from the human enzyme. These values obtained are in the range that was expected compared to the reported values in the literature.

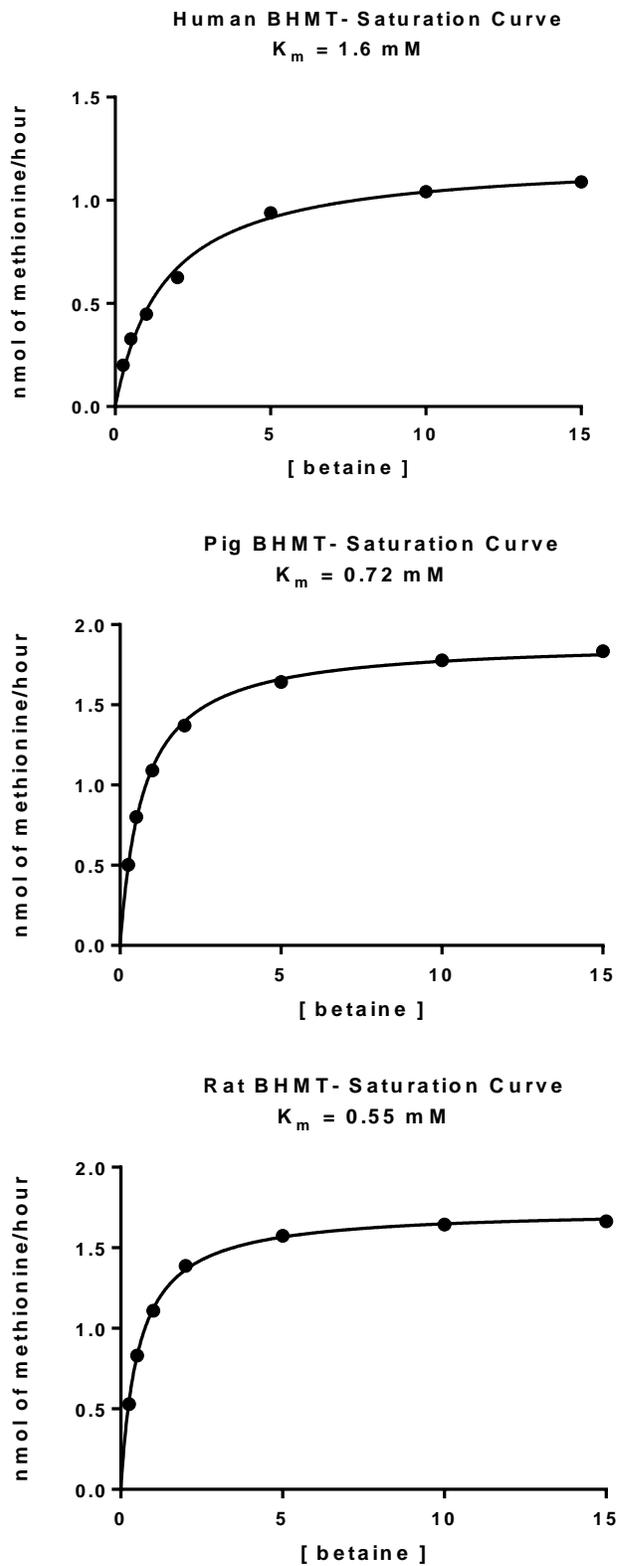


Figure 3.2 Kinetic rates for purified human BHMT, pig and rat liver BHMT

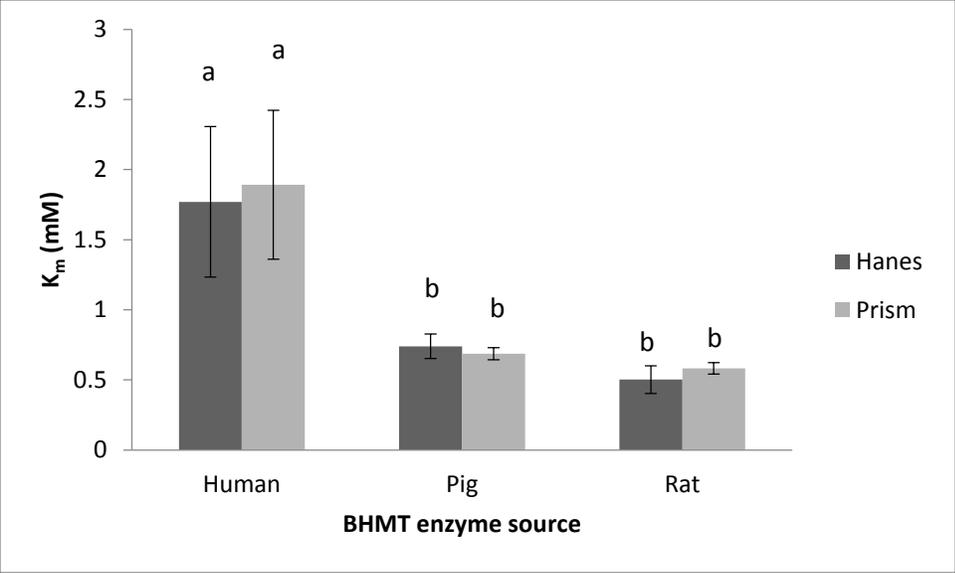


Figure 3.3 K_m^{betaine} Hanes Plot vs Graph Pad Prism

Discussion

It is important when doing enzyme kinetic measurements to do so at initial rate conditions. This means, ideally, that the consumption of substrate must be kept to a minimum so there is no accumulation of products and the substrate concentrations do not significantly change over the assay period. In this assay, the substrate (betaine) concentration was as low as 250 μM and at this low substrate concentration about 10% of the substrate was converted to product, and much less at the higher substrate concentrations tested. As betaine concentrations increased, Met production increased until saturation was reached. The data reported here indicate that the variation in the K_m for betaine toward various BHMT enzymes is not nearly as great as reported in the literature.

The inability of betaine treatment to normalize tHcy in homocystinurics has led to the search for alternative methyl donors that might be better substrates for BHMT, as determined by high *k_{cat}* values, and whose demethylated products might be weaker inhibitors of the enzyme. A primary objective of this study was to characterize two sulfonium analogs of betaine, DMSA and DMSP, to see if they have higher turnover rates compared to betaine, but this objective was not met due to technical difficulties and time allotted to this project. These studies will be continued by other personnel.

Summary

This work demonstrated that the K_m of betaine for the human, rat and pig BHMT enzymes are similar and range between 0.5 to 1.8 mM. The differences in K_m values between these enzymes are relatively small compared to the values previously reported in the literature, and support the hypothesis that some experimental values were in error most likely due to the overconsumption of betaine at low substrate concentrations. A primary objective of this research was to characterize the kinetic properties of DMSA and DMSP as a first step to determine if they might be useful alternatives to betaine treatment for the treatment of homocystinuria, but due to time limitation and technical difficulties, it was only possible to determine the K_m for betaine. Future studies will complete these unmet objectives.

REFERENCES

1. Fryar, C. D., Chen, T. C., & Li, X. (2012). Prevalence of uncontrolled risk factors for cardiovascular disease: United States, 1999-2010. US Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Health Statistics.
2. Allen, R. H., Stabler, S. P., & Lindenbaum, J. (1993). Serum betaine, N, N-dimethylglycine and N-methylglycine levels in patients with cobalamin and folate deficiency and related inborn errors of metabolism. *Metabolism*, 42(11), 1448-1460.
3. Boushey, C. J., Beresford, S. A., Omenn, G. S., & Motulsky, A. G. (1995). A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: probable benefits of increasing folic acid intakes. *Jama*, 274(13), 1049-1057.
4. Millian, N.S. 2000. Recombinant human BHMT discovery as a zinc metallo-enzyme and regulation of activity by redox status (Doctoral dissertation). Retrieved from University of Illinois library.
5. Malinow, M. R., Bostom, A. G., & Krauss, R. M. (1999). Homocyst (e) ine, diet, and cardiovascular diseases a statement for healthcare professionals from the Nutrition Committee, American Heart Association. *Circulation*, 99(1), 178-182.
6. Kang, S. S., Wong, P. W., & Malinow, M. R. (1992). Hyperhomocyst (e) inemia as a risk factor for occlusive vascular disease. *Annual review of nutrition*, 12(1), 279-298.
7. Guttormsen, A. B., Ueland, P. M., Svarstad, E., & Refsum, H. (1997). Kinetic basis of hyperhomocysteinemia in patients with chronic renal failure. *Kidney international*, 52(2), 495-502.

8. McCully, K. S., & Wilson, R. B. (1975). Homocysteine theory of arteriosclerosis. *Atherosclerosis*, 22(2), 215-227.
9. Finkelstein, J. D., & Martin, J. J. (2000). Homocysteine. *The international journal of biochemistry & cell biology*, 32(4), 385-389.
10. Maclean, K. N., Jiang, H., Greiner, L. S., Allen, R. H., & Stabler, S. P. (2012). Long-term betaine therapy in a murine model of cystathionine beta-synthase deficient homocystinuria: decreased efficacy over time reveals a significant threshold effect between elevated homocysteine and thrombotic risk. *Molecular genetics and metabolism*, 105(3), 395-403.
11. Townsend, D. M., Tew, K. D., & Tapiero, H. (2004). Sulfur containing amino acids and human disease. *Biomedicine & pharmacotherapy*, 58(1), 47-55.
12. Book Medicine haha
13. Reo, N. V., Adinehzadeh, M., & Foy, B. D. (2002). Kinetic analyses of liver phosphatidylcholine and phosphatidylethanolamine biosynthesis using ¹³C NMR spectroscopy. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1580(2), 171-188.
14. Garrow. T.A. (2010). Choline. Rucker, R. B., Zemleni, J., Suttie, J. W., & McCormick, D. B. (Eds.). *Handbook of vitamins* (pp. 450-487} CRC Press.
15. Chiuve, S. E., Giovannucci, E. L., Hankinson, S. E., Zeisel, S. H., Dougherty, L. W., Willett, W. C., & Rimm, E. B. (2007). The association between betaine and choline intakes and the plasma concentrations of homocysteine in women. *The American journal of clinical nutrition*, 86(4), 1073-1081.

16. Kotb, M., Mudd, S. H., Mato, J. M., Geller, A. M., Kredich, N. M., Chou, J. Y., & Cantoni, G. L. (1997). Consensus nomenclature for the mammalian methionine adenosyltransferase genes and gene products. *Trends in Genetics*, 13(2), 51-52.
17. Liau, M. C., Chang, C. F., & Becker, F. F. (1979a). Alteration of S-adenosylmethionine synthetases during chemical hepatocarcinogenesis and in resulting carcinomas. *Cancer research*, 39(6 Part 1), 2113-2119.
18. Liau, M. C., Chang, C. F., Belanger, L., & Grenier, A. (1979b). Correlation of isozyme patterns of S-adenosylmethionine synthetase with fetal stages and pathological states of the liver. *Cancer research*, 39(1), 162-169.
19. Sullivan, D. M., & Hoffman, J. L. (1983). Fractionation and kinetic properties of rat liver and kidney methionine adenosyltransferase isozymes. *Biochemistry*, 22(7), 1636-1641.
20. Kotb, M., & Geller, A. M. (1993). Methionine adenosyltransferase: structure and function. *Pharmacology & therapeutics*, 59(2), 125-143.
21. Kutzbach, C., & Stokstad, E. L. R. (1967). Feedback inhibition of methylene-tetrahydrofolate reductase in rat liver by S-adenosylmethionine. *Biochimica et Biophysica Acta (BBA)-Enzymology*, 139(1), 217-220.
22. Smith, A. E., & Matthews, R. G. (2000). Protonation state of methyltetrahydrofolate in a binary complex with cobalamin-dependent methionine synthase. *Biochemistry*, 39(45), 13880-13890.

23. Kraus, J., Packman, S., Fowler, B., & Rosenberg, L. E. (1978). Purification and properties of cystathionine beta-synthase from human liver. Evidence for identical subunits. *Journal of Biological Chemistry*, 253(18), 6523-6528.
24. Kashiwamata, S., & Greenberg, D. M. (1970). Studies on cystathionine synthase of rat liver properties of the highly purified enzyme. *Biochimica et Biophysica Acta (BBA)-Enzymology*, 212(3), 488-500.
25. Jencks, D. A., & Mathews, R. G. (1987). Allosteric inhibition of methylenetetrahydrofolate reductase by adenosylmethionine. Effects of adenosylmethionine and NADPH on the equilibrium between active and inactive forms of the enzyme and on the kinetics of approach to equilibrium. *Journal of Biological Chemistry*, 262(6), 2485-2493.
26. Finkelstein, J. D., Harris, B. J., & Kyle, W. E. (1972). Methionine metabolism in mammals: kinetic study of betaine-homocysteine methyltransferase. *Archives of Biochemistry and Biophysics*, 153(1), 320-324.
27. Park, E. I., & Garrow, T. A. (1999). Interaction between dietary methionine and methyl donor intake on rat liver betaine-homocysteine methyltransferase gene expression and organization of the human gene. *Journal of Biological Chemistry*, 274(12), 7816-7824.
28. Atkinson, W., Slow, S., Elmslie, J., Lever, M., Chambers, S. T., & George, P. M. (2009). Dietary and supplementary betaine: effects on betaine and homocysteine concentrations in males. *Nutrition, Metabolism and Cardiovascular Diseases*, 19(11), 767-773.

29. Mudd, S. H., Skovby, F., Levy, H. L., Pettigrew, K. D., Wilcken, B., Pyeritz, R. E., & Schweitzer, L. (1985). The natural history of homocystinuria due to cystathionine β -synthase deficiency. *American journal of human genetics*, 37(1), 1.
30. Uchida, A., Ooguri, T., Ishida, T., Kitaguchi, H., & Ishida, Y. (1996). Biosynthesis of dimethylsulfoniopropionate in *Cryptothecodinium cohnii* (Dinophyceae). In *Biological and Environmental Chemistry of DMSP and Related Sulfonium Compounds* (pp. 97-107). Springer US.
31. Marc J. E. C. van der Maarel, Peter Quist, Lubbert Dijkhuizen, Theo A. Hansen
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32. Finkelstein, J. D., & Mudd, S. H. (1967). Trans-sulfuration in Mammals The Methionine-Sparing Effect of Cystine. *Journal of Biological Chemistry*, 242(5), 873-880.
33. Duerre, J. A., & Miller, C. H. (1966). Preparation of L-homocysteine from L-homocysteine thiolactone. *Analytical biochemistry*, 17(2), 310-315.
34. Sebaugh, J. L. (2011). Guidelines for accurate EC50/IC50 estimation. *Pharmaceutical statistics*, 10(2), 128-134.
35. Garrow, T. A. (1996). Purification, kinetic properties, and cDNA cloning of mammalian betaine-homocysteine methyltransferase. *Journal of Biological Chemistry*, 271(37), 22831-22838.
36. Schwahn, B. C., Hafner, D., Hohlfeld, T., Balkenhol, N., Laryea, M. D., & Wendel, U. (2003). Pharmacokinetics of oral betaine in healthy subjects and patients with homocystinuria. *British journal of clinical pharmacology*, 55(1), 6-13.

37. Slow, S., Lever, M., Lee, M. B., George, P. M., & Chambers, S. T. (2004). Betaine analogues alter homocysteine metabolism in rats. *The international journal of biochemistry & cell biology*, 36(5), 870-880.
38. Collinsova, M., Strakova, J., Jiracek, J., & Garrow, T. A. (2006). Inhibition of betaine-homocysteine S-methyltransferase causes hyperhomocysteinemia in mice. *The Journal of nutrition*, 136(6), 1493-1497.
39. Steele, R. D., Barber, T. A., Lalich, J., & Benevenga, N. J. (1979). Effects of dietary 3-methylthiopropionate on metabolism, growth and hematopoiesis in the rat. *The Journal of nutrition*, 109(10), 1739-1751.
40. Harter, J. M., & Baker, D. H. (1978). Factors affecting methionine toxicity and its alleviation in the chick. *The Journal of nutrition*, 108(7), 1061-1070.
41. Blom, H. J., Boers, G. H., Van Den Elzen, J. P., Gahl, W. A., & Tangerman, A. (1989). Transamination of methionine in humans. *Clinical science (London, England: 1979)*, 76(1), 43-49.