THE EFFECT OF SUPPLEMENTARY DIETARY SULFUR AMINO ACIDS ON HEPATIC SULFUR AMINO ACID METABOLISM IN RATS

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The Effect of Supplementary Dietary Sulfur Amino Acids on Hepatic Sulfur Amino Acid Metabolism In Rats

By

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Abstract

Dietary cysteine can replace up to 40% of the methionine requirement in the diets of both men and rats. Various experiments were designed to examine this phenomenon, including measurements of transsulfuration flux in isolated hepatocytes, hepatic enzyme and substrate analysis of sulfur amino acid metabolism, and cystathionine β-synthase (CBS) mRNA. In adult rats fed a high cystine diet for one week, transsulfuration flux was decreased by 40% as compared to animals fed a basal diet. This change in flux was mirrored by a similar change in CBS activity and mRNA. In young rats fed sulfur amino acid supplemented diets for one week, transsulfuration flux measured with 0.1 mM methionine fell by over 50% in cystine supplemented animals as compared to basal and methionine supplemented rats. Among the variety of results that followed, CBS activity decreased in rats supplemented with cystine compared to high methionine supplemented rats by 45%, while CBS mRNA decreased by almost 65% in these two groups. We also examined the effect that allosteric regulators S-adenosylmethionine and S-adenosylhomocysteine had on the enzymes of methionine metabolism. Our results indicated that SAM played a crucial role in regulating transsulfuration and remethylation of homocysteine via folate.

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Apo E	Apolipoprotein E	LDH	Lactate Dehydrogenase
ATP	Adenosine Triphosphate	MAT	Methionine Adenosyltransferase
BCAA	Branched Chain Amino Acids	Met	Methionine
BHMT	Betaine:Homocysteine Methyltransferase	mRNA	Messenger Ribonucleic Acid
CBS	Cystathionine β-Synthase	MTHF	Methylenetetrahydrofolate
CHDH	Choline Dehydrogenase	MTHFR	Methylenetetrahydrofolate Reductase
CGL	Cystathionine γ-Lyase	NADH	Nicotinamide Adenine Dinucleotide
CVD	Cardiovascular Disease	P5P	Pyridoxal 5-Phosphate
Cys	Cystine	Pi	Phosphate
DMG	Dimethylglycine	PPi	Pyrophosphate
DNA	Deoxyribonucleic Acid	PPPi	Tripolyphosphate
DTT	Dithriothreitol	SAA	Sulfur Amino Acids
EDTA	Ethylenediaminetetraacetic acid	SAH	S-adenosylhomocysteine
FAD	Flavin Adenine Dinucleotide	SAM	S-adenosylmethionine
GNMT	Glycine N-Methyltransferase	TCA	Trichloroacetic acid
HPLC	High Performance Liquid Chromatography	THF	Tetrahydrofolate
kDa	kilodalton	UV	Ultraviolet
Km	Michaelis-Menton constant		

Chapter 1

1. Introduction to Homocysteine

The interest in homocysteine metabolism and related fields of research has grown almost exponentially in the past several decades (Finkelstein, 2000a). Aside from the interrelationship between methionine, homocysteine, and cysteine, the areas of interest arise from the roles sulfur amino acid metabolism play in folate metabolism, antioxidant effects, and the majority of the methylation reactions.

The metabolism of methionine begins with the transfer of an adenosyl group onto methionine via Methionine Adenosyltransferase (MAT), creating S-Adenosylmethionine (SAM). SAM is known as the body's most biologically available methyl group donor, meaning that it can transfer its methyl group to a variety of methyl group acceptors, creating S-Adenosylhomocysteine (SAH) and adenosine in the process (Fontecave *et al*, 2004). SAH Hydrolase rapidly converts SAH to homocysteine (Stipanuk, 1986). Together, these reactions make up the transmethylation pathway.

The metabolism of sulfur amino acids can branch at this point in the pathway. One branch is known as the remethylation pathway, which, in itself, contains two different reactions. The first reaction involves folate metabolism, as a methyl group from methyltetrahydrofolate is transferred to homocysteine by methionine synthase, recreating methionine. Methyltetrahydrofolate itself is produced by the action of Methylenetetrahydrofolate Reductase (Finkelstein *et al*, 1978). The alternate routing of remethylation arises from the donation of a

methyl group from betaine to homocysteine via Betaine:Homocysteine Methyltransferase, producing dimethylglycine and methionine (Pajares and Perez-Sala, 2006).

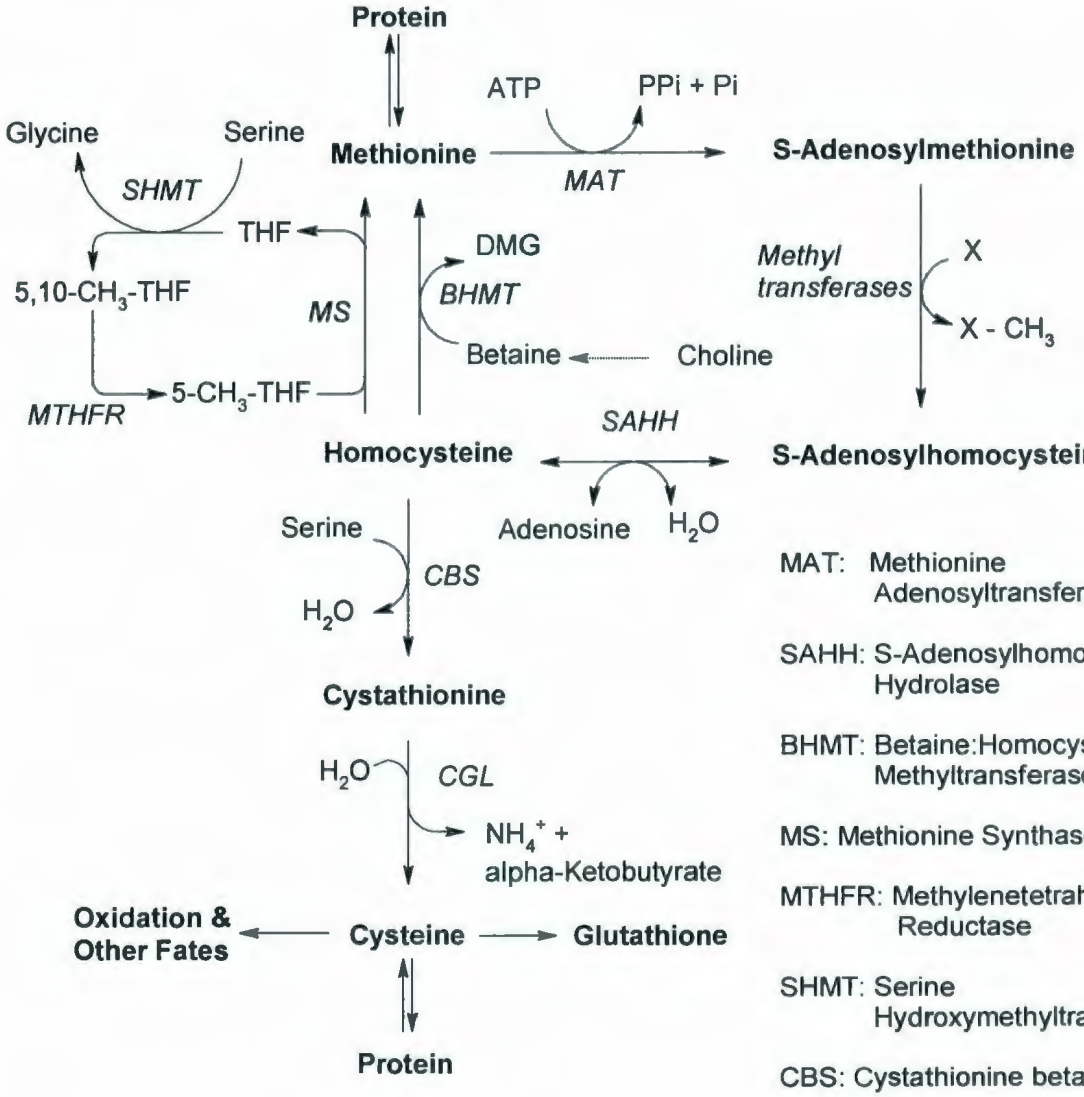
The other branch that homocysteine can traverse is that of the irreversible transsulfuration pathway. Homocysteine is condensed with serine to produce cystathionine through the action of Cystathionine β-Synthase (Finkelstein *et al*, 1974). This cystathionine is then acted upon by Cystathionine γ-Lyase, producing cysteine, which can then proceed into a variety of different fates (Finkelstein *et al*, 1974). The whole of methionine metabolism can be seen in Figure 1.1

1.1 Homocysteine and Health

Another reason for the increase in homocysteine research has been the links discovered between increased plasma homocysteine concentrations and a variety of diseases, such as cardiovascular disease (Humphrey *et al*, 2008), renal disease, and different cognitive disorders.

In a metabolic pathway as complex as homocysteine's, a variety of inborn errors can occur, leading to a build-up of homocysteine with deleterious health effects. The most common of these inborn errors is a deficiency in CBS activity, first reported by Carson (Bittles and Carson, 1981), resulting in excessively increased concentrations of homocysteine and methionine. In addition to the abnormal plasma thiol concentrations, CBS deficient patients also tend to suffer from dislocations of the ocular lens, mental retardation, skeletal deformations,

Figure 1.1: Mammalian hepatic methionine metabolism.



MAT: Methionine Adenosyltransferase

SAHH: S-Adenosylhomocysteine Hydrolase

BHMT: Betaine:Homocysteine Methyltransferase

MS: Methionine Synthase

MTHFR: Methylenetetrahydrofolate Reductase

SHMT: Serine Hydroxymethyltransferase

CBS: Cystathionine beta-Synthase

CGL: Cystathionine gamma-Lyase

THF: Tetrahdrofolate

DMG: Dimethylglycine

and cardiovascular lesions, the latter of which are frequently a cause of death (Mudd *et al*, 1995).

The second most common types of errors in homocysteine metabolism occur in the remethylation pathway. The errors in this pathway can take several forms ranging from a genetic defect in metabolism to defects in folic acid or cobalamin transport. Whatever the defect, the displayed symptoms are essentially the same as the CBS deficiencies, with the exception of low methionine concentrations versus the high methionine concentrations of CBS deficiency.

Whether or not the defect is in transmethylation or in transsulfuration, both errors in homocysteine metabolism result in vascular lesions, leading to the death of the patient. This finding led to the speculation that the cause of the vascular lesions was, in fact, due to high circulating homocysteine concentrations. This speculation was tested by examining two types of CBS deficient patients, those that responded to pyridoxine treatment and those that did not. The patients that responded to pyridoxine treatment had decreased plasma homocysteine concentrations when compared to the non-responders (Mudd *et al*, 1985). In these non-responding patients, there was also a 50% higher risk of a vascular event occurring after thirty years. Multiple other studies have since concluded that high concentrations of homocysteine are linked to an increased risk of vascular disease (Wilcken *et al*, 1997; Yap *et al*, 2000).

With the discovery that very high plasma homocysteine concentrations caused blood vessel lesions in a small population of patients with inborn errors, it

was considered that mild increases in homocysteine concentrations could potentially increase the risk of cardiovascular disease in the population at large. This mild homocysteinemia was defined as a plasma homocysteine concentration of 15 to 25 μM, as compared to accepted normal homocysteine concentration ranges of 5 to 15 μM (Green and Jacobson, 1995).

In 1995, a meta-analysis of 27 studies provided a strong link between mild homocysteinemia and increased risk of CVD (Boushey *et* al, 1995). This meta-analysis of over 4000 subjects concluded that increased homocysteine concentrations were a risk factor for cardiovascular disease, independent of all other known risk factors; in fact, mild homocysteinemia has been classified as having the same risk as diabetes (Sparks *et* al, 2003) and mild increases in plasma cholesterol concentrations (Boushey *et* al, 1995). Further studies have indicated that 20-30% of all cardiovascular disease, cerebrovascular disease, and peripheral vascular disease sufferers have a mild elevation in homocysteine.

The mechanism by which a mild elevation in homocysteine can increase risk for a thrombotic event occurring is still unclear, although many mechanisms have been proposed. These mechanisms have been proposed to include homocysteine-induced oxidative stress (Wilcken *et al*, 2000), reduced endothelial-dependant vasodilation (Tawakol *et al*, 1997), protein homocysteinylation and/or acylation (Jakubowski, 2000), or an inhibition of the synthesis of nitric oxide (Upchurch *et al*, 1997).

Despite these theories, it is possible that any or none of them are true – in fact, the increased homocysteine concentrations could simply be an

epiphenomenon that reflects the real underlying mechanism. One study which used apolipoprotein E (ApoE) deficient mice, which develop spontaneous atherosclerotic lesions, thereby allowing the investigators to examine the effect of nutrition on the progression of lesion formation, suggests that it may not be high homocysteine concentrations that increase lesion formation, but high methionine concentrations. The lesions formed in the high methionine groups, but with normal plasma homocysteine concentrations, were far larger than those lesions found in the high homocysteine, low methionine mice, indicating that the high methionine concentrations accelerated lesion formation (Troen *et al*, 2003). Whether homocysteine concentrations exacerbate this condition, or remain just a marker of lesion development, has yet to be determined.

In addition to any effects that homocysteine concentrations may have on cardiovascular disease, there is also mounting evidence that homocysteinemia may be linked to a decrease in cognitive function and an increase in Alzheimer's disease and dementia. Riggs *et al* (1996) discovered that an increase in total plasma homocysteine concentrations was associated with a decrease in spatial copying abilities. In 1997, Joosten *et al* found that there were increased plasma homocysteine concentrations in Alzheimer's patients compared to both healthy, elderly patients and control hospitalized subjects. Finally, it was also found that there was a positive correlation with depressed elderly patients and plasma homocysteine concentrations (Bell *et al*, 1992). Whether the total plasma homocysteine is a cause of the neurodegenerative damage or simply an indicator

of folate status, which is also important for brain function (Rosenburg and Miller, 1992), remains to be seen.

1.2 The Metabolism of Sulfur Amino Acids

The metabolism of methionine and its related compounds is extremely complex. For simplification, the pathway has been broken into three different sections – the transmethylation pathway, the remethylation pathway, and the transsulfuration pathway.

1.2.1 Transmethylation Pathway

The transmethylation pathway denotes the reactions that produce and use SAM, a major biological methyl group donor. These reactions include those catalyzed by Methionine Adenosyltransferase, a variety of methyltransferases, and SAH Hydrolase.

1.2.1.1 Methionine Adenosyltransferase

The first reaction of the transmethylation pathway is the synthesis of S-Adenosylmethionine (SAM) by Methionine Adenosyltransferase (MAT) from methionine and adenosine triphosphate (ATP). The production of SAM is a two-step process that begins with the synthesis of SAM and tripolyphosphate (PPPi). Both products remain attached to the enzyme until the completion of the second step, which is the hydrolysis of the tripolyphosphate to inorganic phosphate (Pi) and pyrophosphate (PPi) (Mudd; 1963). This second step is the rate-limiting step in the overall reaction (Sanchez del Pino *et al*; 2000).

Two genes are responsible for the coding of MAT: MAT1A and MAT2A (Mato *et al*; 1997). MAT2A encodes for the MAT II isoenzyme, and is present

only in extrahepatic tissues and in fetal liver (Gil *et al*, 1996). MAT II is a tetramer of two catalytic α_2 subunits and two regulatory β subunits (Halim *et* al, 1999) that is inhibited by its product, SAM, and has a low Km for methionine (Finkelstein, 2000b). In fact, at physiological concentrations of methionine, MAT II is saturated with its substrate, which results in a relatively steady concentration of SAM in extrahepatic tissues (Finkelstein, 2000b).

The second gene responsible for MAT synthesis, MAT1A, is present in the adult liver, and produces two isoenzymes, MAT I and MAT III (Kotb, M and Geller, AM, 1993; Torres *et al*, 2000). MAT I has a low Km for methionine in the liver, and is responsible for the basal level of SAM synthesis to support polyamine synthesis, etc (Finkelstein, 1990). This particular isoenzyme is a tetramer of α1 subunits, and is inhibited by SAM (Hoffman, 1983).

The other hepatic isoenzyme, MAT III, is a dimer of α1 subunits (Hoffman, 1983). In contrast to MAT I and II, MAT III has a high Km for methionine, and is activated by its product, SAM (Finkelstein, 2000b). During times of high methionine, such as the ingestion of a high protein meal, this enzyme is responsible for the clearance of this amino acid. In fact, approximately 50% of ingested methionine is catabolized by the MAT I/III combination (Corrales *et al*, 2002). The SAM activation further allows an increase in the activity of MAT III in order to clear the excess methionine. Once the methionine becomes relatively depleted in the liver, the MAT III activity and SAM concentrations will decrease.

1.2.1.2 S-Adenosylmethionine

The product of the MAT reaction, SAM, has been estimated to be the second most common biological reaction co-factor, after ATP (Lu, 2000). The majority of biological methylation reactions (approximately 85%) (Mudd and Poole, 1975), occur in the liver, where most of methionine catabolism occurs. SAM is also chemically inclined to donate its methyl groups, as its high-energy sulfonium ion results in a predisposition of the attached carbons towards nucleophilic attack (Lu, 2000). These methylation reactions result in the creation of S-Adenosylhomocysteine (SAH), which then acts as feedback to inhibit most of the enzymes responsible for methylation (Mato *et al*, 1997).

Apart from the methylation reactions, SAM metabolism is also involved in polyamine synthesis. In this pathway, decarboxylated-SAM is produced via SAM decarboxylase. The aminopropionyl group of the decarboxylated SAM can then be transferred to putrescine to produce spermidine, and to spermidine, to produce spermine (Finkelstein, 1990). A product of these reactions is methylthioadenosine, which can eventually be recycled back to methionine. Polyamine synthesis usually accounts for no more than 5% of methionine catabolism in liver (Mudd and Poole, 1975).

Recently, much work has been done on the clinical utility of SAM as a treatment, especially in diseases of the liver, such as alcoholic cirrhosis. A decrease in MAT activity has been found in cirrhotic liver. Altered DNA methylations may also occur which may also contribute to the etiology of the

disease (Avila *et al,* 2000; Krahenbuhl *et al*, 2000; Lieber *et al*, 1994; Varela-Moreiras *et al*, 1995).

1.2.1.3 Glycine N-Methyltransferase

Despite the fact that there are a large number of methyltransferases that require SAM as a methyl group donor, the enzyme Glycine N-Methyltransferase (GNMT) stands out for the role it plays in the clearance of high methionine loads, as well as its role in the regulation of SAM:SAH ratios. Like other methyltransferases, GNMT catalyzes the transfer of a methyl group from SAM. In this instance, GNMT transfers the methyl group onto glycine, which forms both SAH and sarcosine. The SAH that is produced will continue on through the transmethylation pathway, while sarcosine, which currently has no known physiological role, can be recycled by sarcosine oxidase back into glycine (Cook and Wagner, 1984).

The rat isoform of the GNMT enzyme has a significantly lower Km for both SAM and glycine than its counterpart in other species. For example, in the rat, the Km for SAM and glycine are 0.05 mM and 0.29 mM, respectively; however, for humans, the Km for SAM and glycine are 0.27 mM and 6.3 mM (Ogawa *et al*, 1993).

GNMT activity can theoretically be altered via the binding of 5-methyltetrahydrofolate. The binding of this folate co-factor causes a decrease in the GNMT activity (Wagner *et al*, 1985), possibly due to the 5-methyltetrahydrofolate competing for the SAM binding site (Ogawa *et al*, 1998). The folate binding will also prevent phosphorylation of GNMT, which would

normally double the activity of the enzyme (Wagner *et al*, 1989). It is thought that this binding may prevent unnecessary breakdown of SAM by GNMT in order to preserve methyl groups for other essential transmethylations at times when methionine is low.

1.2.1.4 SAH and SAH Hydrolase

SAH, the product of all SAM methylation reactions, is an inhibitor of the majority of these reactions; therefore, there must be mechanisms by which to eliminate the increasing amounts of SAH in order to ensure that the SAM methylation reactions can continue. Currently, there are two means by which SAH is eliminated from cells.

The first of these means occurs when SAH is transported out of the cells into the blood, where it can be cleared by the kidney (Duerre *et al*, 1969). Secondly, SAH Hydrolase can act upon SAH. SAH is also known to bind to intracellular proteins, thereby decreasing the amount of free SAH (Svardel and Ueland, 1987).

SAH Hydrolase catalyzes the reversible reaction that converts SAH and a water molecule to homocysteine and adenosine. The SAH Hydrolase contains NAD^+. Though this reaction is freely reversible, its equilibrium heavily favours SAH synthesis; however, *in vivo*, both adenosine and homocysteine are rapidly metabolized (de la Haba and Cantoni, 1959), with adenosine being metabolized by adenosine kinase and adenosine deaminase, and homocysteine entering either the transsulfuration pathway or the remethylation pathway, or being exported out of the cell. These factors ensure that SAH is efficiently removed.

Due to the fact that SAH Hydrolase is heavily involved in the reduction of SAH concentrations intracellularly, it has become a favoured target of pharmaceutical treatments. By inhibiting SAH Hydrolase, a patient's intracellular SAM:SAH ratio is lowered, thereby causing an inhibition of methylation reactions, which results in various effects, including antiviral (Bader *et al*, 1978), antiparasitic (Tseng *et al*, 1989), and possibly anti-cancer effects (Chiang, 1981).

1.2.1.5 Homocysteine

Once homocysteine is produced by SAH Hydrolase, it has several possible fates. Firstly, homocysteine can be transported in the plasma. Homocysteine has several forms in plasma, including a free form, a mixed disulfide, and bound to albumin. Secondly, the homocysteine can proceed to cysteine through the transsulfuration reactions, and from cysteine, can lead to other areas of metabolism, such as taurine synthesis, glutathione synthesis, or protein synthesis. Alternatively, homocysteine can be directed towards the remethylation pathway, which recycles homocysteine back to methionine.

1.2.2 Remethylation Pathway

There are two mechanisms by which methionine can be regenerated. The first involves folate metabolism, while the second mechanism uses a methyl group from betaine.

1.2.2.1 Methionine and Folate Metabolism

Methionine synthase catalyzes the transfer of a methyl group from MTHF to homocysteine, resulting in the formation of tetrahydrofolate (THF) and methionine. This large, cytoplasmic protein is present in most cells in the body,

and requires cobalamin as a cofactor (Chen *et al*, 1995). The THF that is formed can then be used by the cells for the synthesis of purines and pyrimidines.

The MTHF necessary for methionine synthase can come from intracellular sources as well as from the blood. The enzyme Methylenetetrahydrofolate Reductase produces the intracellular MTHF from methylenetetrahydrofolate, which can be produced from THF by Serine Hydroxymethyltransferase (SHMT).

MTHFR is an important regulatory enzyme in both folate and homocysteine metabolism. This dimer, which contains identical 77 kDa subunits (Daubner and Matthews, 1982), contains a catalytic region in the N-terminal, which can be activated allosterically through the binding of SAM to a specific regulatory region in the C-terminal. Each subunit is also associated with a non-covalently bound flavin adenonucleotide molecule (FAD), which is necessary for the transfer of a methyl group from methylenetetrahydrofolate (Daubner and Matthews, 1982).

While a large number of mutations in the MTHFR enzyme have been discovered in humans, the Ala222Val mutation is particularly common in Caucasians, with an incidence of approximately 12% of the Quebec population (Frosst *et al*, 1995). This mutation results in decreased activity in fibroblasts and increased susceptibility to heat-sensitive deactivation (Engberson *et al*, 1995). Additionally, this mutation also leads to the dissociation of the FAD compounds associated with MTHFR (Matthews *et al*, 1998). This form of MTHFR has been definitively linked to an increased incidence of neural tube defects (Whitehead *et al*, 1995), and potentially linked to an increased incidence of cardiovascular

disease. Individuals with this particular mutation have a higher dietary folate requirement.

1.2.2.2 Methionine and Choline Metabolism

While the folate-dependent homocysteine remethylation pathway takes place in most cells throughout the body, the liver contains an additional enzyme (Betaine:Homocysteine Methyltransferase), which is able to remethylate homocysteine (Garrow, 1996). This enzyme is also found, in small amounts, in the kidney and the brain of humans (Gaull *et al*, 1973). BHMT catalyzes a methyl transfer from betaine to homocysteine, producing methionine and dimethylglycine; dimethylglycine is a potent inhibitor of BHMT (Finkelstein *et al*, 1972). DMG is further demethylated by DMG dehydrogenase, producing sarcosine, which can be converted into glycine. BHMT is a hexamer of a 45 kDa protein that has been shown to have a Km of 15-20 μM for homocysteine and 50-60 μM for betaine (Stipanuk, 1986).

In addition to its effects in the homocysteine remethylation pathway, BHMT also plays a central role in choline oxidation. Choline, which arises either from the diet or as a consequence of the methylation of phosphatidylethanolamine by SAM, has three fates: phospholipid incorporation (Li and Vance, 2008), acetylcholine synthesis (Amenta and Tayebati, 2008), and oxidation to eliminate excess choline (Barak and Tuma, 1983). The committed step of choline oxidation is the first step in the pathway, whereby choline dehydrogenase (or choline oxidase) coverts choline to betaine aldehyde, which is converted to betaine by betaine aldehyde dehydrogenase.

1.2.3 Transsulfuration Pathway

1.2.3.1 Cystathionine β-Synthase

The alternative pathway for homocysteine metabolism is through the transsulfuration pathway, which irreversibly removes the sulfur from the methionine cycle. The first step in the transsulfuration pathway is catalyzed by cystathionine β-synthase (CBS), which condenses the homocysteine with serine to produce cystathionine. *In vivo*, this reaction is irreversible, which, therefore, explains why cysteine is considered a conditionally essential amino acid, as cysteine may be produced from methionine if sufficient methionine is present.

In fresh liver extracts, CBS has been shown to exist in a tetrameric form, with each subunit having a molecular weight of roughly 63 kDa (Taoka *et al*, 1998). This enzyme has a Km of 1.5-5.0 mM for homocysteine (Kery *et al*, 1998) and Km of 2.0-3.0 mM for serine (Taoka *et al*, 1999). Methionine synthase, however, has a Km of approximately 60 μM for homocysteine (Burke, Mangum, and Brodie, 1971), which is significantly lower than the CBS Km. Due to this difference in Km's, it would seem that, *in vivo*, remethylation to methionine is favoured over transsulfuration to cysteine. In fact, CBS would only be highly active in times of excess homocysteine concentrations, thereby making it an essential mechanism for removing excess homocysteine.

Two necessary co-factors for CBS activity are pyridoxal 5-phosphate (P5P) and a heme group. P5P binds serine, thereby increasing its reactivity, and allowing for easier transfer of the sulfur group (Mudd *et al*, 1970). The heme

group interacts with the homocysteine thiol group, which will potentially allow it to act with the serine bound to P5P (Taoka *et al*, 1998).

In addition to the tetrameric form of CBS, a dimer of this enzyme, consisting of two 48 kDa subunits, was originally discovered in liver samples that had been frozen for several weeks (Skovby *et al*, 1984). Initially, it was believed that this form of the CBS enzyme did not exist *in vivo*, and was simply a result of limited proteolysis in the frozen sample. Recently, however, evidence has been brought forward demonstrating that the dimer may be present *in vivo* under oxidizing conditions. The subunits of this dimeric form of CBS have been cleaved so that they have lost their C termini, which are responsible for the allosteric regulation of CBS by SAM (Zou and Banerjee, 2003). This cleaved CBS, therefore, no longer responds to SAM, thereby decreasing the activity of the tetrameric CBS by about 70% (Shan and Kruger, 1998).

A mutation in CBS has been found to be the cause of the most common form of homocysteinuria (Mudd and Levy, 1969). Almost 50% of these mutations are a direct result of a mutation in CBS that prevents any interaction with P5P; in such patients, supplementation with vitamin B6 is effective in lowering high plasma homocysteine levels (Mudd, 1989).

1.2.3.2 Cystathionine γ-Lyase

The second transsulfuration enzyme is cystathionine γ-lyase (CGL), which cleaves cystathionine into cysteine and α-ketobutyrate (Braunstein and Goryachenkova, 1984). CGL also requires P5P as a co-factor for activity, and has a Km for cystathionine of 3.5 mM (Kato *et al*, 1966).

CGL is absent from certain tissues that contain the CBS enzyme, such as, the rat brain and rat adipose tissue (Mudd *et al*, 1965). The function of CBS in these tissues remains unclear, although a role in H_2S production has been proposed.

The cysteine produced by transsulfuration can have several fates. A portion will be directed towards protein synthesis, while up to 70% of the excess cysteine may be used to make glutathione (Rao *et al*, 1990). Glutathione is a key agent in the prevention of oxidative damage in the body; in fact, it is the main anti-oxidant in cells. Therefore, the increase in transsulfuration activity under oxidative conditions to produce cysteine most likely occurs to create glutathione, which would combat the oxidative stress. The majority of the remaining cysteine will be metabolized to sulfates by the cysteinesulfonate pathway. This pathway can lead to one of two different directions; either the cysteinesulfonate can be broken into sulfate and pyruvate, which can then be fully oxidized in the Krebs' cycle, or cysteinesulfonate will be converted to taurine via a decarboxylation reaction.

1.3 Regulation of Homocysteine Metabolism

1.3.1 Enzyme Kinetics

A variety of mechanisms regulate homocysteine and methionine metabolism. The first and most basic of these mechanisms focuses on the enzyme kinetics of the methionine pathways. From the Km's of the enzymes involved, it is apparent that there are two classes of enzymes in respect to methionine metabolism – those that strive to conserve methionine, and those that

strive to catabolize methionine. Enzymes in each of these groups share common characteristics that allow them to be categorized.

The methionine conservation enzymes all have low Km's for their specific sulfur-containing substrates. These enzymes include MAT I and II, SAH Hydrolase, Methionine Synthase, MTHFR, and BHMT. On the other hand, the methionine catabolizing enzymes have a high Km for sulfur-containing substrates, and include MAT III, CBS, CGL, and GNMT.

The different Km's provide a simple regulatory mechanism. At times when methionine concentrations are low within a cell, the methionine conservation enzymes will function. The transmethylation pathway will synthesize only enough SAM for necessary transmethylation reactions, while homocysteine will be directed towards remethylation and methionine synthesis, not towards transsulfuration. At times when intracellular methionine concentrations are high, the methionine catabolizing enzymes will function to ensure that methionine is efficiently metabolized. The transmethylation pathway will synthesize SAM in excess of what is needed for essential transmethylation reactions; much of this excess SAM is used by GNMT. The excess homocysteine that is produced will be directed towards transsulfuration, thereby irreversibly removing these sulfur-containing compounds from methionine metabolism.

1.3.2 Allosteric Regulation

SAM is considered to be the allosteric regulator with the greatest impact on methionine metabolism. Currently, it is known that high levels of SAM will inhibit MAT I/II (Finkelstein, 1990), BHMT (Finkelstein and Martin, 1984), and

MTHFR (Jencks and Matthews, 1987), while activating MAT III (Finkelstein, 1990) and CBS (Finkelstein *et al*, 1975). This interplay of SAM and the various enzymes of methionine metabolism has led to the theory of the "SAM switch" – high levels of SAM will lead to the suppression of the remethylation pathway, and a net flux of sulfur into the transsulfuration pathway. A figure representing SAM allosteric regulation in methionine metabolism can be seen in Figure 1.2.

Additionally, it has been shown that SAH also has some allosteric effects on the methionine metabolic pathway. SAH inhibits the majority of the transmethylases; however, SAH has also been reported to inhibit BHMT while activating CBS (Finkelstein, Kyle and Harris, 1974), thereby leading to a net flux away from remethylation of homocysteine, and, like SAM, an influx of sulfur will enter the transsulfuration pathway. Additionally, it is known that MTHF inhibits GNMT in an allosteric manner (Wagner *et al*, 1985).

1.3.3 Redox Regulation

One of the major results of the transsulfuration pathway is the production of glutathione from cysteine. As previously mentioned, glutathione is the major intracellular protective agent against oxidative damage; therefore, it is appropriate to note that flux through the transsulfuration pathway will increase under oxidative stress.

The activity of CBS will decrease when the reaction occurs in the presence of a reducing agent; however, when the "reduced" CBS is placed in an environment promoting oxidation, the activity is restored (Taoka *et al*, 1998). Oxidation has been shown to promote cleavage of the CBS enzyme from its

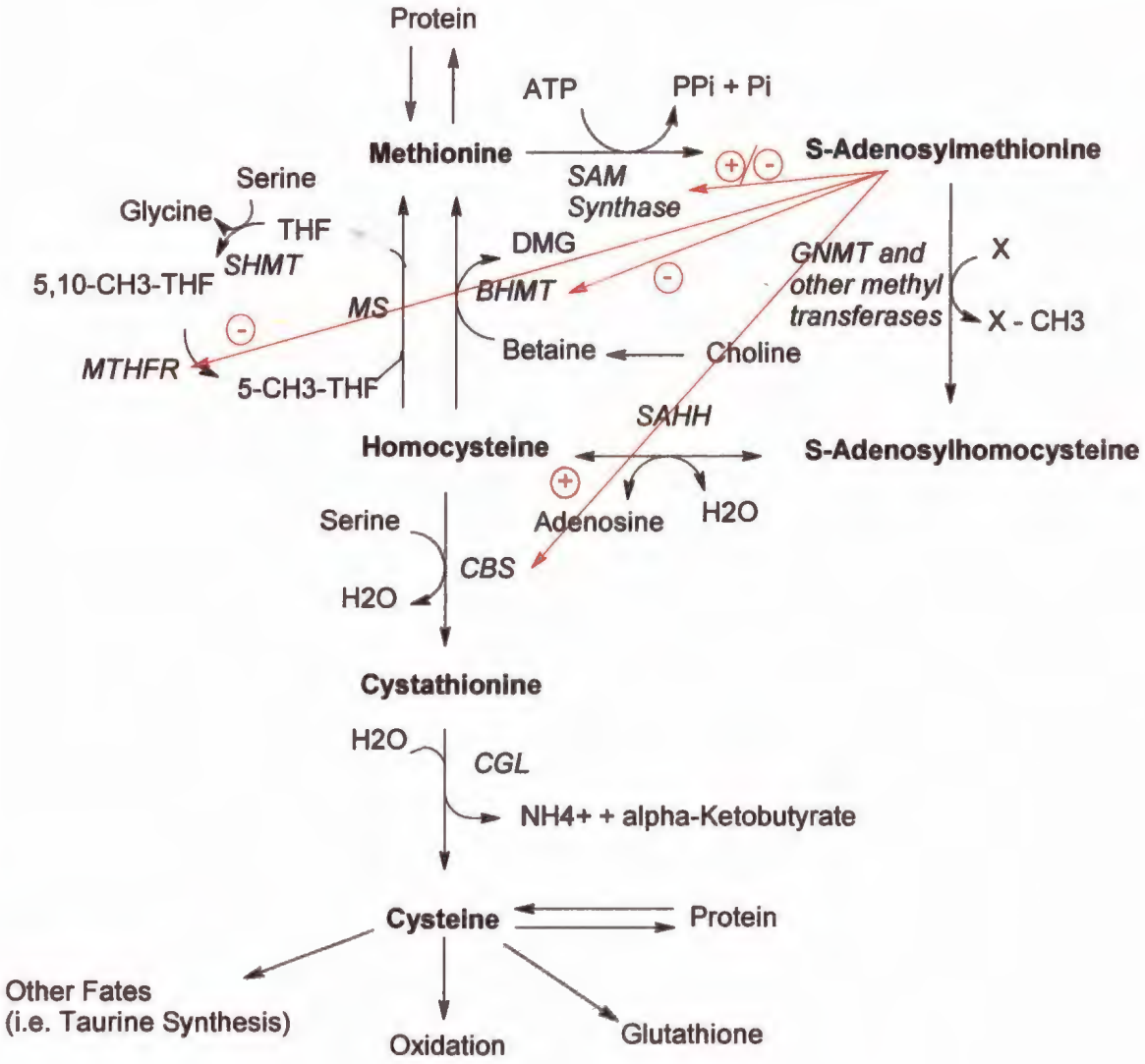
tetrameric form to its dimeric form, thereby creating a CBS enzyme that is highly active and free from allosteric regulation (Zou and Banerjee, 2003).

Additionally, methionine synthase must be maintained in a reduced state, as oxidation of this enzyme will decrease its activity. The cobalamin that is an integral part of methionine synthase activity is active only when reduced, thereby creating the dependency on a reducing environment for methionine synthase (Chen *et* al, 1995). In combination with the CBS redox regulation, an attractive theory would be that oxidation leads to a decreased flux through the remethylation pathway by decreasing methionine synthase activity while increasing the flux through CBS and the transsulfuration pathway, thereby producing more cysteine to be used for glutathione production

1.3.4 Hormonal Regulation

The role of hormones in the regulation of methionine metabolism still remains an active topic of investigation. Recent research has produced new understanding of the effects of glucagon and insulin. For example, our lab has shown that streptozotocin-treated rats have a lower plasma homocysteine concentration than control rats, and that this lowered plasma homocysteine can be returned to normal after treatment with insulin (Jacobs *et al*, 1998). Our lab has further gone on to examine this phenomenon, and has shown that insulin will suppress CBS activity and decrease mRNA levels (Ratnam, *et al*, 2002). On the other hand, glucagon was shown to increase CBS activity and mRNA concentrations, which lowers homocysteine levels (Jacobs *et al*, 2001). Hormonal regulation of other enzymes in the pathway are under investigation.

Figure 1.2: Allosteric regulation of methionine and homocysteine metabolism by SAM. Negative signs indicate that the presence of high SAM concentrations decrease enzyme activity, while positive signs indicate an increase in activity. It should be noted that SAM both increases and decreases SAM Synthase activity, depending on the isoform of the enzyme.


Protein
ATP
PPi + Pi
Methionine
SAM Synthase
S-Adenosylmethionine
Serine
Glycine
THF
SHMT
5,10-CH3-THF
DMG
BHMT
MS
MTHFR
5-CH3-THF
Betaine
Choline
GNMT and other methyl transferases
X
X - CH3
SAHH
Homocysteine
S-Adenosylhomocysteine
Adenosine
H2O
Serine
H2O
CBS
Cystathionine
H2O
CGL
NH4+ + alpha-Ketobutyrate
Cysteine
Protein
Other Fates
(i.e. Taurine Synthesis)
Oxidation
Glutathione

1.4 The Methionine-Sparing Effect of Cysteine

For years, cystine (with cysteine) was known as the only naturally occurring amino acid that contained sulfur. The discovery by Mueller in 1923 (Mueller, 1923; Mueller, 1924) of the existence of methionine, a new sulfur-containing amino acid, caused many biochemists to question the dietary importance of cystine. It was not until 1932, however, that a link was provided between cystine and methionine. Having previously proved that adding methionine to a methionine-deficient diet caused a rapid increase in body weight of rats, Jackson and Block (Jackson and Block, 1932) examined the effect of addition of methionine to a cystine deficient diet. Using young male rats (a body weight of 60-75 g), they followed the changes in body weight. The rats were fed diets that consisted of control amounts of various amino acids but were low in methionine and cystine. Cysteine-supplemented diets contained 1.2 g cysteine/kg diet, while the methionine supplemented diets contained 2 and 4 times the molar equivalents of methionine compared to cystine. It was found that the addition of methionine or cystine to the cystine-deficient diet immediately caused a rapid increase in growth; this was not found in the rats fed diets supplemented with other amino acids. The authors suggested that only one of the amino acids is indispensable and that one amino acid, either cystine or methionine, could be converted by the body into the other.

With mounting evidence that cystine was not as essential in the diet as once thought, Womack, Kemmerer and Rose reported a study in 1937 that would end the debate. Using crystalline amino acid diets, they investigated weight gain

in rats ingesting four different diets. The first diet contained 0.6% *dl*-methionine with 0.3% cystine; the second diet was supplemented with 0.6% cystine; the third diet contained 1.4% *dl*-methionine; and the final diet used only the basal amino acid mixture, and therefore, had no sulfur amino acids present. The rats with the methionine-supplemented diets, diets 1 and 3, grew far better than the rats lacking the additional methionine. In fact, the rats that ate the diet supplemented with cystine alone and the diet lacking sulfur amino acids actually lost weight. Therefore, the authors were finally able to conclude that it was, in fact, methionine that was indispensable, whereas cystine was a non-essential amino acid that could be synthesized by the body. The investigators were also the first to speculate that it was possible that cysteine could potentially replace a portion of methionine in the diet, yet could not replace the entire methionine component.

The final word on this discussion would come from Rama Rao *et al* in 1961, in experiments aimed at determining both the effect of tyrosine supplementation on phenylalanine requirements as well as cystine supplementation on methionine requirements. L-methionine levels were tested at 0.0%, 0.1%, 0.2%, 0.3%, and 0.4% in the presence of 0.5% cystine levels in the diet. This group demonstrated that the dietary requirement of methionine that allowed rats to grow could be reduced from 0.5% to 0.16% of the diet, if cystine were supplemented at a level of 0.34%.

With the relationship between methionine and cystine definitively proven and methionine recognized as the essential sulfur-containing amino acid, investigators examined the evidence surrounding potential pathways that might

be involved in the methionine-sparing effect. Studies by Folin and Marenzi, followed by Butz and du Vigneaud in the 1930's led to the discovery of homocysteine. It was later shown that homocysteine, cysteine, and methionine were linked in a common pathway. Research by du Vigneaud, Dyer, and Harmon (1934) attempted to determine the dietary effect of homocysteine in rats. They employed four different diets; a basal diet, a cystine-supplemented diet (0.3g/100g of diet), a homocystine supplemented diet (0.675 g/100 g of diet), and a methionine supplemented diet (0.750 g/100 g of diet). These supplements were added to the diet of the rats after 4 days on the basal diet. During these initial four days, all of the rats lost weight. However, on the fifth day, rats began receiving the three supplements, and immediately showed an increase in weight, whereas the group of rats still receiving the basal diet continued to lose weight. The investigators concluded that homocysteine was an intermediate step in the pathway between methionine and cystine, as homocystine caused an increase in growth similar to that of both the methionine and cystine supplements.

Finally, Finkelstein and Mudd provided the last piece of the puzzle, in 1967. In a series of experiments designed to determine the enzymatic basis for the methionine-sparing effect of cystine and cysteine, they specifically studied the effect of cystine on cystathionine synthase. As it was already known that homocysteine had the potential to be recycled to methionine or be used in the synthesis of cysteine, investigators had focused on the regulation of the reaction that formed cystathionine. They examined cystathionine β-synthase activity in

young rats (125-150g) fed diets containing a basal level of methionine (2.5 g/kg) with supplements of methionine (8.0 g/kg) or cystine (7.68 g/kg). After 7 days on these diets, the rats on the cystine supplemented diet showed a 40% reduction in the activity of hepatic cystathionine β-synthase compared with those on the basal diet or the methionine-supplemented diet.

While the studies outlined above used laboratory rats as test subjects, experiments have also been performed to determine whether these results are, in fact, also relevant to human dietary needs. In 1955, Rose and Wixom employed nitrogen balance to examine the amino acid requirements of the human diet. They found that cystine could replace up to 89% of the methionine requirement in the diet of human males. In this experiment, the methionine requirement was determined for each subject to be approximately 1.0 g/d. The subjects were then fed a diet containing 0.8 g/d of methionine and 0.81 g/d of cystine. The level of methionine was then decreased until it reached 0.1 g/d of methionine. Below this, the nitrogen balance became negative; thus, an abundant cystine intake could replace up to almost 90% of methionine requirements in humans.

Since 1955, many advances have been made in measuring amino acid metabolism in the body with perhaps the greatest contribution to this field being the use of tracers. In 1997, Raguso *et al* examined the methionine-sparing in humans with the addition of cystine to the diet by administering [1-^{13}C;methyl-$^{2}H_3$]methionine and [$^{2}H_2$]cystine. Groups of 6 healthy, young males were fed one of three diets: the first diet contained high methionine (13.0 mg/kg per day), but

no cystine; the second contained low methionine (6.5 mg/kg per day); and the final diet had low methionine (6.5 mg/kg per day), but was supplemented with cystine (5.6 mg/kg per day). This methodology allowed the investigators to determine a variety of flux measurements throughout the methionine metabolic pathways. The investigators found that there was no change in flux through the transsulfuration pathway when cystine replaced 60% of the methionine requirement; therefore, they concluded that no evidence for the cysteine sparing effect of methionine in humans was found. Using the same methodology, this same research group found similar results once again several years later (Raguso *et al*, 2000).

The result of the above experiment was disputed recently by an investigation using another technique – indicator amino acid oxidation. In this technique, it is assumed that all amino acids enter a hypothetical pool – the only ways into the pool is via ingestion and protein degradation, while the only ways out are through amino acid catabolism and protein synthesis. The rate of entry into and out of the amino acid pool through protein degradation and synthesis are assumed to cancel each other out, thereby leaving the rate of ingestion equivalent to the rate of amino acid catabolism. Once an amino acid becomes deficient, all other amino acids that are still present in excess are catabolized. Therefore, it is possible to determine the concentrations at which a particular essential amino acid becomes deficient by examining the catabolism of an indicator amino acid. The purpose of this experiment by Di Buono *et al* (2001) was to determine new requirements for sulfur amino acids in the diet by using [1-

^{13}C]phenylalanine as the indicator amino acid. In this study, subjects received 0, 2.5, 5.0, 7.5, 10.0, or 13.5 mg/kg per day of dietary methionine, while dietary cystine levels were consistently at 21 mg/kg per day. In the presence of this level of cystine in the diet, it was found that the minimum requirements for methionine in the diet dropped to 4.5 mg/kg per day with a safe limit of 10.1 mg/kg per day, a full 64% difference between methionine requirements without supplementary cystine. Therefore, it can be concluded, that cysteine does have a sparing effect on methionine requirements in humans. The reason that no methionine-sparing is found in previous studies that examined this effect in humans using tracer amino acids is most likely due to an insufficient amount of methionine in the diet.

More current studies now focus on the molecular basis for regulation of cystathionine synthase, as researchers in Japan have discovered an increase in cystathionine synthase mRNA in rats fed diets low in cystine (Yamamoto *et al*, 1995). These studies have focused on six different diets, which can be broken down into two different groupings. The first three diets are supplemented with a high level of methionine (1.0% of the diet) with no cystine, low cystine (0.75% of the diet), or high cystine (2.0% of the diet). The second grouping consisted of diets containing low methionine levels (0.25% of the diet) with no cystine, low cystine (0.75%), or high cystine (2.0%). The addition of either low cystine or high cystine to the low methionine diet caused significant decreases in the activity of cystathionine β-synthase. The addition of cystine to the high methionine diet did not cause significant differences in activity. The novel result from this study was

that changes in cystathionine β-synthase mRNA levels paralleled those in enzyme activity. The author's concluded that the decrease in cystathionine β-synthase activity caused by the addition of cysteine to the diet was mainly the result of alterations in its mRNA levels. It should be noted that this effect was reduced with age.

1.5 Objectives of Investigation and Specific Hypotheses

Despite the growing amount of research on homocysteine metabolism and the associated sparing effect of methionine by cysteine supplementation in the diet, many questions still remain concerning the mechanisms involved. With this in mind, we have completed a series of experiments with the following questions:

1. Can the manipulation of methionine and cysteine concentrations in incubations of isolated hepatocytes mimic the methionine-sparing effect?
2. What are potential mechanisms by which methionine can be spared in both adult and young rats?
3. What is the effect of excess dietary methionine on the metabolism of methionine in both young and adult rats?

The specific hypotheses were:

1. that the sparing effect of cysteine on methionine metabolism will not be evident in short term incubations with hepatocytes
2. that the sparing effect can be explained by a combination of changes in gene expression and in the concentrations of allosteric effectors.

Chapter 2 – Materials and Methods

2.1 Experimental Design

In experiment 1, hepatocytes were incubated with varying concentrations of 1-^{14}C-methionine and cysteine to determine any acute effects of sulfur amino acids on the transsulfuration flux, and if these variations could mimic the methionine-sparing effect.

In experiment 2, purified amino acid diets were fed to adult rats in order to determine the effect that cystine in the diet would have on the methionine-sparing effect in animals fed a high methionine diet. Additionally, diets were also prepared to examine the effect of additional methionine on methionine metabolism. Experiment 3 was similar to experiment 2, except that it employed young rats.

Finally, experiment 4 examined the effects of S-Adenosylmethionine on the kinetics of some of the regulatory enzymes of methionine metabolism.

2.2 Animals

In the first and fourth experiments, 4 Sprague-Dawley rats were used, each of which ranged, in weight, from 250 to 275g. Experiment 2 and 3 each required 16 Sprague-Dawley rats; however, the weight range for experiment 2 was limited to 250-275 g, while the weight range for experiment 3 was 50-75g. The animals of experiment 4 were also Sprague-Dawley rats, and weighed between 250-275 g. All rats were obtained from the Memorial University of Newfoundland Vivarium. All protocols were approved by the Institutional Animal

Care Committee and adhered to the guidelines of the Canadian Council of Animal Care.

In experiments 2 and 3, the animals were randomly separated into four separate groups and assigned to different diets. All animals in these two experiments were housed in individual cages throughout the experiment.

2.3 Diet Composition

In both experiment two and experiment three, four separate diets were used. The basic composition of each diet can be seen in Table 2.1. Each diet differed in their amino acid composition, with the first diet containing deficient levels of sulfur amino acids (Low SAA), the second diet containing a methionine supplement (High Met), the third diet containing a cystine supplement (High Cys), and the final diet consisting of both a methionine supplement and a cystine supplement (High M+C).

All the diet components, as well as the amino acid composition, of each of the four diets were based on the work of Rogers and Harper (1965) and Newberne (1969). A crystalline amino acid mixture was used in place of casein in order to control the amounts of individual amino acids. A basal amino acid mixture was used that contained all amino acids at a level designed to optimize growth, with the exception of the SAA, which were added according to the diet group. A supplement with alanine was used to ensure the four diets were close to isonitrogenous. The amino acid components of the diets can be seen in Table 2.2. The amounts of methionine and cystine added to the diets were equivalent

Table 2.1: Basic Composition of All Experimental Diets

Diet Components	Amount (g/kg diet)
Cornstarch	369.572
Amino Acids (Crystalline Mixture)	232.920
Dextrinized Cornstarch	150.000
Sucrose	100.000
Soybean Oil	50.000
Fiber	50.000
Mineral Mix	35.000
Vitamin Mix	10.000
Choline Bitartrate	2.500
Tertiary-butyl Hydroquinone	0.008
TOTAL	1,000.000

Table 2.2: The Amino Acid Composition of the Diets

Basal Amino Acid Composition			
Amino Acid	Amount (g/kg diet)	Amino Acid	Amount (g/kg diet)
Histidine	3	Lysine	16
Isoleucine	8	Phenylalanine	10
Leucine	10	Tyrosine	4
Threonine	7	Tryptophan	2
Valine	8	Arginine	18
Proline	6	Asparagine	18
Glycine	24	Glutamic Acid	68
Alanine	12.82	Methionine	2.5

Experimental Diet	Supplemental Methionine (g/kg diet)	Supplemental Cystine (g/kg diet)	Supplemental Alanine (g/ kg diet)	Total Supplement (g/kg diet)
Control	0	0	15.6	15.6
High Met.	8.0	0	7.6	15.6
High Cys.	0	7.6	8.0	15.6
Met. + Cys.	8.0	7.6	0	15.6
Total Amino Acids = 232.92g				

in terms of sulfur content, and were based on diets developed by Finkelstein and Mudd (1967).

Once the dry components of the diet had been adequately mixed, an equivalent amount of boiling 3% agar gel solution was added slowly, with heating (Newberne, 1969). Once the solution was completely added, the diet was thoroughly mixed into a slurry and left to cool for several minutes. The diet was then placed in an airtight container, and refrigerated until use.

The animals were first fed an acclimatization diet over a period of three days. This acclimatization diet consisted of chow ground into a powder, and then dissolved in boiling agar to create a gel as described above. After the acclimatization period, the rats received their respective gel diet for a period of seven days. During this time, each rat was weighed once per day. Food intake was determined by measuring the amount of food given and what was left over each day.

2.4 Liver Extraction

On the day of sacrifice, each rat was anaesthetized with 65 mg/kg sodium pentobarbital. Approximately 2-3 mL of blood was drawn from the aorta into a heparinized syringe. A small piece of liver (approximately 0.5 g) was rapidly removed and homogenized in guanidium thiocyanate buffer for mRNA analysis, while a larger piece of liver (approximately 5 g) was homogenized in 50 mM Potassium Phosphate buffer (pH of 7.0) for enzyme assays. The remaining liver was removed and freeze-clamped using aluminum tongs pre-cooled in liquid nitrogen, and then stored at –80° C until analysis.

The blood was centrifuged at 10,000 g for 5 minutes in 1.5mL Eppendorf tubes. The plasma was removed and frozen until required. The liver portions used for fresh enzyme assays were diluted to 1:5 liver:potassium phosphate buffer, and then homogenized with a Polytron homogenizer (Brinkman Instruments, Toronto, Canada) for 20 seconds at an output of 50%. Homogenates were centrifuged at 10,000 g at 4^{O} C for 30 minutes, and the supernatant was retained.

2.5 Enzyme Assays

MAT, CBS, CGL, BHMT, and MS were all assayed using fresh liver samples. The other assays could be performed on frozen liver samples. All enzyme assays were shown to be linear with protein concentration and incubation time under the conditions of our assays.

2.5.1 Methionine Adenosyltransferase

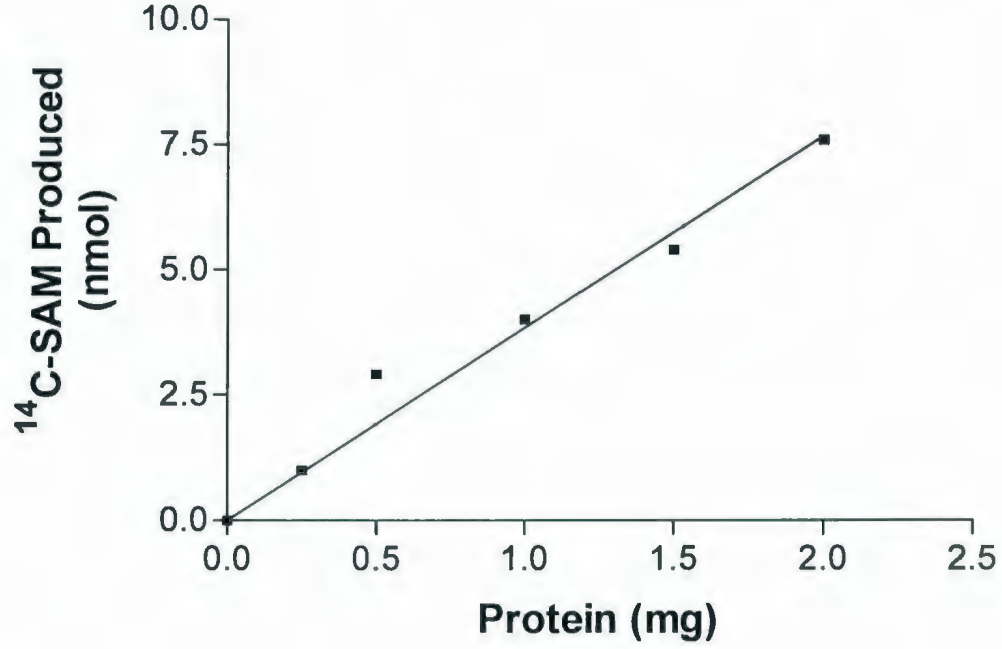
Methionine adenosyltransferase activity was determined using the methodology of Mudd *et al* (1965) and Duce *et al* (1988), and involves the production and measurement of 1-^{14}C-S-Adenosylmethionine from 1-^{14}C-methionine. 1 mg of liver homogenate protein was added to an incubation medium of 100 mM Tris HCl (pH 7.8), 200 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 5 mM ATP, 1 mM benzamidine, and 0.1% ß-mercaptoethanol with a volume of 230 µl. The reaction was incubated at 37^{o} C for 30 minutes upon the addition of 20 µl of 62.5 mM 1-^{14}C-methionine, in a final volume of 250 µL, with a final concentration of 5 µM of methionine. The reaction was stopped after the 30 minute incubation with the addition of 750 µL of ice cold H_2O. From this stopped

reaction mixture, 700 µL was added to a column (0.9 x 2.0 cm) of Dowex 50-X4 (NH_4^+) to separate the labelled SAM from the methionine. The column was washed with 20 mL of H_2O, and the SAM was eluted with the addition of 6 mL of 3 M NH_4OH. 10 mL of Scintiverse was added, and the radioactivity of the sample was determined in a scintillation counter. Protein and time curves can be seen in Figure 2.1.

2.5.2 Glycine N-Methyltransferase

Glycine N-methyltransferase was assayed by means of a modification of the method of Cook and Wagner (1984) which measured the production of ^{3}H-sarcosine from [methyl-^{3}H]-S-adenosylmethionine. Frozen liver samples were homogenized in 50 mM potassium phosphate buffer (pH 7.0), and then diluted to a protein concentration of 0.075 mg per assay. 60 µL of this protein homogenate was added to 100 µL of incubation medium, which consisted of 100 mM Tris HCl (pH 9.0), 2 mM glycine, and 5 mM DTT. To start the reaction, 60 µL of 1 mM [methyl-^{3}H]-S-adenosylmethionine (with a final concentration of 0.3 mM) was added to bring the final volume up to 200 µL. This mixture was incubated for 15 minutes at 25° C, at which point the reaction was stopped with the addition of 100 µL of 10% TCA. 500 µL of 0.1 M acetic acid-washed charcoal suspension containing 950 mg of charcoal was added to each sample to absorb the remaining SAM, and was immediately vortexed. Samples were allowed to stand for 15 minutes, after which they were centrifuged at 3,200 g for 10 minutes. 500 µL of the supernatant was added to 10 mL of Scintiverse, for scintillation counting. Linearity for protein and time can be seen in Figure 2.2.

Figure 2.1: The production of S-adenolsylmethionine by hepatic methionine adenosyltransferase as a function of cytosolic protein concentration and incubation time. The time period used during the protein concentration studies was 30 minutes, while the time curve was determined using a protein concentration of 1.0 mg.


10.0
7.5
5.0
2.5
0.0
0.0
0.5
1.0
1.5
2.0
2.5
^{14}C-SAM Produced (nmol)
Protein (mg)

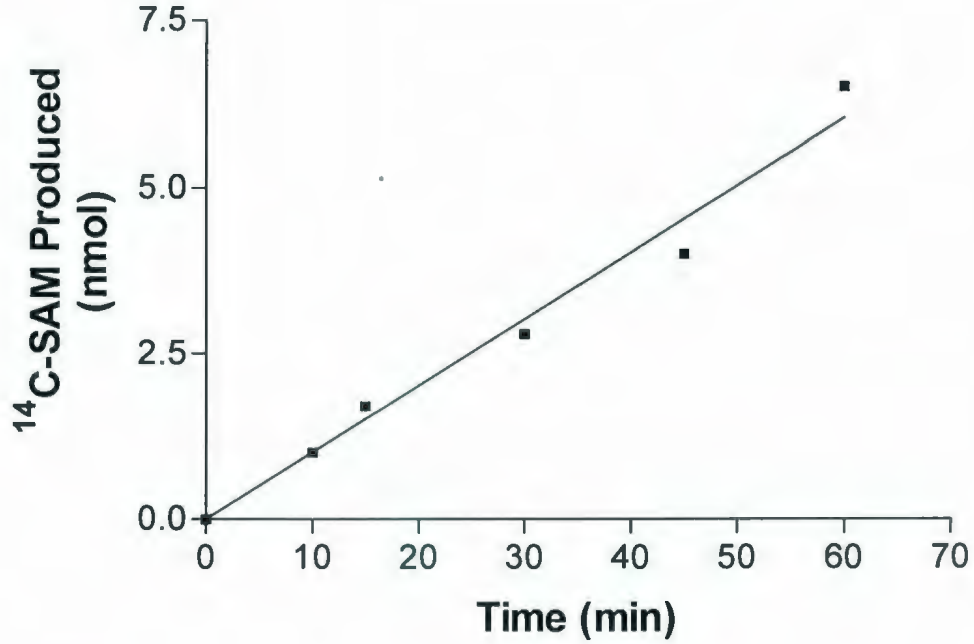
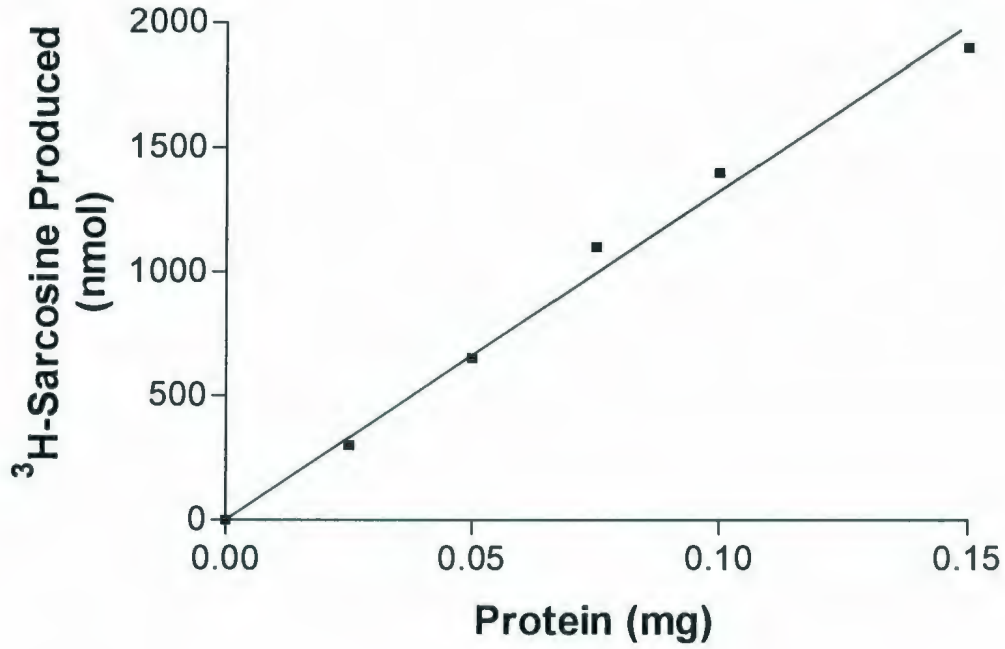
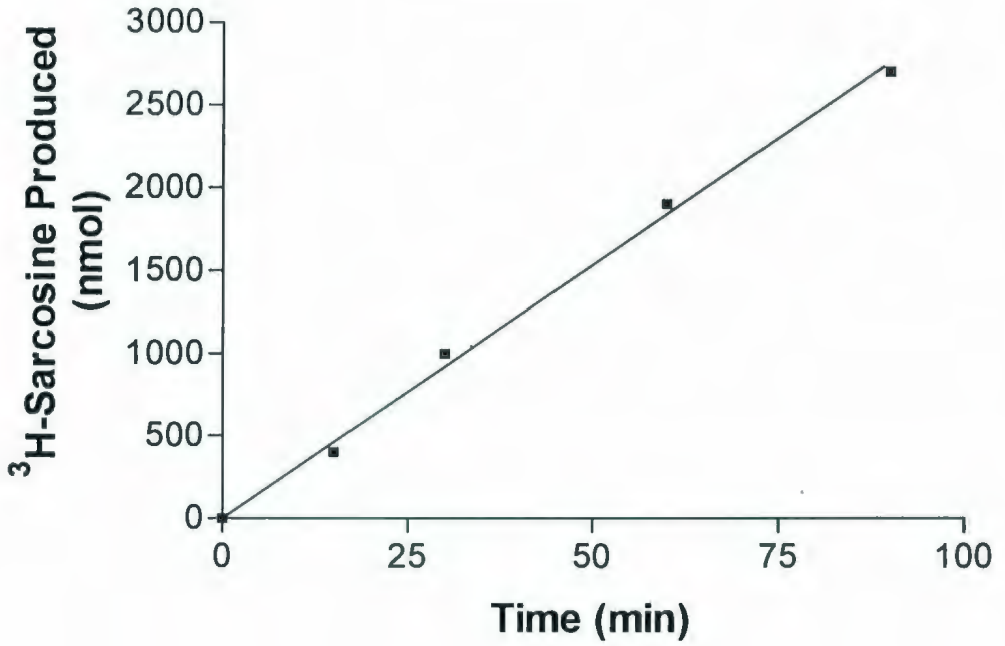

7.5
5.0
2.5
0.0
0
10
20
30
40
50
60
70
^{14}C-SAM Produced (nmol)
Time (min)

Figure 2.2: The production of ^{3}H-sarcosine by glycine N-methyltransferase as a function of hepatic protein concentration and incubation time. The incubation period used during the protein study was 30 minutes, while the protein concentration used in the time determination was 0.075 mg.


^{3}H-Sarcosine Produced (nmol)
2000
1500
1000
500
0
0.00
0.05
0.10
0.15
Protein (mg)


^{3}H-Sarcosine Produced (nmol)
3000
2500
2000
1500
1000
500
0
0
25
50
75
100
Time (min)

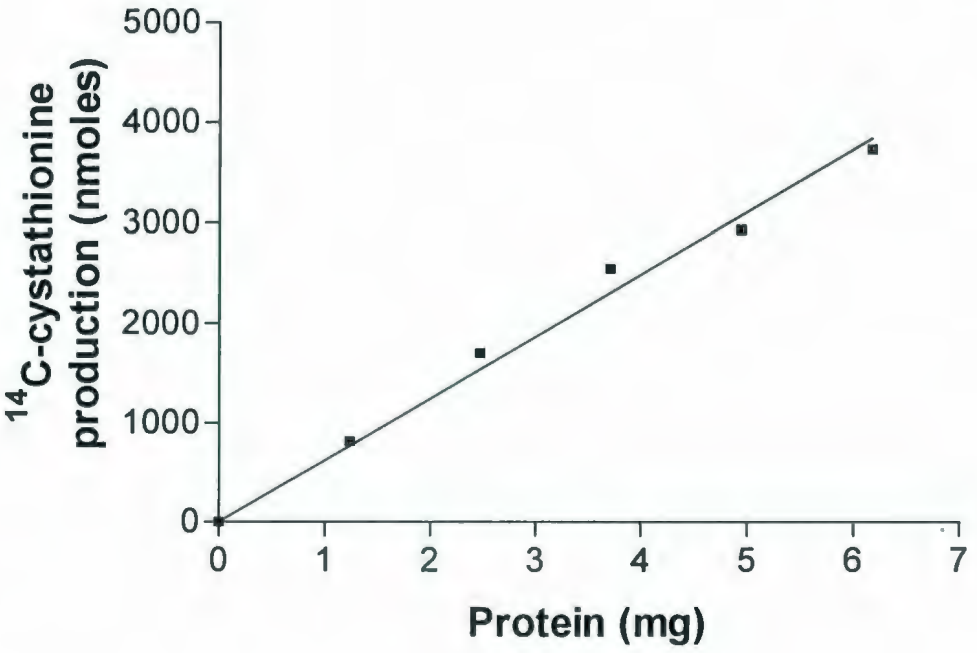
2.5.3 Cystathionine β-Synthase

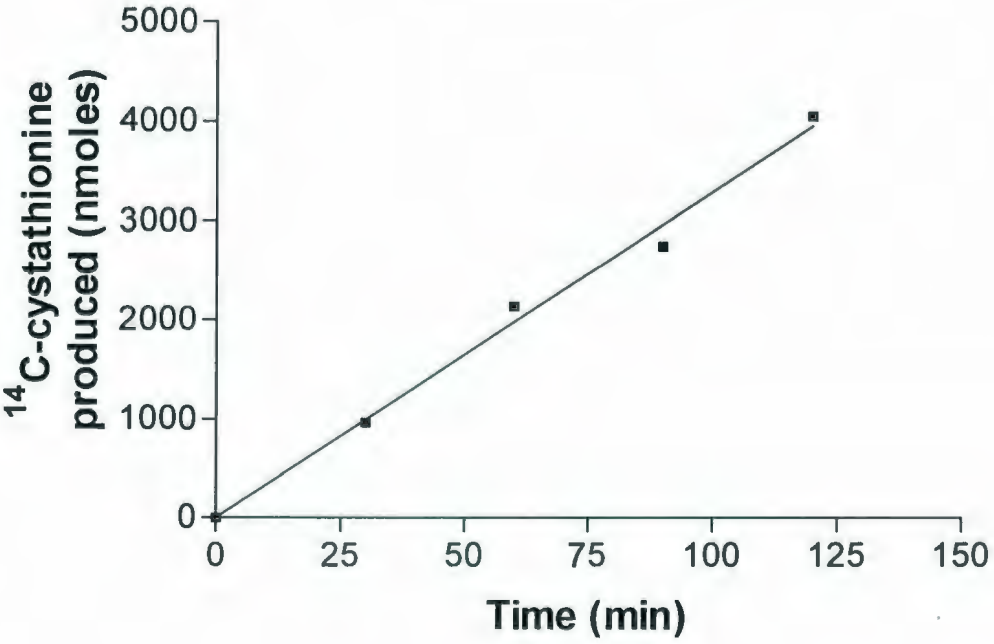
CBS was measured via a variation of the method of Miller *et al* (1994), which measures the production of 1-^{14}C-cystathionine from 1-^{14}C-serine. 3.0 mg of protein were incubated in a reaction mixture containing 187.5 mM Tris HCl, 3.125 mM EDTA, 0.214 mM L-cystathionine, 62.5 mM homocysteine, 0.475 mM SAM, 3.125 mM propargylglycine, and 0.625 mM pyridoxal 5-phosphate. The reaction was started with the addition of 50 µL of 300 mM 1-^{14}C-serine for a total reaction volume of 0.6 mL. The incubation continued for 1 hr. at 37°C, when it was stopped with the addition of 300 µL of ice cold 15% TCA. The samples were centrifuged at about 14,000 g for five minutes, after which, 0.5 mL of supernatant was added to columns (0.9 x 2.0 cm) containing Dowex 50w-x4 resin (200-400 mesh, H^+ form). These columns were washed with 8 mL of water, 24 mL of 1M HCl, and a further 16 mL of water. Finally, the radiolabelled cystathionine was collected from each column with 5 mL of 3M NH_4OH. 1 mL of the eluted solution was added to 10 mL of scintiverse, and the radioactivity was counted in a scintillation counter. The curves depicting cystathionine β-synthase activity as a function of time and protein are shown in Figure 2.3.

2.5.4 Cystathionine γ-Lyase

The method used to determine CGL activity was based on that of Stipanuk *et al* (1979). Cytosolic protein (1.5 mg) was added to a 2 mL cocktail in a cuvette containing 0.125 mM potassium phosphate, 0.32 mM pyridoxal 5-phosphate, 0.32 mM β-NADH, 4.0 mM of L-cystathionine, and approximately 1.5 units of lactate dehydrogenase (LDH) at 37° C in a thermostatted spectrophotometer.

Figure 2.3: The production of cystathionine from hepatic cystathionine β-synthase as a function of protein concentration and incubation time. The incubation period used during the protein determination was 60 minutes, whereas the protein concentration used during the time determination was 3.0 mg.


^{14}C-cystathionine production (nmoles)
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
6
7
Protein (mg)


^{14}C-cystathionine produced (nmoles)
5000
4000
3000
2000
1000
0
0
25
50
75
100
125
150
Time (min)

One of the products of the CGL reaction is α-ketobutyrate, which can act as a substrate for LDH. The decrease in absorbance at 360 nm measures the oxidation of NADH, and is used to determine the activity of CGL. Enzyme activity was determined from the linear portions of the graph. The dependence of CGL on protein concentration can be seen in Figure 2.4.

2.5.5 Methylenetetrahydrofolate Reductase

MTHFR was measured according to the method of Engbersen *et al* (1995). 0.1 mg of frozen liver homogenate protein was incubated at 37°C with 400 µL of reaction cocktail, which consisted of 50 mM potassium phosphate buffer (pH 6.8), 11.5 mM ascorbic acid, 1.15 mM EDTA, 54 µM FAD, and 20 µM [^{14}C-CH_3]methyltetrahydrofolate. 100 µL of menadione was added to bring the total reaction volume to 600 µL and begin the reaction which was at 37°C for 20 minutes, in the dark. After 20 minutes, the reaction was stopped by the addition of a combination of 100 µL of 3 M potassium acetate (pH 4.5), 200 µL of dimedone dissolved in ethanol:water (1:1), and 10 µL of 1.0 M formaldehyde. The samples were immediately heated in a 95°C water bath for fifteen minutes, followed by cooling in an ice bath for ten minutes. After removal from the ice bath, 3 mL of toluene was added to each of the samples, which were all centrifuged at low speed for approximately 5 minutes. 2 mL of the toluene phase was removed and added to 10 mL of Scintiverse for scintillation counting. The dependence of MTHFR activity on protein concentration and incubation time can be seen in Figure 2.5.

Figure 2.4: The dependence of cystathionine γ-lyase activity on protein concentration. The activity was evaluated over 10 minutes.

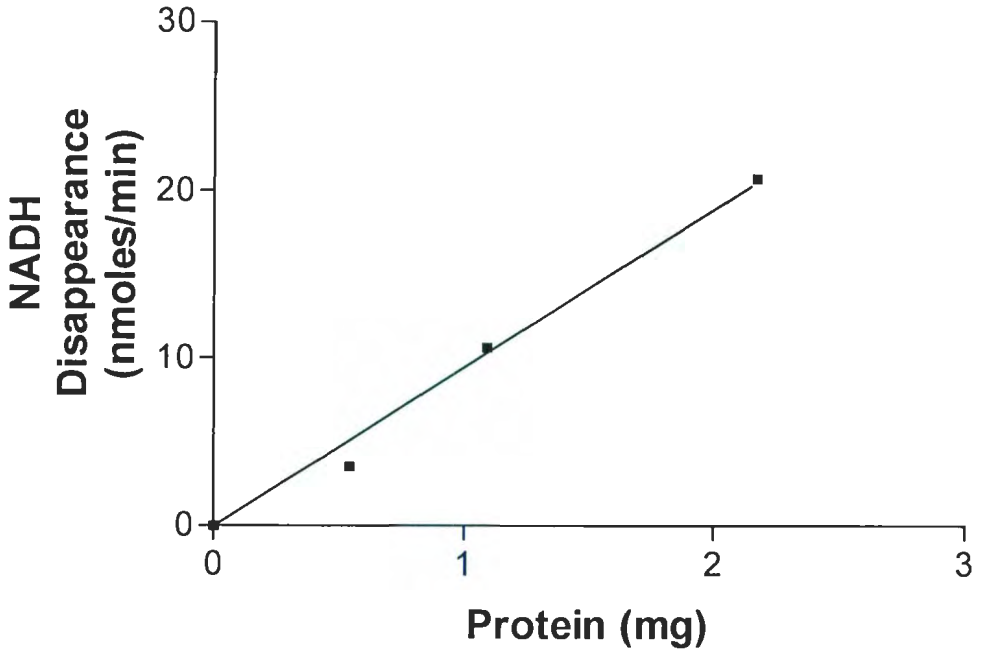
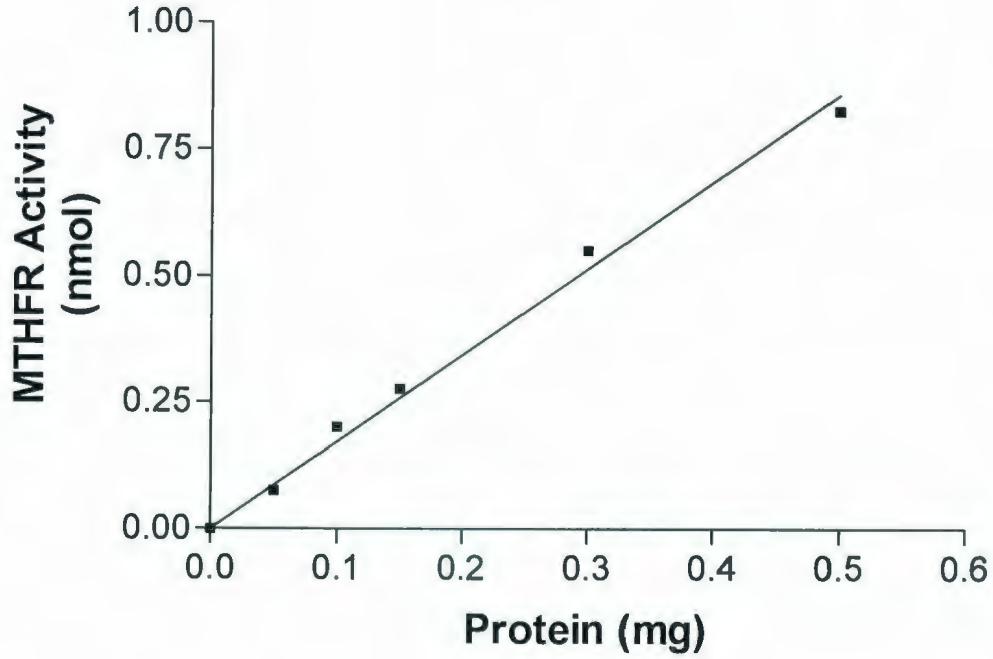
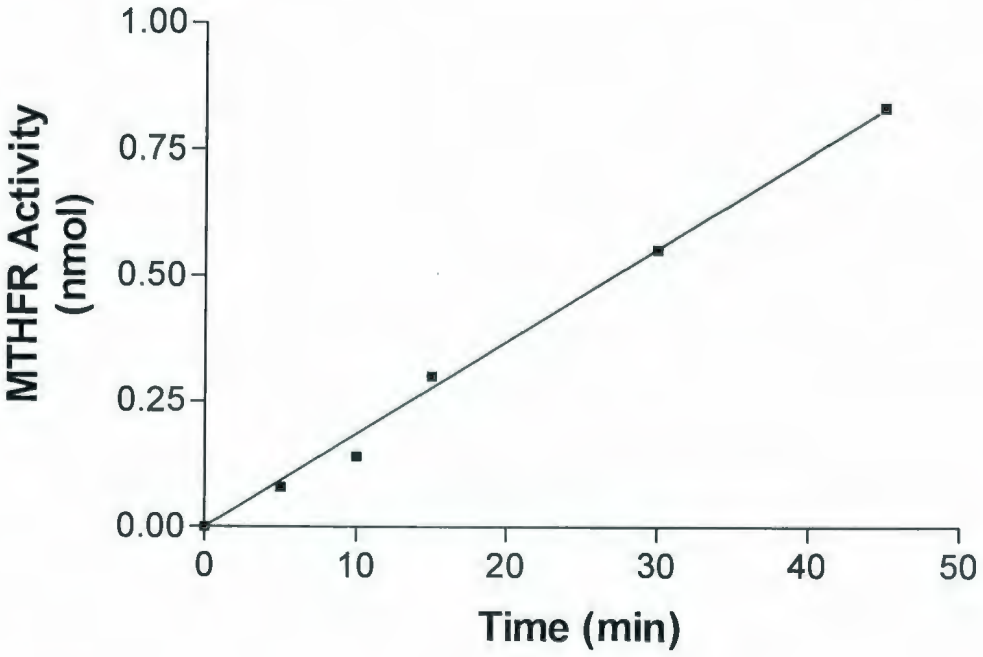

NADH
Disappearance
(nmoles/min)
30
20
10
0
0
1
2
3
Protein (mg)

Figure 2.5: The activity of methylenetetrahydrofolate reductase as a function of protein concentration and incubation time. The protein concentration used during the time determination was 0.15 mg, whereas the incubation period used while protein concentration was examined was 15 minutes.


MTHFR Activity (nmol)
1.00
0.75
0.50
0.25
0.00
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Protein (mg)


MTHFR Activity (nmol)
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
50
Time (min)

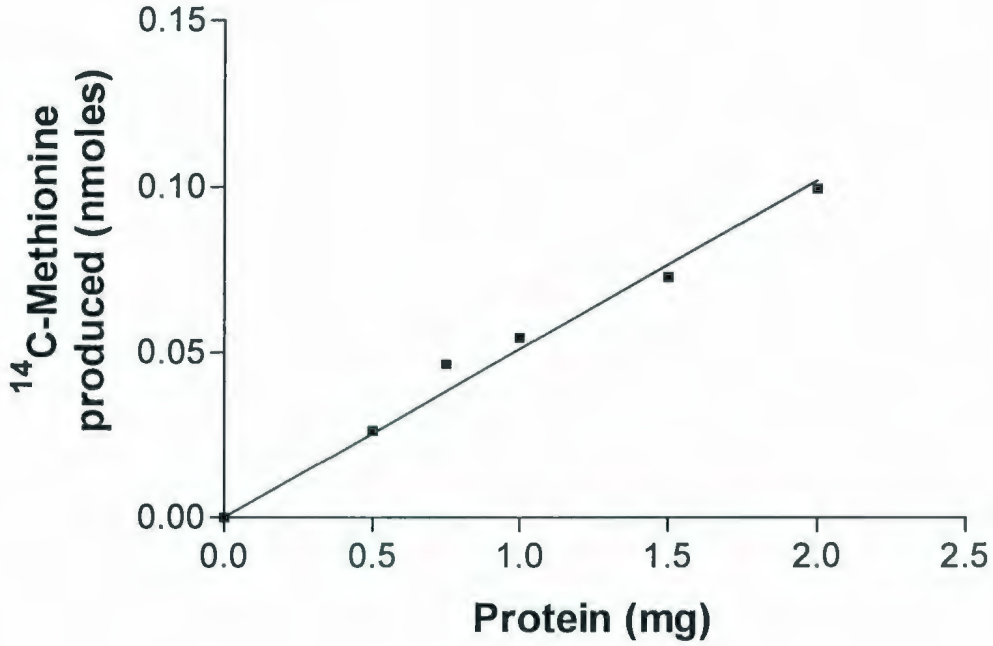
2.5.6 Methionine Synthase

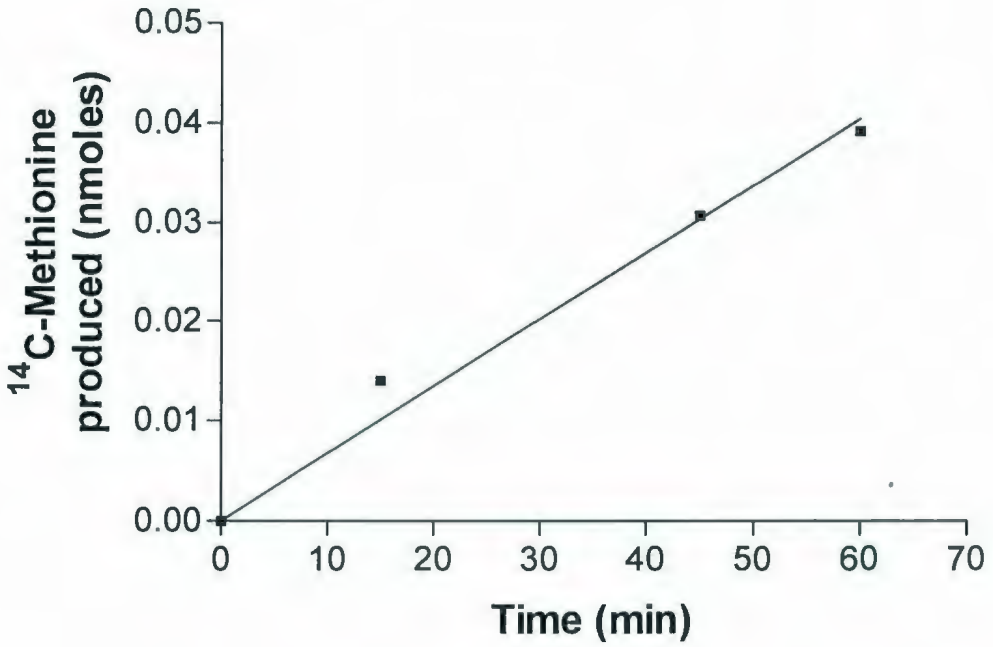
Methionine Synthase was assayed according to the method of Kolbin *et al* (1981). The cytosolic protein (100 µL at 1.0 mg/assay) was added to a reaction mixture (100 µL) containing 87.5 mM potassium phosphate buffer (pH 7.5), 58 mM DTT, 14 mM B-mercaptoethanol, 20 µL of cyanocolbalamin, 15 mM homocysteine, 0.5 mM S-adenosylmethionine, and 1 mM of [5-^{14}C]methyltetrahydrofolate (0.25 µCi) in a total of 200 µL reaction volume. This reaction mixture was incubated in the dark at 37°C for 30 minutes, and was stopped with the addition of 500 µL of ice-cold water. After stopping the reaction, the samples were placed on ice until ready to be added onto a column (0.9 x 2.0 cm) containing Bio-Rad washed with 1.5 mL of water and the effluent was collected. This 1.5 mL of effluent was then added to 10 mL of Scintiverse and the radioactivity determined. The effects of protein and incubation time can be seen in Figure 2.6.

2.5.7 Betaine:Homocysteine Methyltransferase

BHMT was assayed by a modification of the method of Wang *et al* (1991). Cytosolic protein (100 µL of protein at 0.3 mg/assay) was added to a reaction mixture containing 125 mM Tris HCl, 25 mM homocysteine, and 10 mM of 1-^{14}C-betaine, all of which were incubated in at a temperature of 37°C for 1 hour with a total volume of 500 µL. The reaction was stopped with the addition of 2.5 mL of ice-cold water and the samples immediately placed in an ice bath. 2 mL of the stopped

Figure 2.6: The effect of protein concentration and incubation time on methionine production via hepatic methionine synthase. The protein concentration during the incubation investigation was 1.0 mg, while the incubation period during the protein concentration determination was 30 minutes.


0.15
0.10
0.05
0.00
0.0
0.5
1.0
1.5
2.0
2.5
^{14}C-Methionine produced (nmoles)
Protein (mg)


0.05
0.04
0.03
0.02
0.01
0.00
0
10
20
30
40
50
60
70
^{14}C-Methionine produced (nmoles)
Time (min)

reaction was added to columns containing Dowex 1-X4 resin (OH^-; 200-400 mesh). The columns were then washed with 15 mL of water and eluted with 3 mL of 1.5 M HCl. The elutant was added to 10 mL of Scintiverse. The effect of protein concentration and time can be seen in Figure 2.7.

2.5.8 Choline Dehydrogenase

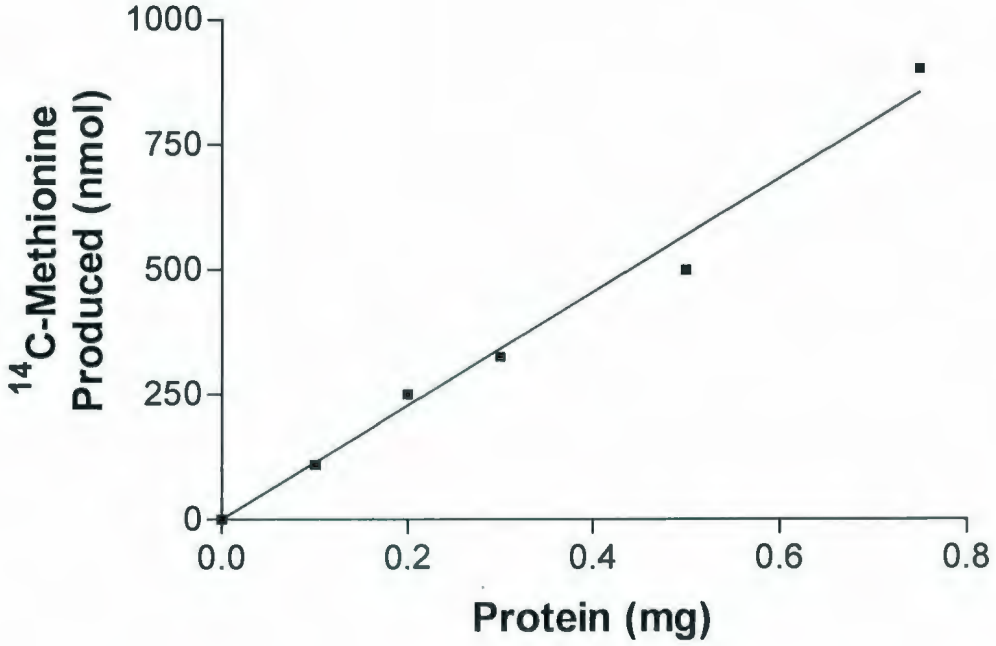
Choline dehydrogenase activity was assayed by Grossman and Hebert's (1989) modification of the method of Haubrich and Gerber (1981). Frozen liver homogenate was diluted to 1.0 mg protein / mL, and incubated at a temperature of 37°C in a cocktail of potassium phosphate (pH 7.0, 0.5 M), 0.7 mM choline, 1.0 mM phenazine methosulfate and 1.0 mM $CaCl_2$. After a ten minute incubation, the samples were placed on ice after the addition of 1N NaOH and 30% H_2O_2. The samples were then incubated for another hour at room temperature The figures depicting the effect of protein concentration and incubation time on CHDH activity can be seen in Figure 2.8.

2.4.9 SAM-dependent Enzyme Assays

The fourth experiment focused on the effects of SAM on allosteric control of a number of enzymes. The enzymes examined were CBS, BHMT, and MTHFR; the first two assays required fresh liver homogenate, while the MTHFR assay used frozen liver samples.

All enzyme assays were completed as above with some modifications. The first modification was that after each sample had been homogenized and diluted to a 1:4 liver:homogenization buffer ratio, the samples were run through a PD-10 column. This column retains all small molecules and salts, allowing the

Figure 2.7: The effect of protein concentration and incubation time on methionine production by betaine:homocysteine methyltransferase. The incubation period during protein concentration trials was 1 hour, while the protein concentration during the incubation period trials was 0.3 mg.


1000
750
500
250
0
0.0
0.2
0.4
0.6
0.8
^{14}C-Methionine Produced (nmol)
Protein (mg)

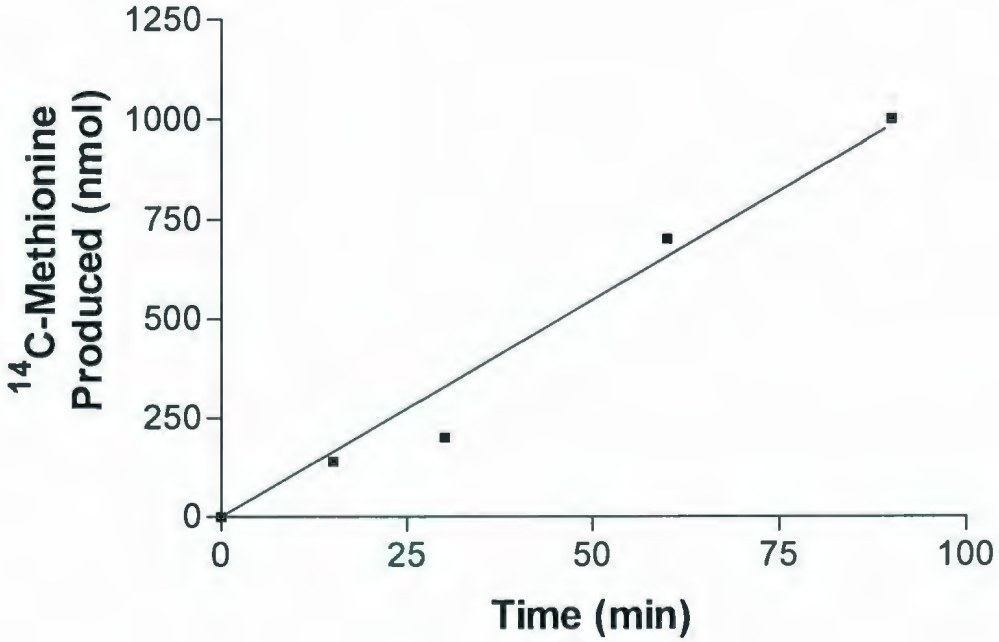
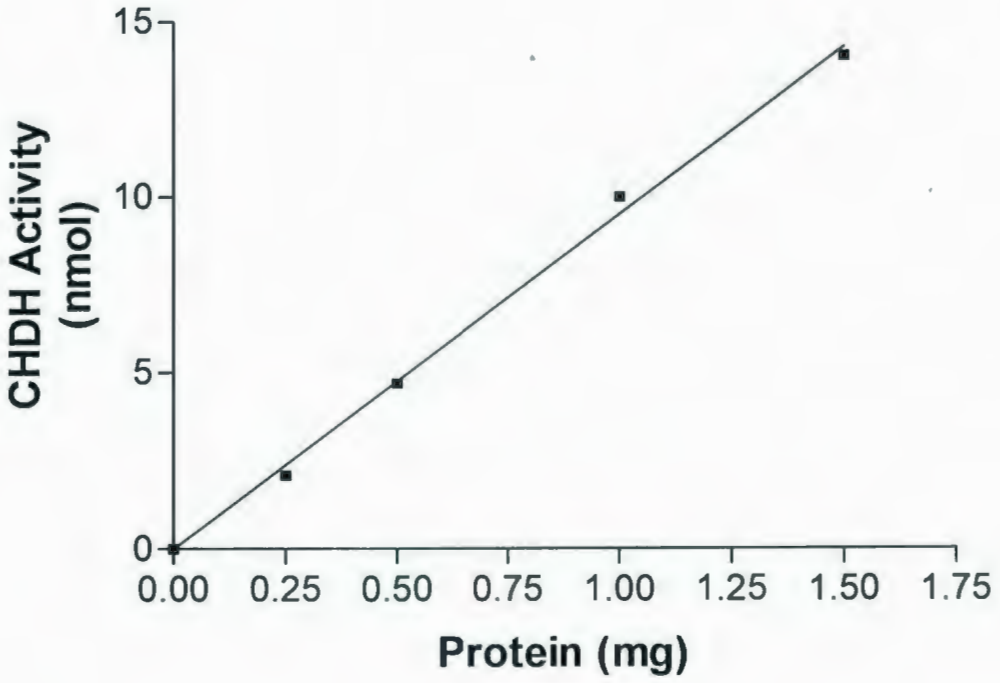
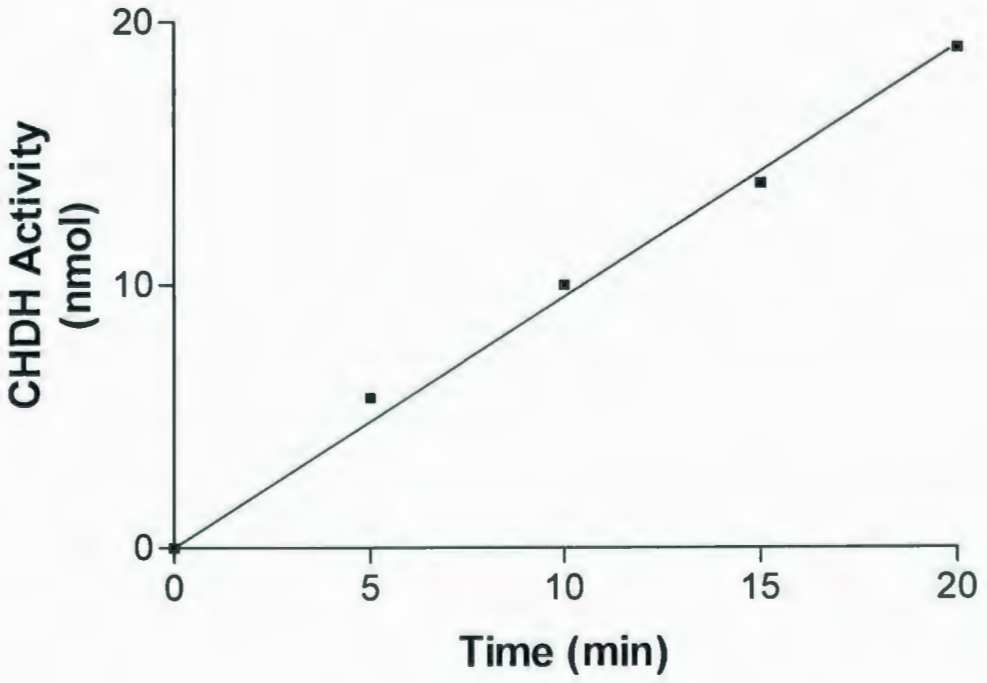

1250
1000
750
500
250
0
0
25
50
75
100
^{14}C-Methionine Produced (nmol)
Time (min)

Figure 2.8: The effect of protein concentration and incubation period on choline dehydrogenase activity. During the protein concentration determination, the incubation period was 10 minutes, whereas the protein concentration was 1.0 mg during the incubation period determination.


15
10
5
0
CHDH Activity
(nmol)
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
Protein (mg)


20
10
0
CHDH Activity
(nmol)
0
5
10
15
20
Time (min)

homogenate to become free of endogenous SAM and SAH. Approximately 20 mL of water were run through the column before 2.5 mL of each liver sample was placed on a column. The 2.5 mL of SAM-free homogenate was eluted by the addition of an additional 5.0 mL of water. A fraction of the liver homogenate was saved for SAM and SAH analysis.

2.6 Substrate Concentration Determination

2.6.1 Amino Acid Determination

Both plasma and liver amino acids were analyzed using the Department of Biochemistry Amino Acid Facility. 10% sulfosalicylic acid was added to plasma samples, following which the samples were allowed to cool on ice for 30 minutes. After the cooling period, 0.025 mL of an internal standard (AEC) and 0.275 mL of lithium citrate buffer (pH 2.2) were added to the samples. The samples were then spun at 11,000 rpm for 5 minutes, and the supernatant was saved for analysis.

For liver samples, 4 mL of ice cold 6% $HClO_4$ was added to 1 g of finely ground, frozen liver and homogenized at 4°C. The liver sample was spun at 17,000 g for 20 minutes, and the supernatant saved. 0.020 mL of dilute universal indicator and 0.1 mL K_2CO_3 (50%) were added to each sample, and the pHs of the samples neutralized with the addition of KOH. After centrifugation to remove the precipitated $KClO_4$, the supernatants were then frozen. When samples were ready to be analyzed, the pH was adjusted to 2.2 using concentrated HCl.

Both the liver and the plasma samples were analyzed in the MUN Biochemistry Amino Acid Facility, using a Beckman 121 MB Amino Acid Analyzer

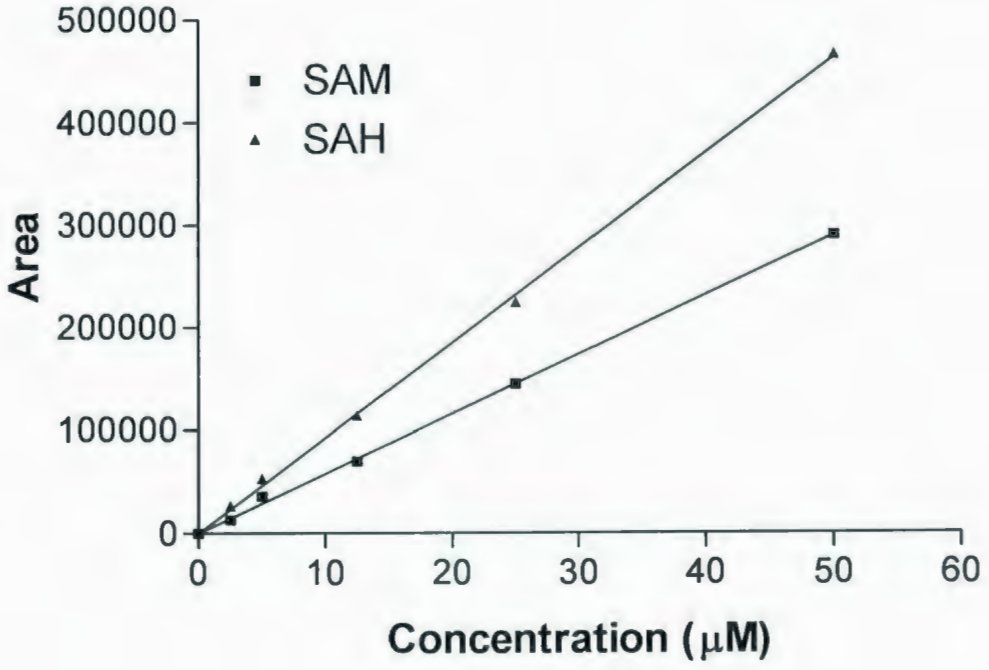
with a Benson D-X, 0.25 cation Xchange Resin and a single column, three buffer lithium method as per Beckman 121MB-TB-017 application notes. The results were then quantified with a Hewlett Packard Computing Integrator Model 3395A.

2.6.2 SAM and SAH Determination

Approximately 1 g of frozen liver sample was homogenized in 5 mL of ice cold 8% TCA, and then centrifuged at 12,500 g. The supernatant was collected and analyzed via HPLC using a VYDAC column (#2187P54) that was equilibrated with 96% 50 mM NaH_2PO_4, 10 mM heptane sulfonic acid (which was adjusted to a pH of 3.2 with concentrated sulphuric acid) and 4% acetonitrile. A 20 minute linear acetonitrile gradient (4% to 20%) was used to separate the SAM and the SAH. Peaks were determined at 258 nm with a UV detector; these peaks were then quantified with a 3390A Hewlett Packard Integrator. The concentrations of SAM and SAH were determined by reference to a standard curve (Figure 2.9).

Our lab modified the HPLC method of Vester and Rasmussen (1991) for determining the concentrations of plasma and liver homocysteine, cysteine and glutathione. 20 µL of 10% tri-n-butyl-phosphine in dimethylformamide was added to 150 µL of either plasma or frozen liver samples. After the thiols were allowed to become reduced during a thirty minute period at 4° C, the samples were deproteinized by the addition of 125 µL of 0.6 M perchloric acid. 50 µL of 0.2 mM 8-aminonaphtalene-1,3,6-trisulfonic acid was added as a standard, and the tubes were centrifuged at 14,000 g for 5 minutes at 4°C. After the centrifugation,

Figure 2.9: Standard curve for S-adenosylmethionine and S-adenosylhomocysteine on HPLC with UV detection.


500000
400000
300000
200000
100000
0
Area
SAM
SAH
0
10
20
30
40
50
60
Concentration (μM)

100 µL of supernatant was added to an Eppendorf tube containing 200 µL of 2 M potassium borate (pH 10.5) containing 5 mM EDTA and 100 µL of ammonium 7-flurobenzo-2-oxo-1,3-diazole-4-sulphonate solution (1.0 g/l in 2 M potassium borate, pH 9.5). After a 60 minute incubation period at 60°C and a cooling period of fifteen minutes in an ice bath, 20 µL where injected into an HPLC.

2.6.3 Homocysteine and Other Thiol Determination

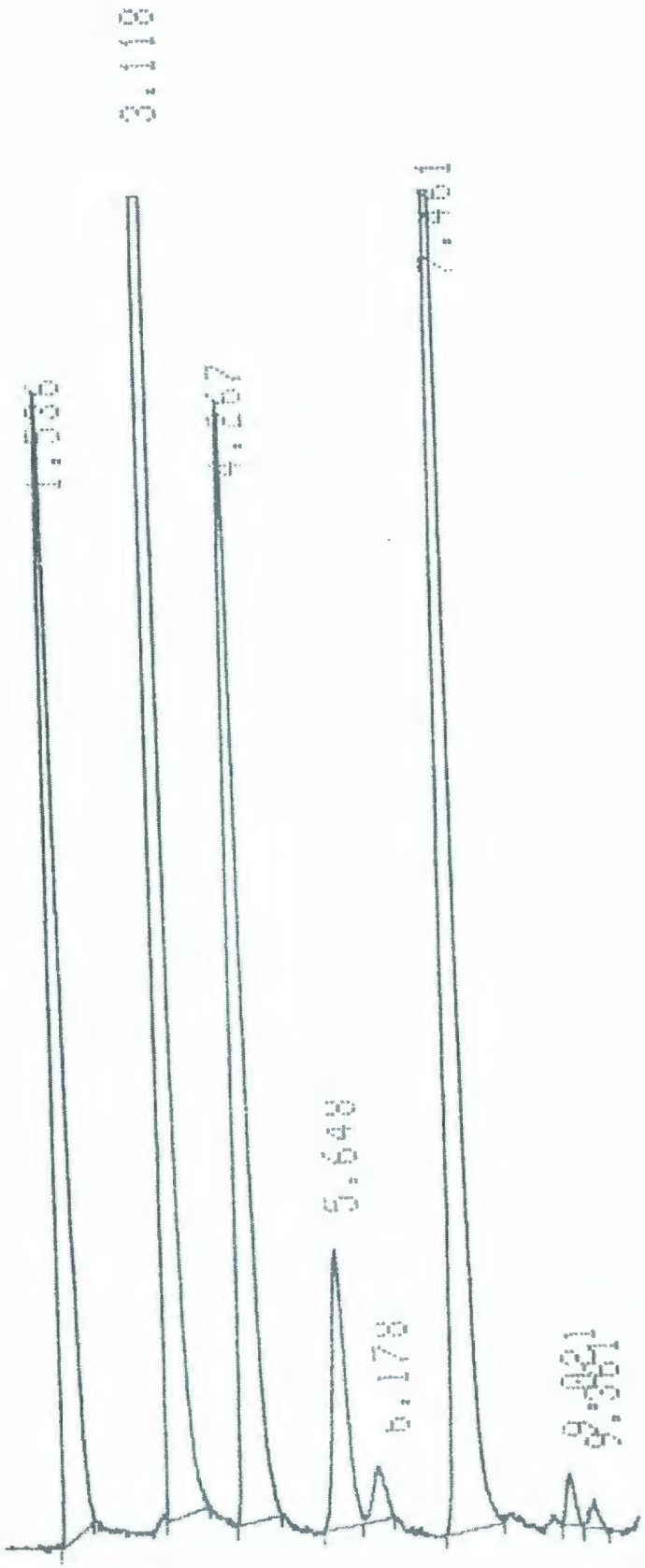
All samples were analyzed using a Shimadzu system (Pump GT-104, SCL-10A Controller, SIL-10A Auto Injector, LC-10AD Liquid Chormatograph, RF-535 Fluorescence Monitor) equipped with a Phase Separations Hypersil C18-ODS analytical column (4.6 x 150 mm). The fluorescence intensities were measure with excitation at 385 nm and emission at 515 nm. The detection signal was recorded and the peaks were quantified using a Man-Tech Shimadzu CR501 Chromatopac integrator. Figure 2.10 is an example of a typical plasma chromatogram.

2.7 Transsulfuration Flux Determination

2.7.1 Isolation of Primary Hepatocytes

Primary hepatocytes were prepared using the method of Berry *et al* (1991). Rats were anesthetized with 65 mg/kg sodium pentobarbital, and then injected with 100 µL of heparin (1000 units/mL) through the femoral vein. A cannula was inserted into the portal vein, and perfusion was quickly begun with 500 mL of calcium-free Krebs-Henseleit medium (144 mM Na^+, 6 mM K^+, 1.2 mM Mg^{2+}, 126 mM Cl^-, 1.2 mM $H_2PO_4^-$, 1.2 mM SO_4^{2-}, 25 mM HCO_3^-) containing

Figure 2.10: A sample chromatogram of plasma analysis for homocysteine, cysteine, cys-gly, and glutathione. The peak for cysteine appears at 3.1 minutes; the peak for homocysteine appears at 4.2 minutes; the peak for cys-gly appears at 5.5 minutes; and the peak for glutathione appears at 7.3 minutes. The standard, 8-aminonaphthalene-1,3,6-trisulfonic acid, appears at 1.5 minutes.



2 mM EGTA, 20 mM glucose, 2.1 mM lactate, and 0.3 mM pyruvate (pH 7.4) and gassed, for 20 minutes, with 19:1 O_2/CO_2. The perfusion flow rate was 40 mL/min. Following a 12-minute flow-through period, the medium was switched to 500 mL Krebs-Henseleit solution containing 1.3 mM Ca^{2+}, 20 mM glucose, 2.1 mM lactate, and 0.3 mM pyruvate, which was also gassed with O_2/CO_2 for 20 minutes.

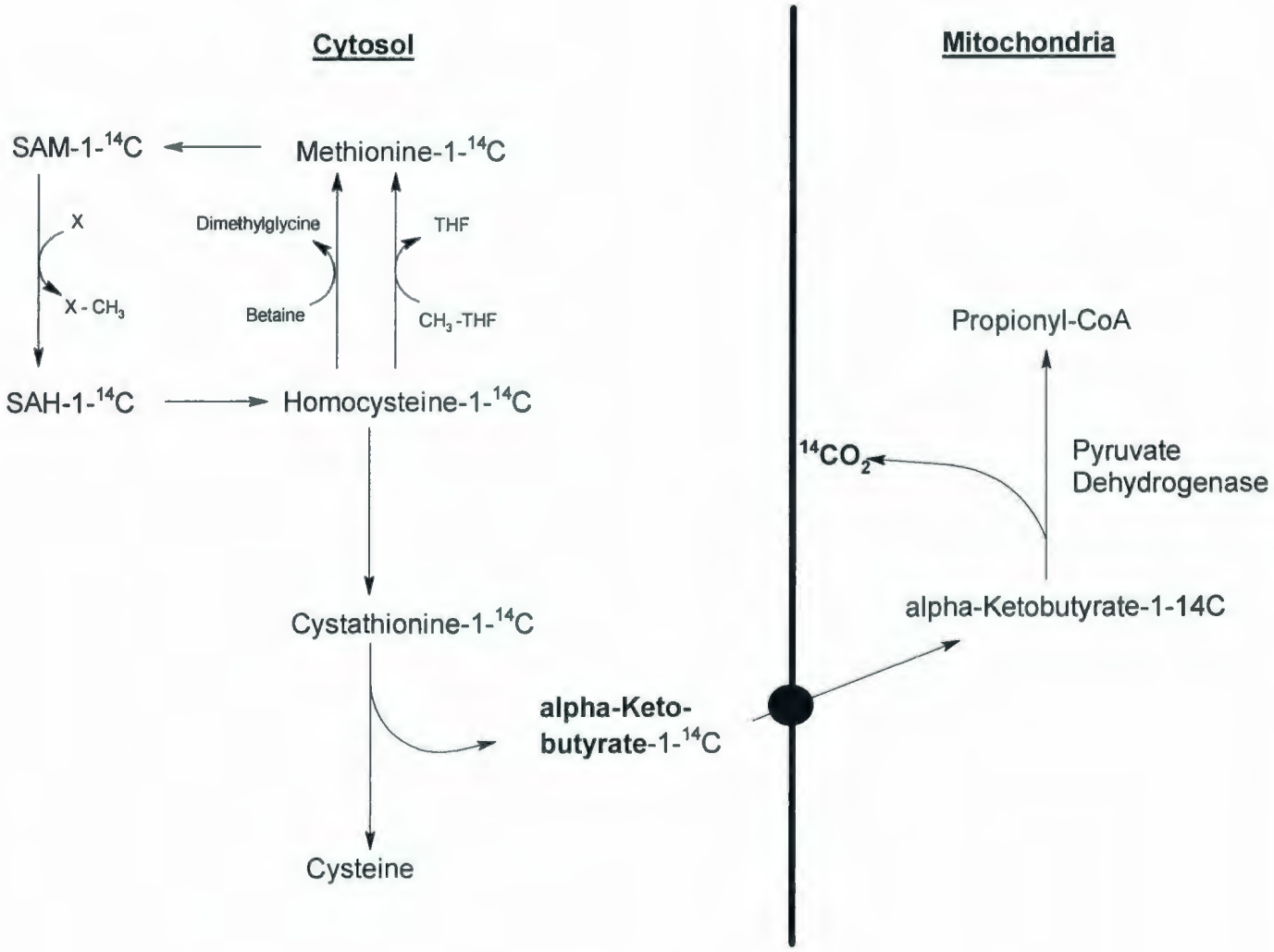
A cannula was inserted through the right atrium and into the inferior vena cava. Krebs-Henseleit medium supplemented with 0.25% BSA and collagenase (50 mg/100 mL) was then recirculated through the liver. To begin the flow through the liver, the inferior vena cava was cut below the kidney. The recirculation of perfusate continued until the liver became soft (approximately 20 minutes), at which point the liver was transferred to a petri dish containing collagenase medium and massaged gently to liberate cells. The resulting suspension was incubated at 37°C for 10 minutes in a shaking water bath under constant gassing with O_2/CO_2. Following filtration through a cheesecloth, the cells were centrifuged at 600 rpm for 2 minutes. The cells were resuspended in Krebs-Henseleit solution containing calcium, and the filtration was repeated. After a final wash with Krebs-Henseleit medium containing 2.5% BSA, the cell pellet was resuspended in this medium. Hepatocytes were quantified by drying 3.0 mL of resuspended cells and 3.0 mL of 2.5% BSA Krebs-Henseleit medium in tared vessels at 50 degrees for 24 hours. The difference in the weights represented the dry weight of the cells. Cell viability was determined by means of 0.1% trypan blue exclusion.

2.7.2 $^{14}CO_2$ recovery from isolated hepatocytes

The determination of the flux through the transsulfuration pathway takes advantage of the fact that the CGL reaction produces α-ketobutyrate. Incubation of the primary hepatocytes with [1-^{14}C]methionine will result in the production of [1-^{14}C]α-ketobutyrate, which can act as a substrate for pyruvate dehydrogenase, liberating $^{14}CO_2$ in the process. [1-^{14}C]α-ketobutyrate is treated with hydrogen peroxide, which decarboxylates it. After determining the amount of $^{14}CO_2$ that was released, the sum of the two values results in a measure of the flux through transsulfuration, as can be seen in Figure 2.11.

The primary hepatocytes were incubated with 1 mM L-[1-^{14}C]methionine (American Radiolabeled Chemicals, Inc., St. Louis, MO) for 30 minutes. At the end of the incubation period, Incubations were terminated with the injection of 0.3 mL of 30% (wt/vol) perchloric acid via a syringe through rubber septa with suspended centre wells containing NCS tissue solubilizer, and the $^{14}CO_2$ was collected for one hour. Each of the centerwells was added to scintillation vials containing Omnifluor scintillation fluid. New centerwells were added to each flask and 0.3 mL of 30% (wt/vol) hydrogen peroxide was added to the samples in the same way as the perchloric acid. Once again, the $^{14}CO_2$ was collected for one hour, and the centerwells added to Omnifluor scintillation fluid-containing scintillation vials. The radioactivity was then determined using a scintillation counter.

Figure 2.11: Transsulfuration flux determination in isolated hepatocytes. The radiolabelled carbon atom proceeds from the transmethylation pathway into the transsulfuration pathway, where the action of CBS removes the label in the form of α-ketobutyrate. Some of the α-ketobutyrate does move into the mitochondria, where it acts as a substrate for pyruvate dehydrogenase (PDH), which produces CO_2. Any unreacted 1-^{14}C-α-ketobutyrate can be decarboxylated with H_2O_2.


Cytosol
Mitochondria
SAM-1-14C
Methionine-1-14C
X
X - CH3
Dimethylglycine
Betaine
THF
CH3-THF
SAH-1-14C
Homocysteine-1-14C
Propionyl-CoA
14CO2
Pyruvate
Dehydrogenase
alpha-Ketobutyrate-1-14C
Cystathionine-1-14C
alpha-Keto-
butyrate-1-14C
Cysteine

2.8 RNA Isolation and Northern Blot Analysis

Total RNA was isolated by the rapid guanidinium isothiocyanate method as described by Chomczynski and Sacchi (1987). RNA was denatured in formamide (50%), formaldehyde (6.5%) sample buffer at 65°C for 15 minutes. The RNA was then separated on formaldehyde (1.8%), agarose (0.8%), and transferred to a nylon (S & S Nytran) membrane (Schleicher & Schuell). RNA was fixed by UV cross-linking. The membranes were prehybridized and then hybridized with a cDNA probe specific for rat CBS (2.3 kb *Eco*RI fragment of rat CBS cDNA, which was random-primer-labelled with [α-^{32}P]dCTP (3000 Ci/mmol; PerkinElmer Life Sciences) using the MegaPrime labelling kit (Amersham Biosciences) according to the manufacturer's instructions). The membranes were stripped and hybridized with a random primer radiolabelled β-actin cDNA probe (CLONTECH Laboratories, Palo Alto, CA) and washed at high stringency (final wash, 0.1% SSC, 0.1% SDS at 53°C). Autoradiography was carried out by exposure of the blot to Kodak XAR or Biomax film. Autoradiographs were scanned on Chemi-Imager 4000, and RNA levels were quantitated with Alpha Ease software (Alpha Innotech Corporation, San Leandro, CA).

2.9 Statistical Analysis

All results are expressed as mean $\pm$ standard deviation. Statistical analysis between means was determined by means of a one-way ANOVA followed by a Neuman-Keul's multiple means test. In each analysis, a P value < 0.05 indicated a significant difference.

Chapter 3: Results and Discussion

3.1 Transsulfuration Flux in Hepatocytes – Effect of Cysteine

3.1.1 Background

It is known that the composition of the diet affects the metabolism of methionine in mammals. One of the most important of these dietary effects is the methionine-sparing effect, whereby the requirements for dietary methionine are reduced by cysteine. While this effect is well known *in vivo*, the effect of acute addition of cysteine to incubated hepatocytes has not been investigated. To examine this effect, hepatocytes from 4 Sprague-Dawley rats were incubated with different concentrations of 1-^{14}C-methionine and unlabelled cysteine, and the transsulfuration flux determined. Both radiolabelled α-ketobutyrate and carbon dioxide were measured as a measure of total transsulfuration flux (Stead *et al,* 2001). If the methionine-sparing effect is evident in hepatocytes, we would see a decrease in the transsulfuration flux upon addition of cysteine.

3.1.2 Results

Two concentrations of methionine were used – 1.0 mM 1-^{14}C-methionine and 0.1 mM 1-^{14}C-methionine. For each of these groups, hepatocytes were also incubated with and without 1.0 mM cysteine or with and without 0.1 mM cysteine. The recovered label was measured as nmol ^{14}C/30 min incubation time/mg dry weight of the hepatocytes. As both $^{14}CO_2$ and ^{14}C-α-ketobutyrate are components of transsulfuration flux, both of these are presented in Table 3.1.

Table 3.1 shows that, at 1.0 mM methionine, the addition of cysteine, at either concentration, was without effect. At 0.1 mM methionine, however, the

addition of an unphysiologically high cysteine concentration (1.0 mM) results in a 30% decrease in transsulfuration flux.

Our results show that, at physiological concentrations of methionine and cysteine, no sparing effect is evident. This result suggests that the sparing effect *in vivo* does not occur by an acute mechanism.

1.0 mM methionine	1-^{14}C-methionine	1-^{14}C-methionine + 0.1 mM cysteine	1-^{14}C-methionine + 1.0 mM cysteine
CO_2	4.18 ± 0.53	3.96 ± 0.29	3.94 ± 0.50
α-ketobutyrate	1.48 ± 0.29	1.40 ± 0.30	1.24 ± 0.39
Total	5.66 ± 0.66[a]	5.35 ± 0.25[a]	5.18 ± 0.71[a]
0.1 mM methionine	**1-^{14}C-methionine**	**1-^{14}C-methionine + 0.1 mM cysteine**	**1-^{14}C-methionine + 1.0 mM cysteine**
CO_2	1.66 ± 0.22	1.67 ± 0.12	1.23 ± 0.36
α-ketobutyrate	0.94 ± 0.17	0.98 ± 0.29	0.59 ± 0.07
Total	2.60 ± 0.22[b]	2.65 ± 0.29[b]	1.82 ± 0.33[c]

Table 3.1: The effect of varying concentrations of methionine and cysteine on transsulfuration flux in isolated rat hepatocytes. The values are presented in terms of nmol/30 min/mg dry weight of hepatocytes.

3.2 The Effect of Varying Concentrations of Dietary Methionine and Cysteine on Hepatic Sulfur Amino Acid Metabolism in Adult Rats

3.2.1 Background

The sparing effect on methionine, an essential amino acid in mammals, by the addition of cysteine to the diet has been shown several times (Finkelstein, Martin & Harris, 1986; Finkelstein, Martin & Harris, 1988). To investigate this mechanism, we fed rats one of four different diets. The first diet contained only methionine as a sulfur amino acid, which was provided at an insufficient amount (Low SAA); the second diet contained a supplement of methionine (High Methionine); the third diet contained an insufficient amount of methionine with a supplement of cystine (High Cystine); while the fourth diet contained a supplement of both sulfur amino acids (High Methionine + Cystine). Rats were fed these diets *ad libitum* for one week, after which plasma and liver metabolites, as well as enzyme activities were determined.

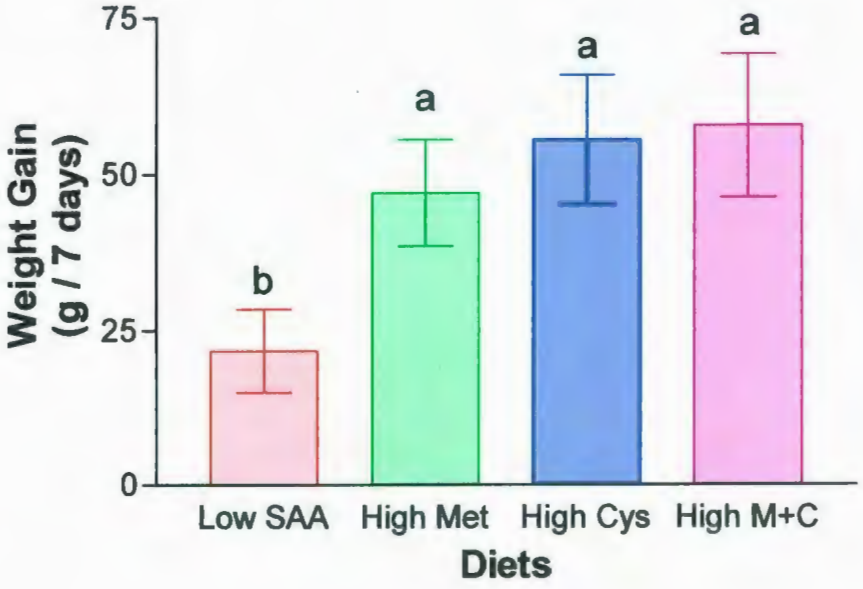
3.2.2 Results

3.2.2.1 Body Weight

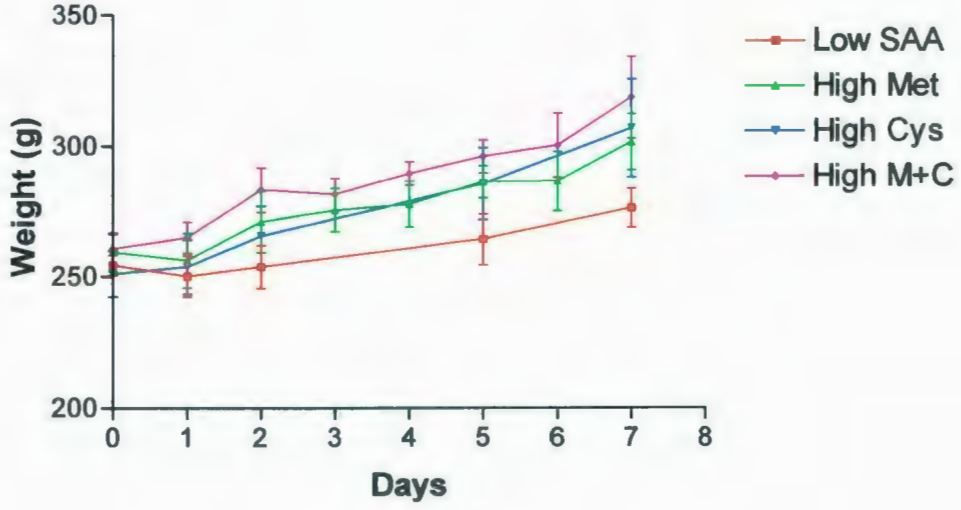
Sprague-Dawley rats with an initial weight of approximately 250 g each were used. The rats were weighed every day. At the end of the feeding experiment, the Low SAA group had gained an average of 21.75 g, whereas the High Methionine group, the High Cystine, and High Methionine + Cystine groups had all gained significantly more (mean of 47.00 g, 55.30 g, and 57.75 g respectively). Weight gain is presented in Figure 3.1.

Figure 3.1: The effect of varying dietary methionine and cystine on weight gain in adult Sprague-Dawley rats. Figure 3.1a demonstrates the average weight gain in grams of each of the four diet groups over the 7 day experiment. Figure 3.1b displays daily weight gain in grams of the rats over the seven-day feeding period. Different subscripts indicate significantly different results.

a.


75
50
25
0
Weight Gain
(g / 7 days)
b
a
a
a
Low SAA
High Met
High Cys
High M+C
Diets

b.


350
300
250
200
Weight (g)
0
1
2
3
4
5
6
7
8
Days
Low SAA
High Met
High Cys
High M+C

3.2.2.2 Transmethylation and Transsulfuration

Table 3.2 shows that increased methionine intake, but not cystine intake, increased hepatic MAT activity. Table 3.3 shows that increased dietary methionine, not cystine, results in a massive increase in SAM levels. There were also very large increases in hepatic SAH levels. Together with the elevated hepatic methionine levels, these results indicate a very large increase in transmethylation. If this is so, the question arises: which transmethylation reaction(s) is (are) increased? The conventional wisdom is that the glycine-N-methyltransferase reaction acts as an over-flow methylation reaction for the disposal of excess methionine. Evidence that this is occurring in this situation is provided in Tables 3.4 and 3.5, where a substantial increase in both plasma and liver sarcosine is evident in the animals fed the high methionine diets. Increased flux through the transmethylation pathway will generate increased quantities of homocysteine. Unfortunately, we were unable to measure the hepatic content of this amino acid but the massively increased plasma tHcy provides evidence for the increased synthesis of this amino acid. Increased catabolism of methionine occurs via increased flux through the transsulfuration pathway. The ingestion of the high methionine diets did not result in increased CBS activity when measured *in vitro*; however, the elevated SAM levels would be expected to activate CBS and promote increased transsulfuration. Evidence that this has actually occurred is provided by two observations – the increased plasma and liver levels of cystathionine and of 2-aminobutyrate, which is produced by the transamination of α-ketobutyrate (Table 3.2).

Assay	Low SAA	High Methionine	High Cystine	High Methionine + Cystine
MAT				
nmol/min/mg protein	3.50 ± 0.47 [a]	5.75 ± 0.30 [b]	3.00 ± 0.24 [a]	6.14 ± 0.39 [b]
nmol/min/g liver	335.45 ± 38.9 [a]	539.02 ± 40.45 [b]	292.28 ± 33.67 [a]	556.88 ± 78.82 [b]
CBS				
nmol/min/mg protein	11.47 ± 0.81	11.17 ± 1.56	6.86 ± 1.21	12.82 ±1.52
nmol/min/g liver	1.11 ± 0.14	1.05 ± 0.16	0.66 ± 0.10	1.12 ± 0.23
CGL				
nmol/min/mg protein	16.32 ± 2.11 [a]	27.83 ± 4.32 [b]	22.15 ± 1.53 [b]	28.83 ± 1.23 [b]
nmol/min/g liver	1578 ± 293.5 [a]	2598 ± 306.6 [b]	2158 ± 251.0 [b]	2600.1 ± 174.4 [b]
BHMT				
nmol/min/mg protein	1.73 ± 0.21 [a]	1.07 ± 0.10 [b]	1.92 ± 0.28 [a]	1.80 ± 0.20 [a]
nmol/min/g liver	167.00 ± 23.87 [a]	101.20 ± 14.03 [b]	186.13 ± 17.19 [a]	162.75 ± 24.72 [a]
MS				
nmol/min/mg protein	0.084 ± 0.008	0.089 ± 0.008	0.099 ± 0.002	0.102 ± 0.004
nmol/min/g liver	8.08 ± 0.43	8.33 ±1.13	9.67 ± 0.45	9.22 ± 0.44
MTHFR				
nmol/min/mg protein	0.20 ± 0.02	0.21 ± 0.04	0.21 ± 0.03	0.20 ± 0.04
nmol/min/g liver	0.042 ± 0.009	0.043 ± 0.008	0.044 ± 0.007	0.042 ± 0.008

Table 3.2: The Effect of Varying Dietary Sulfur Amino Acid Concentrations on the Activity of Enzymes in the Methionine Metabolic Pathway in Adult Sprague-Dawley Rats. Data are presented in nmol/min/mg protein and nmol/min/g liver. Each superscript represents data that is significantly different from the other.

Metabolite	Low SAA	High Methionine	High Cystine	High Methionine + Cystine
Methionine				
Plasma (μM)	53.9 ± 4.4 [a]	478.6 ± 166.7 [b]	52.6 ± 4.5 [a]	488.3 ± 150.9 [b]
Liver (nmol/g)	30.7 ± 7.8 [a]	167.1 ± 40.6 [b]	35.4 ± 2.5 [a]	146.1 ± 107.1 [b]
SAM (nmol/g)	81.5 ± 3.4[a]	577.8 ± 72.1[b]	80.0 ± 10.0[a]	351.0 ± 24.1[c]
SAH (nmol/g)	6.8 ± 0.9[a]	78.4 ± 15.8[b]	7.0 ± 1.6[a]	80.1± 6.6[b]
Total Homocysteine				
Plasma (μM)	15.6 ± 0.8 [a]	217.9 ± 46.6 [b]	8.1 ± 2.0 [a]	379.4 ± 71.4 [c]
Cystathionine				
Plasma (μM)	Trace [a]	8.3 ± 1.4 [b]	Trace [a]	15.4 ± 3.1 [c]
Liver (nmol/g)	32.9 ± 8.2 [a]	79.6 ± 48.7 [b]	20.8 ± 1.2 [c]	80.9 ± 27.1 [b]
Total Cysteine				
Plasma (μM)	169.3 ± 14.2	163.3 ± 22.0	207.8 ± 66.1	186.7 ± 50.0

Table 3.3: The Effect of Varying Dietary Sulfur Amino Acid Concentrations on Intermediates of Methionine Metabolism Substrates in Adult Male Sprague-Dawley Rats.

Table 3.4: The Effect of Varying Dietary Sulfur Amino Acids on Plasma Amino Acid Concentrations in Adult Male Sprague-Dawley Rats. All measurements are in μM.

Plasma Metabolite	Low SAA	High Methionine	High Cystine	High Methionine + Cystine
Taurine	92.9 ± 7.7 [a]	301.3 ± 24.5 [b]	310.6 ± 53.9 [b]	369.1 ± 89.1 [b]
Urea	6943.5 ± 1004.3	21757.6 ± 16200.6	26144.8 ± 17116.8	105959.0 ± 56817.0
Aspartic Acid	19.9 ± 2.5 [a]	28.7 ± 6.1 [ab]	27.8 ± 3.5 [ab]	41.5 ± 16.2 [b]
Hydroxyproline	36.8 ± 6.8	34.9 ± 2.4	45.5 ± 11.5	46.8 ± 8.5
Threonine	227.7 ± 17.1 [ab]	194.5 ± 17.5 [b]	268.6 ± 41.6 [ab]	267.9 ± 48.1 [ab]
Serine	166.0 ± 12.2	159.0 ± 23.6	199.0 ± 11.1	168.4 ± 39.0
Asparagine	89.0 ± 11.2	79.7 ± 18.7	120.2 ± 20.2	118.2 ± 41.8
Glutamic Acid	206.2 ± 49.2	146.5 ± 9.4	196.4 ± 49.8	168.5 ± 52.8
Glutamine	436.5 ± 49.7 [a]	401.7 ± 45.6 [a]	333.9 ± 13.5 [b]	447.7 ± 48.0 [a]
Sarcosine	2.7 ± 2.3 [a]	16.3 ± 8.7 [b]	2.3 ± 1.5 [a]	18.5 ± 5.0 [b]
Proline	148.4 ± 18.3	152.2 ± 10.6	168.8 ± 16.3	176.5 ± 28.2
Glycine	443.1 ± 53.4 [a]	310.1 ± 81.8 [b]	521.6 ± 54.2 [a]	526.0 ± 124.4 [a]
Alanine	654.3 ± 76.7	670.6 ± 123.1	725.6 ± 84.6	722.9 ± 148.6
Citruline	54.2 ± 6.2 [a]	71.2 ± 8.0 [b]	52.8 ± 8.3 [a]	85.9 ± 5.2 [c]
a-Amino-n-butyric Acid	7.8 ± 4.1 [a]	32.4 ± 12.4 [b]	5.4 ± 3.6 [a]	33.9 ± 6.7 [b]
Valine	201.8 ± 16.5 [a]	117.1 ± 6.5 [b]	209.7 ± 39.6 [a]	122.6 ± 36.5 [b]
Cystine	Below detection levels [a]	Below detection levels [a]	Below detection levels [a]	18.4 ± 7.4 [b]
Methionine	53.9 ± 4.4 [a]	476.8 ± 166.7 [b]	52.6 ± 4.5 [a]	488.3 ± 150.9 [b]
Isoleucine	108.0 ± 11.2 [a]	61.3 ± 9.0 [b]	115.1 ± 18.9 [a]	61.0 ± 14.2 [b]
Leucine	97.3 ± 8.1 [a]	65.6 ± 16.9 [b]	114.4 ± 28.4 [a]	55.5 ± 22.5 [b]
Tyrosine	62.2 ± 5.5 [a]	48.9 ± 7.2 [a]	61.5 ± 5.5 [a]	109.3 ± 24.1 [b]
Phenylalanine	69.4 ± 3.5 [a]	53.5 ± 1.7 [b]	61.7 ± 8.3 [ab]	57.9 ± 10.5 [ab]
Tryptophan	107.7 ± 5.5 [a]	92.4 ± 9.0 [ab]	93.3 ± 12.5 [ab]	78.7 ± 12.7 [b]
Ethanolamine	15.4 ± 9.2	16.0 ± 5.0	8.5 ± 2.3	14.8 ± 1.7
Ammonia	125.3 ± 7.2 [a]	105.7 ± 5.8 [b]	104.6 ± 5.2 [b]	107.1 ± 7.0 [b]
Hydroxylysine	1.0	1.0	1.0	1.0
Ornithine	71.4 ± 9.1 [a]	80.6 ± 18.3 [a]	83.9 ± 15.9 [a]	113.7 ± 8.2 [b]
Lysine	442.5 ± 92.7	515.4 ± 73.5	532.5 ± 99.2	622.6 ± 101.7
Histidine	35.3 ± 7.1 [a]	44.6 ± 2.9 [b]	49.4 ± 5.4 [b]	53.1 ± 6.4 [b]
3-Methylhistidine	1.3 ± 0.6 [a]	1.0 ± 0.7 [a]	1.0	0
Arginine	162.0 ± 17.4	183.6 ± 41.5	170.1 ± 17.5	187.1 ± 63.0
Cystathionine	Trace [a]	8.3 ± 1.4 [b]	Trace [a]	15.4 ± 3.1 [c]
Homocysteine	Below detection levels [a]	Below detection levels [a]	Below detection levels [a]	10.7 [b]

Liver Metabolite	Low SAA	High Methionine	High Cystine	High Methionine + Cystine
Taurine	4505.3 ± 281.7 [a]	10137.7 ± 5589.9 [b]	10169.2 ± 1159.5 [b]	10791.4 ± 964.8 [b]
Urea	24655.9 ± 11080.7 [a]	1554335.1 ± 623944.6 [b]	2056561.5 ± 167259.8 [b]	2705963 ± 515397.4 [c]
Aspartic Acid	2386.8 ± 413.7	3004.3 ± 1430.7	2402.9 ± 1478.0	1181.3 ± 1131.5
Hydroxyproline	Below detection levels	Below detection levels	Below detection levels	Below detection levels
Threonine	269.6 ± 50.1 [a]	143.1 ± 40.4 [b]	226.5 ± 32.0 [a]	223.4 ± 49.6 [a]
Serine	539.0 ± 164.8 [a]	86.9 ± 31.1 [b]	416.4 ± 62.3 [a]	111.0 ± 25.1 [b]
Asparagine	81.1 ± 27.4	44.2 ± 20.2	74.5 ± 20.3	73.2 ± 34.9
Glutamic Acid	2784.1 ± 690.8 [a]	854.6 ± 386.4 [b]	1442.4 ± 135.2 [b]	873.4 ± 365.2 [b]
Glutamine	4536.3 ± 839.7	3041.2 ± 1821.9	3115.6 ± 403.2	3201.4 ± 374.9
Sarcosine	24.2 ± 21.6 [a]	87.0 ± 51.8 [ab]	57.8 ± 23.6 [a]	112.6 ± 43.3 [b]
Proline	646.2 ± 244.8	193.4 ± 178.3	583.5 ± 317.8	632.4 ± 159.8
Glycine	4199.8 ± 1129.4 [a]	732.0 ± 394.1 [b]	3128.6 ± 540.9 [c]	1273.4 ± 152.3 [b]
Alanine	2993.1 ± 951.	1967.5 ± 1502.2	2413.9 ± 266.7	2558.6 ± 352.7
Citruline	50.2 ± 4.2 [abc]	22.0 ± 14.4 [ab]	43.5 ± 15.8 [abc]	66.2 ± 22.9 [ac]
a-Amino-n-butyric Acid	12.9 ± 9.4	51.7 ± 43.3	11.1 ± 5.4	43.3 ± 35.1
Valine	140.8 ± 14.3 [a]	64.5 ± 33.3 [b]	134.2 ± 20.9 [a]	66.0 ± 30.0 [b]

Table 3.5a: The Effect of Varying Dietary Sulfur Amino Acid Concentrations on Hepatic Amino Acid Concentrations in Adult Male Sprague-Dawley Rats.

All metabolites are expressed as µmol/g wet weight.

Liver Metabolite	Low SAA	High Methionine	High Cysteine	High Methionine + Cystine
Cysteine	Below detection levels	155.7 ± 138.2 [a]	Below detection levels	257.7 ± 62.7 [a]
Methionine	30.7 ± 7.8 [a]	167.1 ± 40.6 [b]	35.4 ± 2.5 [a]	146.1 ± 107.1 [b]
Isoleucine	101.2 ± 23.6 [a]	39.0 ± 25.6 [b]	89.7 ± 14.3 [a]	59.1 ± 11.8 [b]
Leucine	121.6 ± 23.5 [a]	59.8 ± 31.4 [b]	101.2 ± 29.8 [a]	55.2 ± 28.4 [b]
Tyrosine	24.6 ± 11.1	22.6 ± 21.2	36.9 ± 9.1	46.2 ± 11.5
Phenylalanine	61.6 ± 16.1	63.5 ± 30.3	47.6 ± 8.2	65.3 ± 18.1
Tryptophan	Below detection levels	Below detection levels	Below detection levels	Below detection levels
Ethanolamine	30.1 ± 13.0	12.9 ± 1.0	17.0 ± 7.2	21.0 ± 18.3
Ammonia	720.2 ± 223.0	549.0 ± 120.1	592.4 ± 74.8	643.4 ± 79.2
Hydroxylysine	Below detection levels	Below detection levels	Below detection levels	Below detection levels
Ornithine	290.3 ± 74.9	182.7 ± 85.2	165.7 ± 33.7	231.3 ± 71.4
Lysine	625.6 ± 132.4	601.7 ± 311.9	753.0 ± 138.1	727.8 ± 167.7
Histidine	Below detection levels	Below detection levels	Below detection levels	Below detection levels
3-Methylhistidine	502.1 ± 112.8	349.0 ± 178.0	463.8 ± 25.9	377.3 ± 40.4
Arginine	3.5 ± 0.5 [a]	7.1 ± 4.5 [a]	37.4 ± 22.6 [b]	43.1 ± 17.7 [b]
Cystathionine	32.9 ± 8.2 [a]	79.6 ± 48.7 [b]	20.8 ± 1.2 [c]	80.9 ± 27.1 [b]
Homocysteine	Below detection levels	Below detection levels	Below detection levels	Below detection levels

Table 3.5b: The Effect of Varying Dietary Sulfur Amino Acid Concentrations on Hepatic Amino Acid Concentrations in Adult Male Sprague-Dawley Rats.

All metabolites are expressed as µmol/g wet weight.

3.2.2.3 Remethylation

Few conclusions can be drawn regarding remethylation rates. The high methionine diets did not alter the measured *in vitro* activity of methionine synthase or of MTHFR; however, it is likely that, *in vivo,* the elevated SAM levels would have inhibited MTHFR and, therefore, curtailed folate-dependent remethylation. The high methionine diet decreased the activity of BHMT, but this was not evident in the animals fed the high methionine plus cystine diets. The relevance of this observation is not immediately apparent.

3.2.2.4 Amino Acids

The High Methionine group, the High Cystine group, and the High Methionine + Cystine group all displayed an increase of approximately 125% in hepatic taurine concentrations over the Low SAA group. These differences are also represented in the plasma taurine levels. The increased taurine reflects the increased availability of cysteine in these groups.

While examining our amino acid data, it was discovered that the branched chain amino acids were seemingly affected by sulfur amino acid supplementation. In plasma, it was found that a methionine supplement decreased valine, isoleucine, and leucine concentrations by almost 35-40% (Table 3.4). This finding was also evident in our hepatic branched chain amino acid analysis (Table 3.5).

Since all three BCAA amino acids decrease with the high methionine supplement, it is likely that some aspect of methionine metabolism affects the common catabolic pathway that the BCAA share. This pathway consists of a

transamination by branched chain aminotransferase, followed by a decarboxylation by the branched chain α-ketoacid dehydrogenase complex; the branched-chain α-keto acid dehydrogenase (BKCD) is recognized as the regulatory enzyme of BCAA catabolism. The BCKD is regulated by phosphorylation, with an active unphosphorylated form (Brosnan and Brosnan, 2006). Interconversion of phosphorylated and unphosphorylated forms is catalyzed by a specific kinase and phosphatase. The kinase is inhibited by the branched-chain α-keto acids, which ensures their catabolism. The kinase is also inhibited by α-ketomethionine and by α-ketobutyrate (Jones and Yeaman, 1986). α-ketomethionine may be expected to increase in rats fed a high methionine diet. So, too, may α-ketobutyrate levels, as may be inferred from the markedly increased levels of α-aminobutyrate (its transamination partner) in both the plasma and liver of these animals (Table 3.4-3.5). One possible suggestion, therefore, is that activation of the BKCD brought about by one or both of these keto-acids may account for the decreased levels of the BCAA.

Another possible reason for this decrease in BCAA in the high methionine diets could be due to the fact that all three are transported across the intestinal epithelial mucosa via the same neutral amino acid transporter (Broen *et al*, 2005). It is possible that the high concentration of methionine could interfere with the absorption of the BCAA in the gut and, since all BCAA are essential and must come from the diet, would result in the lowered levels seen in the results.

3.2.2.5 Cystathionine β-Synthase mRNA

Figure 3.2 shows an increased CBS mRNA in the high methionine-fed group only, which was not reflected by *in vitro* activity. Additionally, a decrease in *in vitro* CBS activity was detected in our cystine-supplemented animals only, but was not reflected in our CBS mRNA analysis. In a paper published by Yamamoto, Tanaka, and Noguchi (1995), the effect of cystine on CBS activity and mRNA was examined. Adult rats (12 weeks) were fed diets containing 1.0% methionine + 0% cystine or 0.25% methionine + 2% cystine. These investigators also found a 40% decrease in activity, without a significant change in CBS mRNA, much the same as the results of our experiment. It would seem, therefore, that the regulation of CBS activity in adult rats by cystine is likely via another mechanism than transcriptional control.

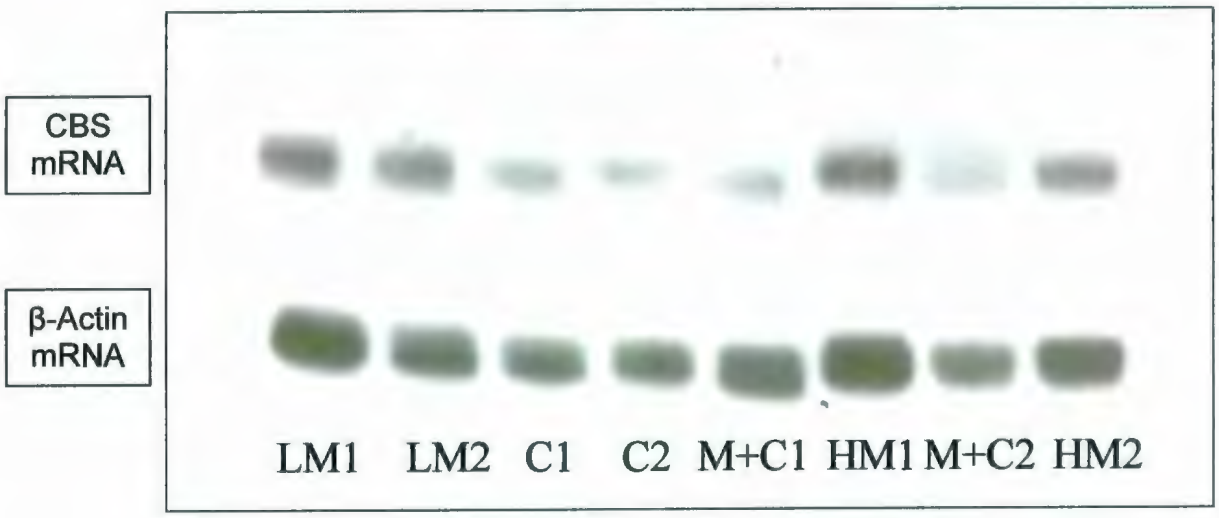
3.2.2.6 Transsulfuration Flux in Hepatocytes

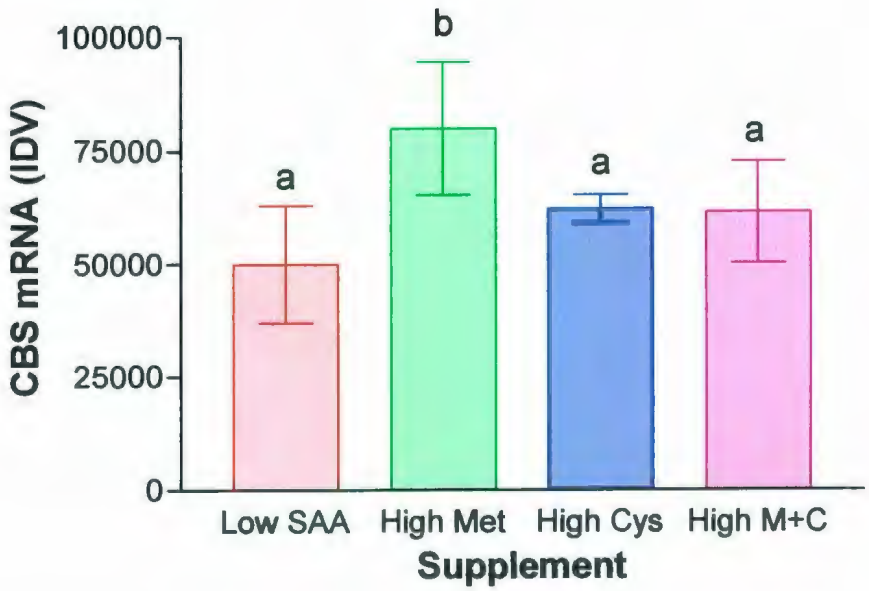
We also examined the transsulfuration flux in hepatocytes from rats fed the low SAA diet and those fed a high cystine diet. There was no significant difference in transsulfuration flux at 1.0 mM 1-^{14}C-methionine; however, when the hepatocytes were incubated with 0.1 mM 1-^{14}C-methionine, there was a significant decrease of 35% in transsulfuration flux in hepatocytes prepared from rats fed the cystine-supplemented diet (Table 3.6).

3.2.3 Conclusion

The addition of a methionine supplement to a diet containing a minimal amount of sulfur amino acids stimulates flux through transmethylation and transsulfuration. Increased transmethylation is suggested by the increase in MAT

Figure 3.2: The Effect of Varying Dietary Sulfur Amino Acid Concentrations on Hepatic Cystathionine β-Synthase mRNA Concentrations. Figure 3.2a represents the Northern Blot, where LM = Low SAA, HM = High Methionine, C = High Cystine, and M+C = High Methionine + Cystine. The numbers 1 and 2 represent sample animals from each diet group. Figure 3.2b represents the statistical analysis of the Northern Blot by comparing integrated density values; differing subscripts indicate significantly different results.


CBS mRNA
β-Actin mRNA
LM1
LM2
C1
C2
M+C1
HM1
M+C2
HM2


CBS mRNA (IDV)
100000
75000
50000
25000
0
a
b
a
a
Low SAA
High Met
High Cys
High M+C
Supplement

Transsulfuration Flux (nmol/30 min/mg dry weight)		
	0.1 mM 1-^{14}C-Methionine	
	Low SAA Diet	**Cystine Supplemented**
α-Ketobutyrate	0.92 ± 0.27 [a]	0.59 ± 0.22 [a]
CO_2	2.22 ± 0.62 [a]	1.45 ± 0.39 [a]
Total	3.11 ± 0.42 [a]	2.01 ± 0.29 [b]
	1.0 mM 1-^{14}C-Methionine	
	Low SAA Diet	**Cystine Supplemented**
α-Ketobutyrate	3.00 ± 0.31 [b]	2.33 ± 0.38 [b]
CO_2	1.72 ± 0.46 [a]	1.57 ± 0.35 [a]
Total	4.72 ± 0.46 [c]	3.89 ± 0.68 [c]

Table 3.6: The Effect of Varying Dietary Sulfur Amino Acid Concentrations on Transsulfuration Flux in Adult Sprague-Dawley Isolated Hepatocytes Incubated with 1.0 mM and 0.1 mM 1-^{14}C-Methionine. Superscripts mark differences between the same groups of measurement.

activity and SAM and SAH concentrations. This increase in SAH would lead to an increase in liver homocysteine, resulting in a number of fates. The first fate that excess homocysteine may face would be transport into the plasma, and we observed that a high methionine supplement in the diet did, in fact lead to an increase in plasma homocysteine concentrations. The second fate would lead homocysteine through the remethylation pathway; however, due to excess methionine present in the cell, it is likely that the remethylation pathway would be inhibited. This would come about both by the decrease in BHMT activity as well as via inhibition of MTHFR by the increased SAM. Finally, homocysteine would be directed towards cysteine production via the transsulfuration pathway; we also saw evidence for this in terms of increased cystathionine and aminobutryate concentrations, CBS mRNA levels and taurine concentrations.

Cystine supplementation in adult rats at these insufficient levels of methionine seemed to have little to no effect on methionine metabolism; however, in adult hepatocytes incubated with physiological levels of methionine, there was a decrease in transsulfuration flux, indicating that a methionine-sparing effect may still be present.

The purpose of this study was to examine the growth rates of adult Sprague-Dawley rats and the possible sparing effect of cysteine on methionine requirements. While an effect on both growth and the sparing effect were noted in this experiment, adult rats do not grow as fast as young rats. Therefore, the question is raised – does the age of the test animal affect the methionine-sparing

effect of cysteine? Would the effect be more prominent in younger animals as compared to their adult counterparts?

3.3 The Effect of Varying Methionine and Cysteine Concentrations in the Diet on Methionine Metabolism in Young Sprague-Dawley Rats

3.3.1 Background

Cysteine spares the requirement for methionine in the adult rat. This sparing effect is likely via an effect on the transsulfuration pathway. The sparing effect is evident *in vivo* from the growth rates of the animals; diets insufficient in methionine result in reduced growth, though when supplemented with cystine, these same diets result in the identical growth as a high methionine diet. These growth studies were examined in adult rats, whereas the growth of young rats, which grow far faster than adult rats, has yet to be studied. Therefore, we examined the sparing effect of cysteine on methionine requirements in young Sprague-Dawley rats in an attempt to determine the potential mechanisms behind the cysteine-sparing effect.

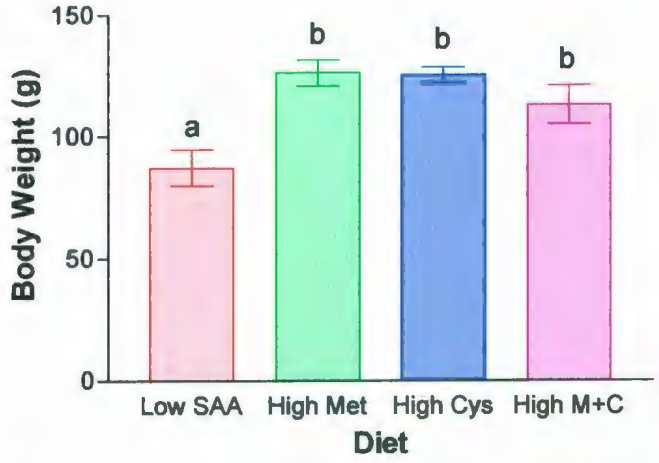
3.3.2 Results

3.3.2.1 Growth Studies

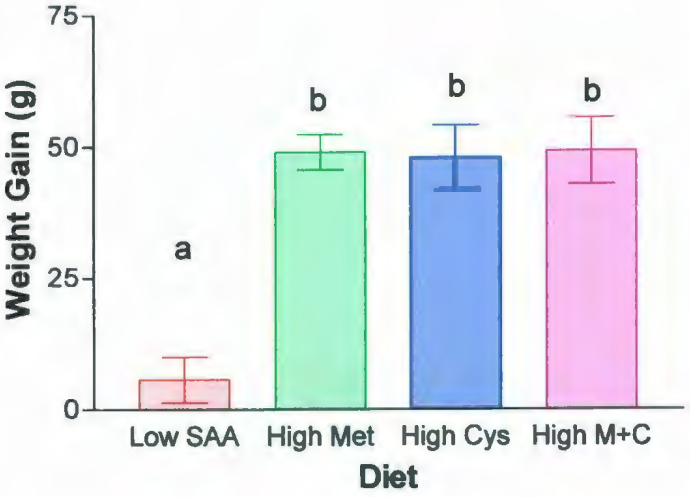
Each of the rats used for this experiment had an initial weight of 50-65g. After a three-day acclimatization period, their average weight was 69.25 $\pm$ 5.95 g, which was followed by the one-week experimental diet-feeding period. On the day of sacrifice, the Low SAA group of rats had grown approximately 6 g from the day they were removed from the acclimatization diet, while the other three animal groups had grown approximately 49 g since that same day. A graphical representation of these results can be seen in Figure 3.3.

Figure 3.3: The Effect of Diets Containing Varying Dietary Sulfur Amino Acid Concentrations on Growth in Young Sprague-Dawley Rats. Figures 3.3a and 3.3b show, respectively, the final body weight and the 7-day weight gain in g; Figure 3.3c demonstrates the growth over the entire feeding period (including the acclimation period). The vertical line in Figure 3.3c represents the end of the acclimation period and the beginning of the seven-day feeding period. Different subscripts represent significant differences among groups.

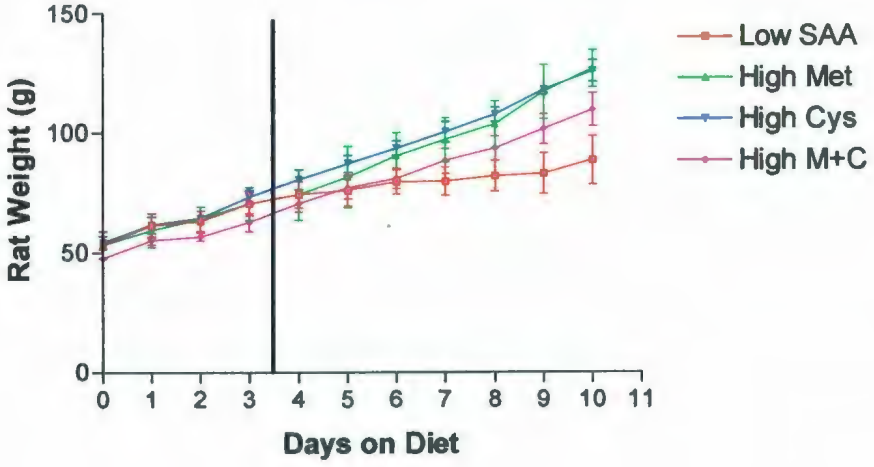
a


150
100
50
0
Body Weight (g)
a
b
b
b
Low SAA
High Met
High Cys
High M+C
Diet

b


75
50
25
0
Weight Gain (g)
a
b
b
b
Low SAA
High Met
High Cys
High M+C
Diet

c


150
100
50
0
Rat Weight (g)
0 1 2 3 4 5 6 7 8 9 10 11
Days on Diet
Low SAA
High Met
High Cys
High M+C

3.3.2.2 Methionine Supplementation

Similar to our adult rats, a methionine supplement increases the activity of MAT (Table 3.7) while simultaneously increasing the hepatic concentrations of SAM, SAH and methionine, as well as plasma tHcy (Table 3.8), which, together, indicates an increase in transmethylation. In contrast to our adult rats, there was no change in hepatic sarcosine levels, and unfortunately, plasma sarcosine concentrations were unable to be measured.

The increased catabolism of excess homocysteine through the transsulfuration pathway is evident in our young rats as well as the adults, as methionine supplements increased hepatic cystathionine levels (Table 3.8). Additionally, though not seen in the adult rats, methionine intake was also associated with increased CBS activity (Table 3.7) and CBS mRNA (Figure 3.4).

Methionine supplementation had no effect on the activity of either MS or MTHFR; however, as with the adult rats, it is likely that increased SAM concentrations would increase MTHFR activity, thereby increasing the remethylation of homocysteine to methionine. Methionine supplementation alone also decreased BHMT activity, thereby slowing the remethylation of homocysteine to methionine.

3.3.2.3 Cystine Supplementation

Cystine supplementation alone has no effect on CBS activity; however, cystine can modify the increase in CBS activity seen with a methionine supplement (Table 3.7). Cystine does have an effect on CBS mRNA that is not

ENZYME ACTIVITY	Low SAA	High Methionine	High Cystine	High Methionine and Cystine
MAT				
nmol/min/g liver	194.5 ± 25.5 a	342.5 ± 65.3 b	190.5 ± 19.6 a	330.0 ± 28.7 b
nmol/min/mg protein	2.23 ± 0.24 a	3.26 ± 0.57 b	1.98 ± 0.16 a	3.46 ± 0.27 b
GNMT				
nmol/min/g liver	854.4 ± 90.1	1030.0 ± 253.7	1031.0 ± 170.3	1125.0 ± 283.4
nmol/min/mg protein	4.35 ± 0.47	5.16 ± 0.57	4.35 ± 0.34	5.34 ± 0.83
CBS				
nmol/min/g liver	0.47 ± 0.07 a	1.04 ± 0.14 b	0.54 ± 0.04 a	0.81 ± 0.02 c
nmol/min/mg protein	5.33 ± 0.67 a	10.83 ± 0.85 b	5.60 ± 0.45 a	7.78 ± 0.59 c
CGL				
nmol/min/g liver	1601 ± 126 a	3322 ± 483 b	2456 ± 207 a	2214 ± 676 a
nmol/min/mg protein	18.17 ± 1.36 a	34.77 ± 4.92 b	25.59 ± 2.89 a	21.20 ± 6.66 a

Table 3.7a: The Effect of Varying Dietary Concentrations on Hepatic Enzyme Activities of Methionine Metabolism in Young Sprague-Dawley Rats. All enzyme activities are presented in both nmol/min/g liver and nmol/min/mg protein. (MAT = Methionine Adenosyltransferase; GNMT = Glycine N-Methyltransferase; CBS = Cystathionine β-Synthase; CGL = Cystathionine γ-Lyase). Different subscripts represent significantly different values within groups.

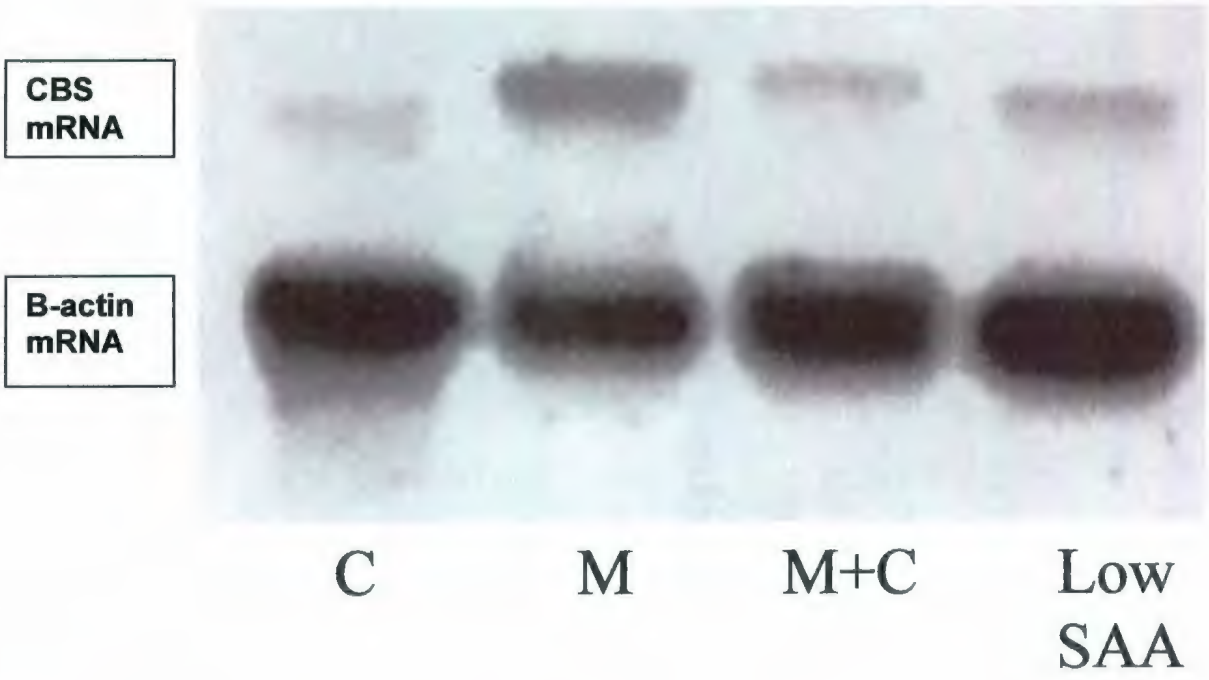
ENZYME ACTIVITY	Low SAA	High Methionine	High Cystine	High Methionine and Cystine
MS				
nmol/min/g liver	11.70 ± 0.53	10.05 ± 0.76	13.24 ± 2.97	10.39 ± 3.01
nmol/min/mg protein	0.133 ± 0.009	0.097 ± 0.011	0.136 ± 0.024	0.109 ± 0.031
MTHFR				
nmol/min/g liver	0.061 ± 0.029	0.086 ± 0.041	0.060 ± 0.046	0.103 ± 0.041
nmol/min/mg protein	0.242 ± 0.061	0.286 ± 0.073	0.231 ± 0.096	0.317 ± 0.068
BHMT				
nmol/min/g liver	80.46 ± 5.29 a	35.58 ± 17.97 b	103.8 ± 14.12 c	136.6 ± 39.28 c
nmol/min/mg protein	0.914 ± 0.621 a	0.332 ± 0.150 b	1.167 ± 0.150 a c	1.441 ± 0.465 c
CHDH				
nmol/min/g liver	8.52 ± 1.69	9.08 ± 2.70	13.01 ± 3.80	9.25 ± 1.00
nmol/min/mg protein	0.240 ± 0.044	0.251 ± 0.051	0.318 ± 0.126	0.249 ± 0.032

Table 3.7b: The Effect of Varying Dietary Concentrations on Hepatic Enzyme Activities of Methionine Metabolism in Young Sprague-Dawley Rats. All enzyme activities are presented in both nmol/min/g liver and nmol/min/mg protein. (MS = Methionine Synthase; MTHFR = Methylenetetrahydrofolate Reductase; BHMT = Betaine:Homocysteine Methyltransferase; CHDH = Choline Dehydrogenase). Different subscripts represent significantly different results within groups.

Substrate	Low SAA	High Methionine	High Cystine	High Methionine and Cystine
Plasma Metabolites (μM)				
Methionine	20.90 ± 3.65 [a]	90.70 ± 20.03 [b]	17.50 ± 6.38 [a]	57.90 ± 8.05 [c]
Homocysteine	8.17 ± 0.42 [a]	19.07 ± 4.56 [b]	6.55 ± 2.87 [a]	22.69 ± 2.92 [b]
Cysteine	56.96 ± 4.85	46.31 ± 7.50	50.68 ± 10.45	44.70 ± 9.75
Cys-Gly	11.81 ± 1.98 [a]	1.66 ± 1.94 [b]	1.99 ± 0.36 [b]	2.06 ± 0.56 [b]
Glutathione	41.58 ± 4.37 [a]	35.99 ± 4.50 [a]	22.19 ± 7.92 [b]	27.09 ± 2.36 [b]
Liver Metabolites (nmoles/g)				
Methionine	56.1 ± 11.6 [a]	121.0 ± 23.8 [b]	80.6 ± 20.9 [a]	118.3 ± 23.4 [b]
SAM	62.1 ± 5.2[a]	155.4 ± 21.6[b]	35.9 ± 20.5[a]	125.1 ± 33.1[b]
SAH	6.7 ± 0.7[a]	14.3 ± 4.3[b]	5.4 ± 3.7[a]	14.6 ± 4.1[b]
Homocysteine	~ 2.5	~ 2.5	~ 2.5	~ 2.5
Cystathionine	68.9 ± 21.5 [a]	225.1 ± 101.5 [b]	44.7 ± 41.5 [a]	218.0 ± 36.3 [b]
Cysteine	25.5 ± 1.9 [a]	69.6 ± 8.0 [b]	98.2 ± 62.8 [b]	92.5 ± 19.5 [b]

Table 3.8: The Effect of Varying Dietary Sulfur Amino Acid Concentrations on Substrates and Products in the Metabolism of Methionine in Young Sprague-Dawley Rats. SAM = S-Adenosylmethionine; SAH = S-Adenosylhomocysteine. All subscripts indicate significantly different results within groups.

Figure 3.4: The Effect of Varying Dietary Concentrations of Sulfur Amino Acids on Hepatic Cystathionine β-Synthase mRNA in Young Sprague-Dawley Rats. Figure 3.4a displays a sample Northern blot. Lane 1 contains the High Cystine sample; Lane 2 contains High Methionine; Lane 3 contains High Methionine + Cystine; and Lane 4 contains Low SAA. Figure 3.4b displays the statistical analysis of the Northern, standardized with β-actin, in terms of IDV.


CBS mRNA
B-actin mRNA
C
M
M+C
Low SAA

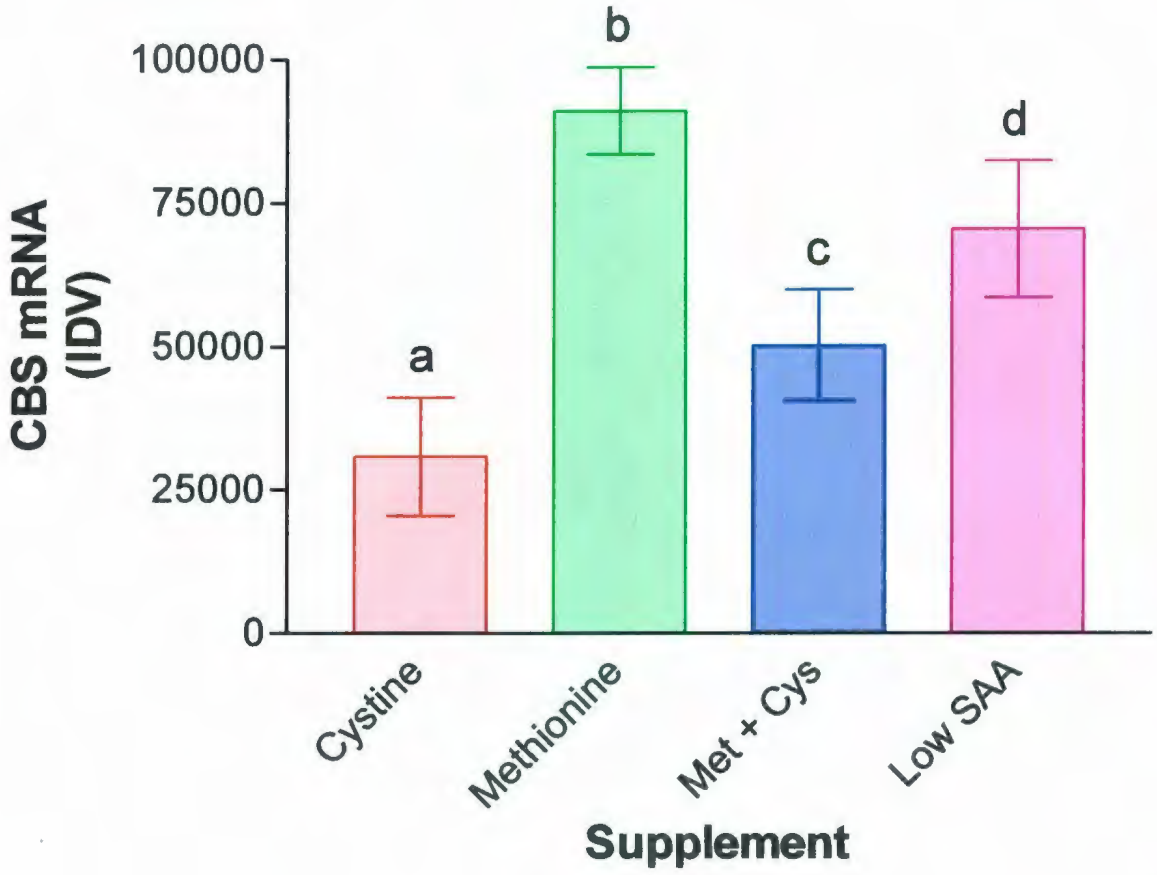

CBS mRNA (IDV)
100000
75000
50000
25000
0
a
b
c
d
Cystine
Methionine
Met + Cys
Low SAA
Supplement

Table 3.9: The Effect of Varying Dietary Sulfur Amino Acid Levels on Plasma Amino Acid Concentrations in Young Sprague-Dawley Rats. All values are presented in terms of μM. Significantly different results within groups are indicated with different subscripts.

Plasma Metabolite	Low SAA	High Methionine	High Cystine	High Methionine and Cystine
Taurine	58.8 ± 2.4 [a]	122.8 ± 20.0 [ab]	179.5 ± 32.4 [b]	223.6 ± 69.3 [c]
Urea	4583.3 ± 745.6	3689.1 ± 627.5	4080.9 ± 1011.8	11292.4 ± 7725.2
Aspartic Acid	16.9 ± 1.9	23.2 ± 5.0	23.6 ± 4.4	20.5 ± 2.9
Hydroxyproline	18.1 ± 5.2	33.1 ± 8.7	31.1 ± 8.3	33.1 ± 13.2
Threonine	394.9 ± 55.1 [a]	219.2 ± 43.1 [b]	168.3 ± 48.0 [b]	174.6 ± 45.4 [b]
Serine	287.8 ± 42.9 [a]	145.8 ± 23.3 [b]	148.7 ± 46.1 [b]	119.3 ± 17.5 [b]
Asparagine	54.8 ± 18.9	91.0 ± 30.6	75.7 ± 31.0	64.3 ± 19.2
Glutamic Acid	72.1 ± 14.6	97.1 ± 21.9	96.3 ± 18.6	90.5 ± 25.9
Glutamine	389.5 ± 44.9	355.4 ± 71.9	329.9 ± 101.7	279.4 ± 49.7
Sarcosine	Below detectable levels	Below detectable levels	Below detectable levels	Below detectable levels
Proline	107.7 ± 21.0	141.9 ± 23.8	113.6 ± 36.9	102.8 ± 21.6
Glycine	445.4 ± 50.5	644.1 ± 153.2	633.9 ± 178.0	575.6 ± 149.3
Alanine	497.1 ± 111.5	639.7 ± 166.4	589.8 ± 133.9 [a]	457.0 ± 100.5 [a]
Citruline	58.1 ± 4.2	58.8 ± 4.0	52.0 ± 11.9	52.9 ± 13.4
a-Amino-n-butyric Acid	8.4 ± 5.0	15.3 ± 4.2	6.4 ± 9.0	8.4 ± 4.9
Valine	156.9 ± 52.1	128.8 ± 8.8	120.9 ± 35.8	126.4 ± 12.0
Methionine	22.2 ± 5.7 [a]	90.7 ± 20.0 [b]	17.5 ± 6.4 [a]	57.9 ± 8.1 [c]
Isoleucine	76.6 ± 24.7	91.9 ± 12.3	80.0 ± 14.9	72.8 ± 5.0
Leucine	98.7 ± 22.9 [a]	50.6 ± 14.3 [b]	44.4 ± 28.1 [b]	61.5 ± 14.4 [b]
Tyrosine	45.6 ± 7.8	56.6 ± 23.7	50.0 ± 14.6	40.8 ± 9.2
Phenylalanine	40.1 ± 9.1	41.9 ± 10.8	37.0 ± 4.8	40.7 ± 6.9
Tryptophan	43.5 ± 15.5	53.4 ± 5.1	47.5 ± 5.6	49.3 ± 7.5
Ethanolamine	9.5 ± 1.3	8.7 ± 0.9	6.8 ± 2.6	7.0 ± 6.5
Ammonia	81.8 ± 12.3	86.0 ± 10.4	165.1 ± 67.5	107.1 ± 76.8
Hydroxylysine	Trace	Trace	Trace	Trace
Ornithine	73.7 ± 13.7	103.8 ± 86.1	67.6 ± 24.2	72.7 ± 61.5
Lysine	409.9 ± 115.1[a]	233.3 ± 89.8 [b]	242.0 ± 20.7 [b]	151.7 ± 101.7 [b]
Histidine	50.9 ± 2.8 [a]	32.6 ± 6.0 [b]	24.3 ± 4.2 [b]	21.3 ± 15.0 [b]
3-Methylhistidine	Trace	Trace	Trace	Trace
Arginine	104.0 ± 33.8	111.3 ± 69.3	116.3 ± 36.0	50.3 ± 45.0
Cystathionine	Trace	2.3 ± 0.5	Trace	2.0 ± 0.8

Liver Metabolite	Low SAA	High Methionine	High Cystine	High Methionine and Cystine
Taurine	1026.1 ± 365.9 [a]	8125.6 ± 2338.9 [b]	8157.1 ± 2608.3 [b]	8227.0 ± 933.4 [b]
Urea	19641.4 ± 2059.4 [a]	243082.2 ± 88302.2 [b]	321586.6 ± 193387.6 [b]	992805.1 ± 184796.1 [b]
Aspartic Acid	1842.4 ± 134.0 [a]	3534.8 ± 353.7 [b]	4012.7 ± 1054.2 [b]	2796.9 ± 1086.5 [b]
Hydroxyproline	Below detectable levels	Below detectable levels	Below detectable levels	Below detectable levels
Threonine	1110.5 ± 279.7 [a]	241.8 ± 77.3 [b]	376.0 ± 148.2 [b]	217.3 ± 47.0 [b]
Serine	2317.0 ± 267.7 [a]	407.2 ± 76.5 [b]	679.9 ± 293.4 [b]	354.0 ± 54.1 [b]
Asparagine	89.3 ± 12.9	59.5 ± 20.8	61.7 ± 13.9	60.2 ± 19.1
Glutamic Acid	1648.4 ± 254.3 [a]	1179.6 ± 159.3 [ab]	1250.0 ± 360.1 [ab]	921.2 ± 217.2 [b]
Glutamine	3999.3 ± 980.5	3984.5 ± 294.2	4459.2 ± 1663.0	3428.8 ± 360.7
Sarcosine	56.0 ± 25.6	42.4 ± 48.9	53.8 ± 44.6	76.8 ± 75.8
Proline	157.3 ± 48.6	306.4 ± 77.1	267.9 ± 174.8	292.7 ± 181.8
Glycine	2468.6 ± 594.2	2398.7 ± 226.7	2874.7 ± 950.5	2022.2 ± 293.7
Alanine	3381.4 ± 1475.3	3159.8 ± 813.4	3502.8 ± 1412.7	2841.9 ± 315.9
Citruline	74.7 ± 16.3 [a]	48.0 ± 15.6 [b]	40.2 ± 4.1 [b]	36.9 ± 5.9 [b]
a-Amino-n-butyric Acid	18.4 ± 5.2	37.0 ± 2.5	28.3 ± 19.7	30.6 ± 7.4
Valine	165.5 ± 32.0	168.8 ± 50.9	201.1 ± 85.5	135.7 ± 34.8

Table 3.10a: The Effect of Varying Dietary Sulfur Amino Acid Concentrations on Hepatic Amino Acid Concentrations in Young Sprague-Dawley Rats. All values are presented in terms of µmol/g wet weight.

Liver Metabolite	Low SAA	High Methionine	High Cystine	High Methionine and Cystine
Cysteine	98.8 ± 9.7	103.7 ± 18.3	105.5 ± 25.4	94.2 ± 25.2
Methionine	22.4 ± 4.6 [a]	48.2 ± 9.8 [b]	32.2 ± 8.4 [a]	47.3 ± 9.4 [b]
Isoleucine	101.8 ± 13.9	116.8 ± 39.1	135.7 ± 43.0	108.5 ± 31.1
Leucine	157.6 ± 15.9	117.6 ± 55.9	134.0 ± 49.9	106.7 ± 49.2
Tyrosine	97.7 ± 12.1	89.8 ± 10.1	94.0 ± 32.9	72.8 ± 16.2
Phenylalanine	65.2 ± 9.1	69.0 ± 17.2	68.7 ± 21.5	63.1 ± 10.8
Tryptophan	Trace	Trace	Trace	Trace
Ethanolamine	Trace [a]	18.0 ± 12.1 [b]	20.6 ± 19.2 [b]	14.7 ± 8.1 [b]
Ammonia	819.6 ± 123.8	711.1 ± 90.8	791.4 ± 256.6	667.1 ± 161.2
Hydroxylysine	Trace	Trace	Trace	Trace
Ornithine	463.8 ± 181.1 [a]	148.9 ± 65.8 [b]	162.1 ± 105.4 [b]	124.1 ± 38.9 [b]
Lysine	792.3 ± 139.4	790.3 ±205.0	821.1 ± 331.7	706.5 ± 115.3
3-Methylhistidine	0	0	0	0
Histidine	50.0 ± 2.8	32.6 ± 6.0	24.3 ± 4.2	21.4 ± 15.0
Arginine	72.7 ± 22.4	66.7 ± 9.1	74.0 ± 27.3	62.5 ± 7.2
Cystathionine	27.6 ± 8.6 [a]	89.8 ± 40.6 [b]	17.9 ± 16.6 [a]	87.2 ± 14.5 [b]
Homocysteine	Trace	Trace	Trace	Trace

Table 3.10b: The Effect of Varying Dietary Sulfur Amino Acid Concentrations on Hepatic Amino Acid Concentrations in Young Sprague-Dawley Rats. All values are presented in terms of µmol/g wet weight.

seen in adult rats; a cystine supplementation decreases CBS mRNA both with (55% decrease) and without (45% decrease) methionine supplementation (Figure 3.3). The decrease in CBS mRNA seen with a cystine supplement is similar to the decrease in CBS mRNA seen by Yamamoto *et al* in 1995, when they demonstrated a 60% decrease in CBS mRNA when comparing rats fed a 1.0% methionine supplemented diet and a 1.0% methionine + 0.25% cystine supplemented diet.

One inexplicable result from our experiment that was not seen in our adult experiment was a decrease in BHMT activity when supplemented with methionine, yet a joint methionine-cystine supplement resulted in an increase in activity. We have no explanation for this discrepancy at this time.

3.2.2.4 Amino Acids

As with the adult rats, both plasma and hepatic taurine concentrations increased with methionine and/or cystine supplementation, which indicates an increase in the availability of cysteine in these groups (Table 3.9 and 3.10).

There was no change in BCAA in the young rats as compared to the decrease in BCAA seen with methionine supplementation with adult rats (Table 3.10). As previously discussed, α-aminobutyrate concentrations can indicate α-ketobutyrate levels, which may, in turn, activate the process by which BCAA are catabolised – in our young rats, α-aminobutyrate concentrations show no significant changes. Additionally, the plasma and hepatic methionine levels in our young rats, though increased with methionine supplementation, do not approach the concentrations seen in adult rats with methionine supplementation.

It is therefore possible that there is insufficient α-ketomethionine and/or α – aminobutyrate formed to activate the BCAA catabolism process.

In this experiment, when supplemental sulfur amino acids were provided, we observed a decrease in plasma threonine, serine, leucine, lysine and histidine (Table 3.10), as well as a decrease in hepatic threonine and serine (Table 3.9). It is possible that this decrease is due to a resultant increase in protein synthesis that would occur when sufficient sulfur amino acids are added to the amino acid pool in the growing rats. This greatly stimulated protein synthesis would also explain why these decreases were not noticed in the adult rats.

3.2.2.5 Transsulfuration Flux in Hepatocytes

There was no difference in transsulfuration flux between the four animal groups when hepatocytes were incubated with 1.0 mM 1-^{14}C-methionine; however, when incubated with the more physiological 0.1 mM concentration, the transsulfuration flux was reduced by about 65% in hepatocytes from rats fed the cystine supplement (Figure 3.5). A similar finding was evident in rats fed a supplement of both amino acids, while the methionine supplement was without effect.

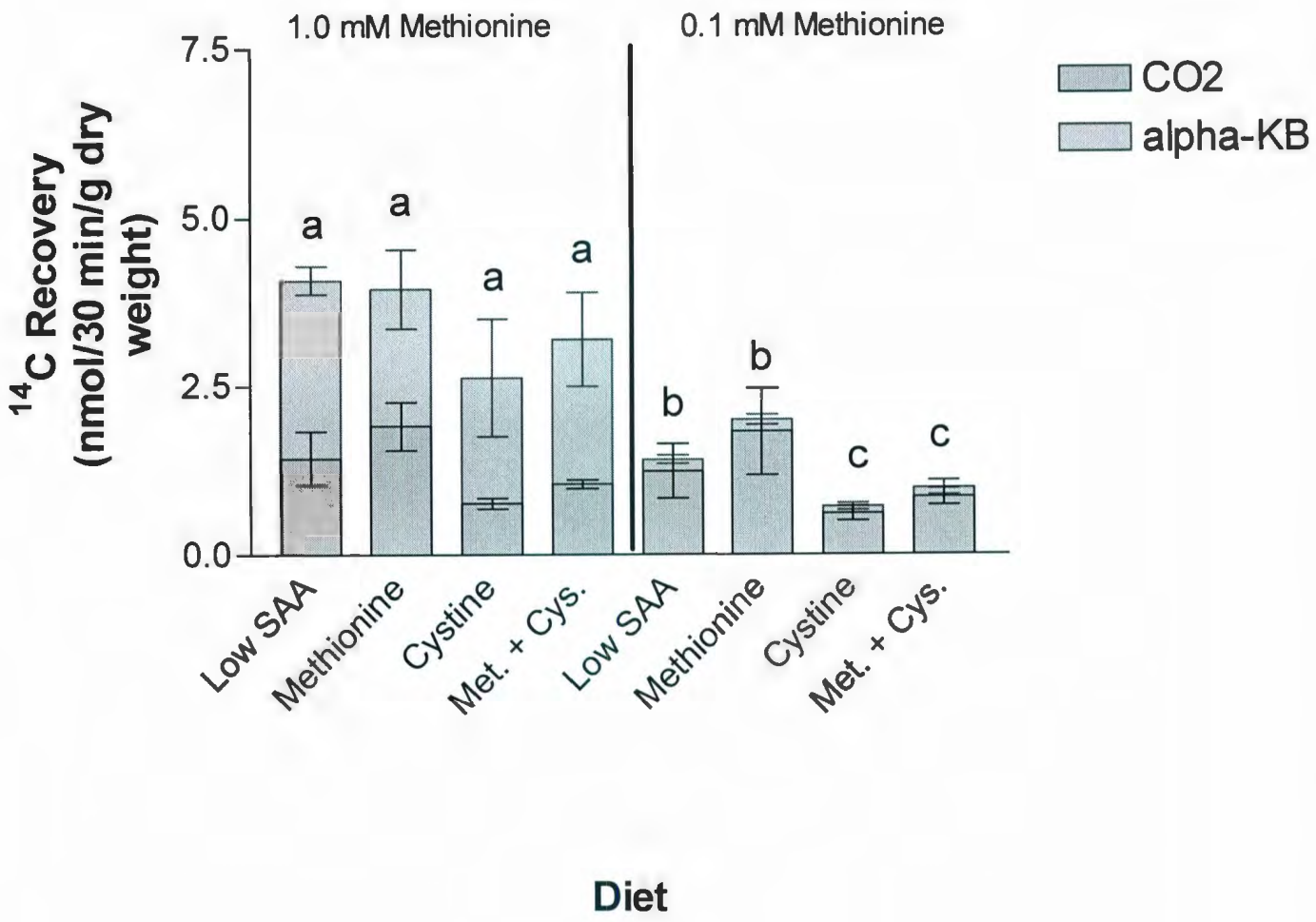
When examining our transsulfuration flux experiments, it is interesting to note that all the changes that occur in our measurements are due to changes in the recovery of $^{14}CO_2$, and not in the recovery of α-ketobutyrate. While it is possible that these changes have some biochemical relationship to the SAA, it is more likely that α-ketobutyrate very efficiently is transported into the mitochondria to be metabolized to CO_2.

3.3.3 Conclusions

When a methionine supplement is added to a Low SAA diet, the metabolism of methionine undergoes a shift towards transsulfuration. The additional methionine results in higher concentrations of methionine in the liver, which, in turn, increases the rate of the transmethylation reactions. This increased transmethylation results in the production of larger concentrations of homocysteine, which are either transported into the plasma or used to synthesis cysteine via the transsulfuration pathway. The remethylation pathway, the third potential fate of homocysteine, will become reduced in activity due to the excess of methionine already present. On the other hand, transsulfuration will increase, as indicated by increased cystathionine, cysteine, and taurine levels.

When cystine is added to the Low SAA diet, there is no effect on the transmethylation pathway. The transsulfuration pathway, however, decreases its activity, as seen by the decrease in CBS mRNA and a decrease in transsulfuration flux in isolated hepatocytes. It seems that the decrease in transsulfuration is responsible for the methionine-sparing effect of cystine supplementation, as can be seen by the comparable growth rates of the High Cystine animals and the High Methionine animals.

Figure 3.5: The Effect of Varying Dietary Sulfur Amino Acid Concentrations on Transsulfuration Flux in Young Sprague-Dawley Rats. The red sections refer to the ^{14}C in α-ketobutyrate, while the blue sections refer to the $^{14}CO_2$ produced. The sum of these two results is the total flux through transsulfuration. The results are produced in terms of nmol$^{14}CO_2$/30 min/g dry liver.


1.0 mM Methionine
0.1 mM Methionine
CO2
alpha-KB
^{14}C Recovery (nmol/30 min/g dry weight)
7.5
5.0
2.5
0.0
a
a
a
a
b
b
c
c
Low SAA
Methionine
Cystine
Met. + Cys.
Low SAA
Methionine
Cystine
Met. + Cys.
Diet

3.4 The Effect of Allosteric Regulators on Key Enzymes of Methionine Metabolism

3.4.1 Background

While metabolism of methionine is regulated by a variety of means, one of the more interesting mechanisms is via allosteric regulation, and specifically, that by SAM and SAH. It has been shown previously that SAM and SAH levels can affect the enzyme activities of several of the key enzymes of methionine metabolism, such as MTHFR (Jencks *et al*, 1987), CBS (Finkelstein *et al*, 1975; Finkelstein *et al*, 1974), and BHMT (Finkelstein and Martin, 1984).

In the previous two experiments, we analyzed both enzyme activities and *in vivo* concentrations of SAM and SAH. Due to the wide range of SAM and SAH concentrations found in the different experiments, we decided to make a definitive study of the effect of these regulators on the enzyme activities. We ran liver cytosol though PD-10 desalting columns. This permits the proteins to be eluted in the void volumes while the columns retain small molecules such as SAM and SAH. MTHFR, CBS, and BHMT were assayed as a function of [SAM] while the dependence of CBS on [SAH] was also examined.

3.4.2 Results

3.4.2.1 SAM and SAH Analysis

We first determined the effectiveness of the PD-10 columns in removing SAM and SAH. This was done by analyzing, via HPLC, a liver cytosol sample that had been through the PD-10 column. Figure 3.6 shows a chromatogram in which just SAM and SAH were run. SAM had a retention time of 16.98 minutes,

while SAH had a retention time of 15.74 minutes. Analysis of the liver cytosol sample that had been run through the PD-10 column showed no discernable SAM and SAH peaks at either 15 or 17 minutes, thereby indicating that SAM and SAH were effectively eliminated by the run through the PD-10 column (Figure 3.7)

3.4.2.2 SAM, SAH and Cystathionine β-Synthase

There was a sigmoidal relationship between [SAM] and CBS activity (Figure 3.8). The Ka of CBS for SAM was determined to be 243.3 μM. There was no significant effect of [SAH] on CBS activity up to 250 μM – this was the maximal concentration that we could use as SAH was not soluble in larger concentrations.

3.4.2.3 SAM and the Remethylation Pathway

MTHFR activity in these liver cytosols was inhibited by SAM with a 50% inhibition evident at 185.6 μM (Fig. 3.9). In contrast to previously published reports, we found no inhibition of BHMT by SAM (Fig. 3.10).

3.4.3 Discussion

The existence of a SAM-regulated metabolic switch has been postulated since Finkelstein (1974) investigated the regulatory properties of SAM. The SAM-switch could determine whether methionine metabolism is functioning in a methionine catabolism mode (where the enzymes are designed to send methionine through the transmethylation and transsulfuration pathways) or in methionine conservation mode, where the flux through the transmethylation pathway is reduced, and flux through the remethylation pathway is increased.

Figure 3.6: Measurement of a standard 10 μM solution of S-Adenosylmethionine and S-Adenosylhomocysteine by HPLC. SAH appears at a retention time of 15.74, while SAM appears at a retention time of 16.98.

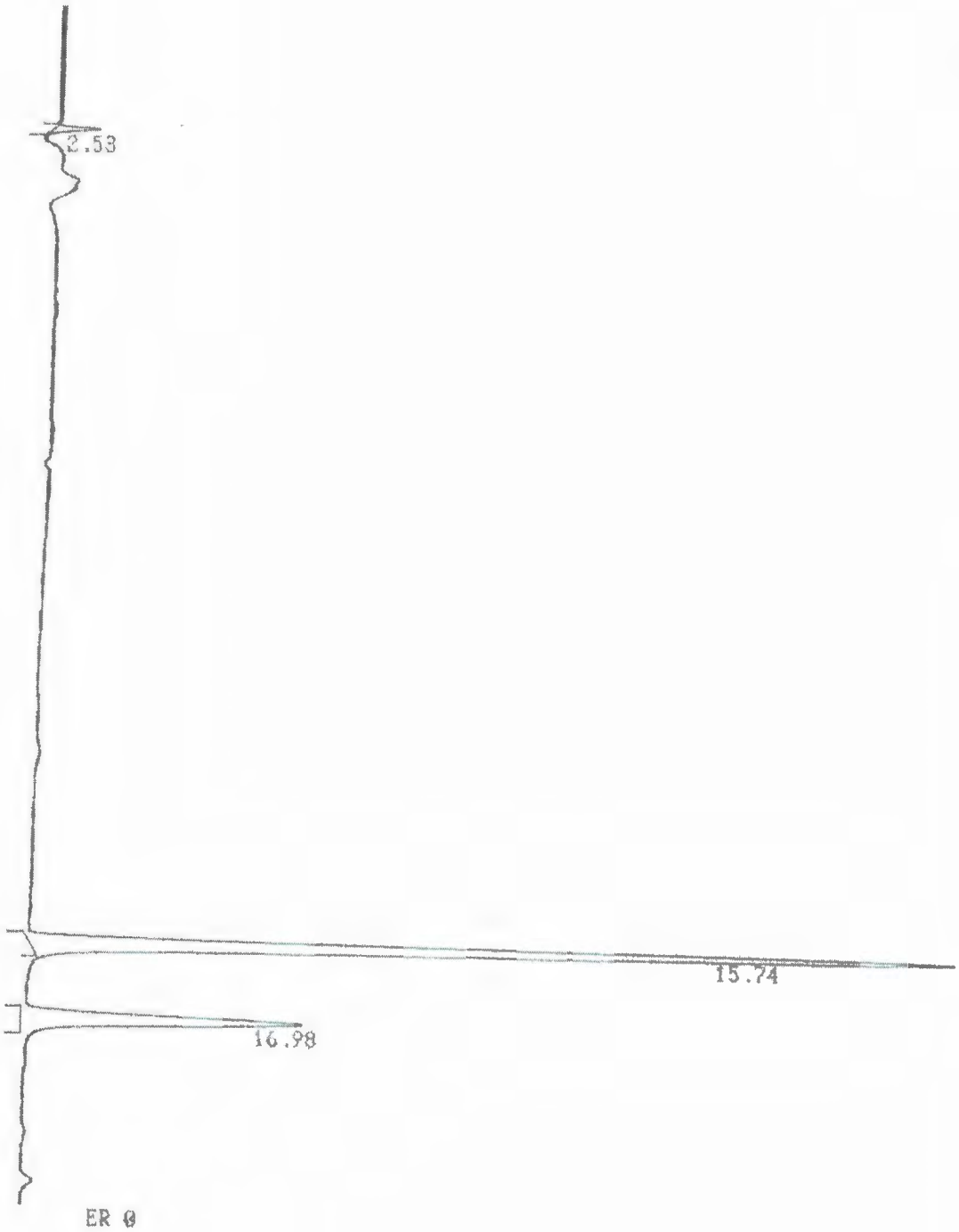

Std
2.53
15.74
16.98
ER 0

Figure 3.7: Determination of SAM and SAH Concentrations in Liver Cytosol after Elution from a PD-10 Desalting Column. As previously shown, SAM and SAH have a retention time of 16.98 and 15.74, respectively; neither peak appears on this sample readout.

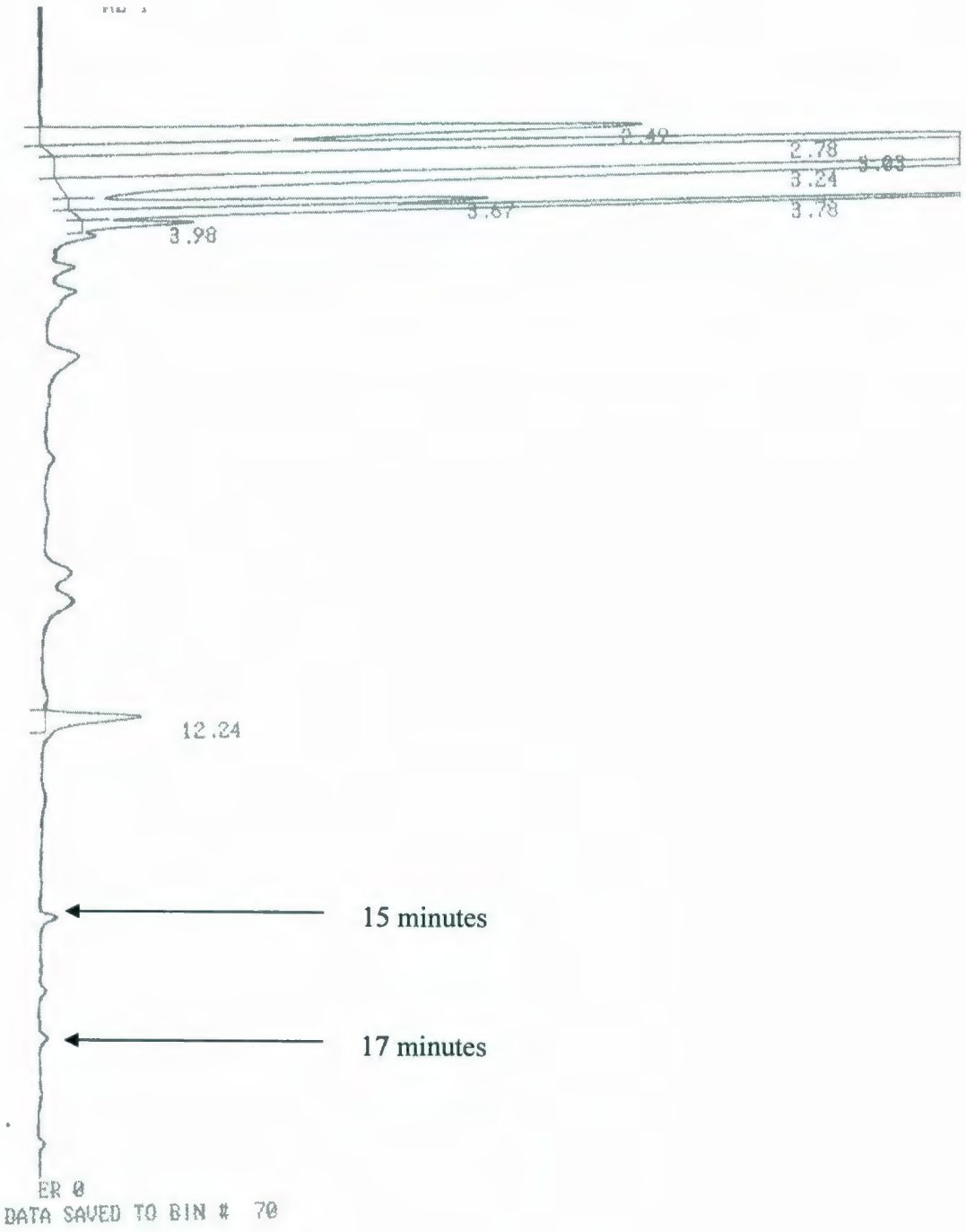
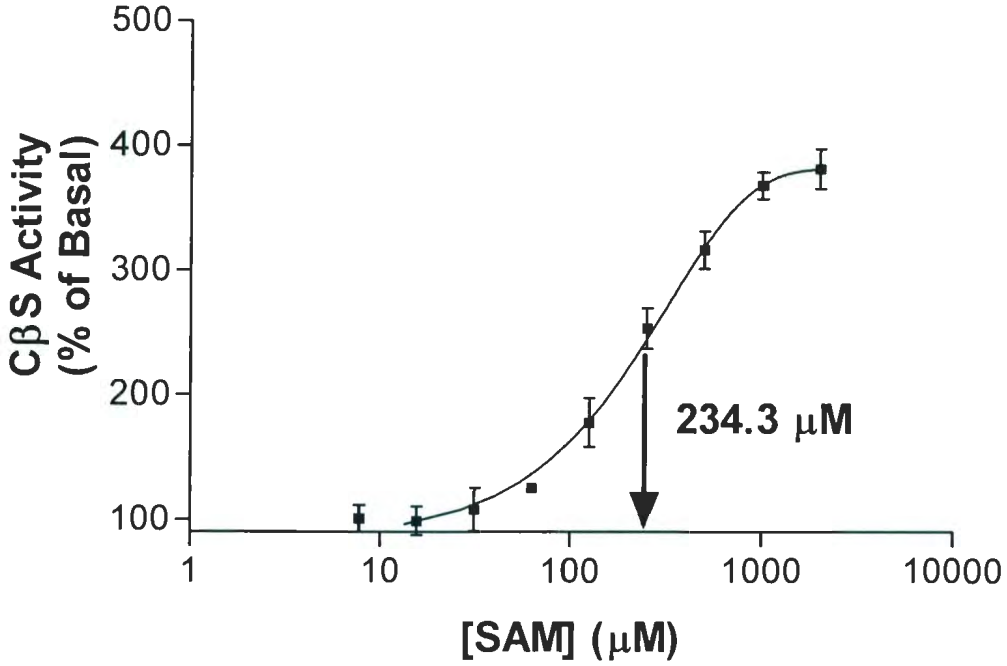

2.49
2.78
3.03
3.24
3.57
3.78
3.98
12.24
15 minutes
17 minutes
ER 0
DATA SAVED TO BIN # 70

Figure 3.8: The Effect of Increasing Concentrations of SAM and SAH on Cystathionine β-Synthase Activity. Figure 3.8a shows the effect of SAM on CBS activity; note that the Ka was determined to be 243.3 μM. Figure 3.8b demonstrates the effect that SAH concentrations have on CBS activity. (n = 4; error bars indicate standard deviation)


500
400
300
200
100
CβS Activity
(% of Basal)
234.3 μM
1
10
100
1000
10000
[SAM] (μM)

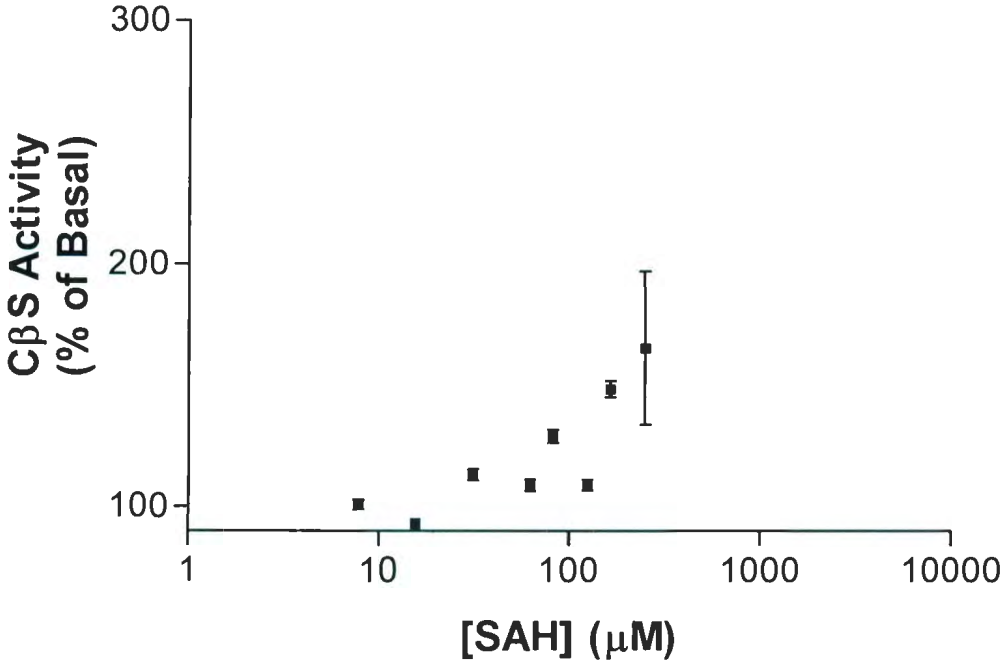

300
200
100
CβS Activity
(% of Basal)
1
10
100
1000
10000
[SAH] (μM)

Figure 3.9: The Effect of Varying SAM Concentrations on Methylenetetrahydrofolate Reductase Activity. MTHFR activity is measured as a percentage of basal activity, while SAM concentrations are measured in μM. (n = 4; error bars indicate standard deviation)

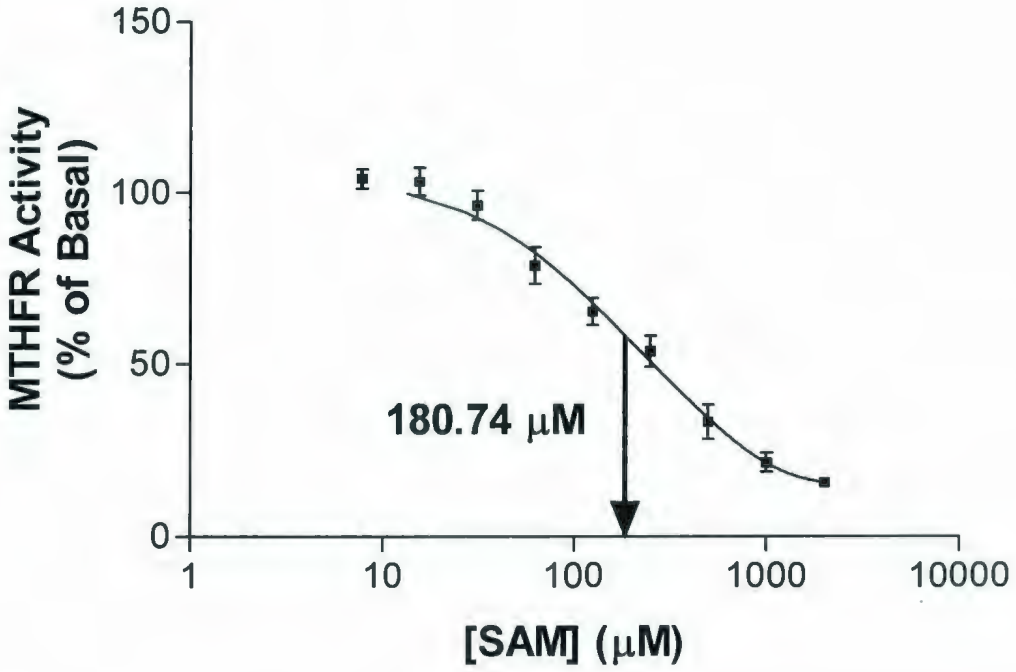
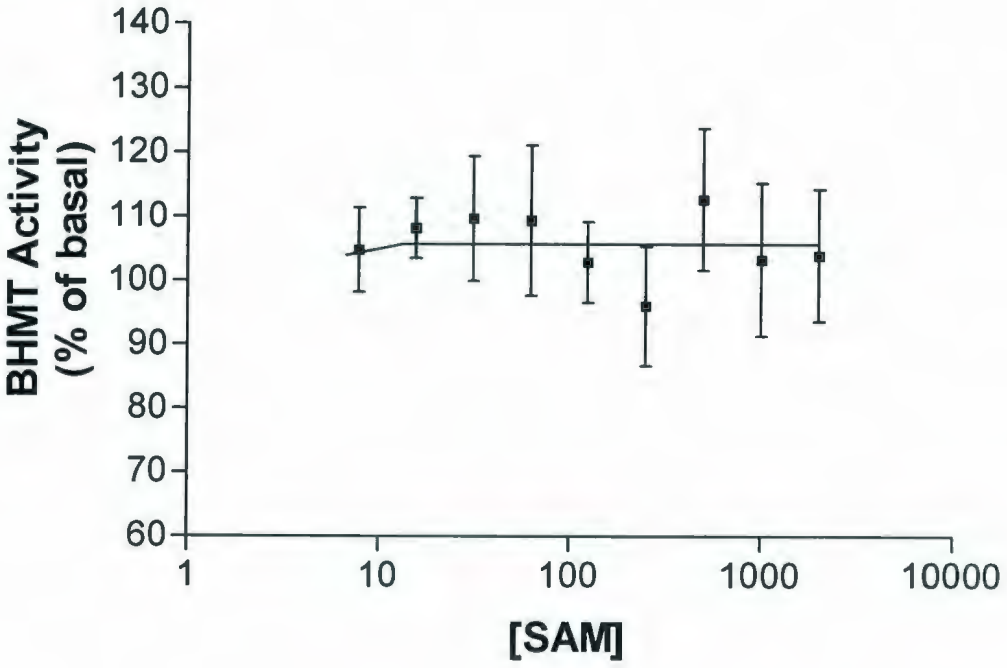

150
100
50
0
MTHFR Activity
(% of Basal)
180.74 μM
1
10
100
1000
10000
[SAM] (μM)

Figure 3.10: The Effect of SAM Concentrations on Betaine:Homocysteine Methyltransferase. Enzyme activity is measured in terms of percentage of basal activity, while SAM concentrations are measured in μM. (n = 4; error bars indicate standard deviation)


BHMT Activity
(% of basal)
140
130
120
110
100
90
80
70
60
1
10
100
1000
10000
[SAM]

It is instructive to apply these data on the sensitivity of key enzymes to SAM concentrations to the levels of these metabolites reported earlier in this thesis. So as to convert our earlier data to intracellular concentrations, we will employ the data of Quan and Brown (1996) that reported that one gram of rat liver contains about 0.45 ml of intrahepatocellular water. Our data in Table 3.3 showed that hepatic SAM levels are about 80 nmole/g and, in those adult rats fed the methionine supplement, about 575 nmole/g. These would translate into intracellular concentrations of 180 μM and 1300 μM, respectively, assuming that SAM were equally distributed through intracellular water. The corresponding concentration for the young rats (Table 3.8) would be about 150 μM in the rats fed no supplemental methionine and about 300 μM in those who received this supplement. Comparing these numbers to the data in Figures 3.8 and 3.9, it is apparent that the allosteric effects of SAM would be manifest in all of these animals. Even in rats fed a SAA deficient diet, sufficient SAM would be present in hepatocytes to activate CBS to about 175% of the basal rate and to inhibit MTHFR by about 50%. When rats were fed the methionine-supplemented diets, there would be sufficient intracellular SAM to fully activate CBS (to almost 300% of the basal rate) and to inhibit MTHFR by more than 60%. Of course, the actual activities of these enzymes will depend on other factors, in particular on substrate concentrations. Nevertheless, these calculations clearly demonstrate the importance of intracellular SAM levels to the regulation of these pathways and, somewhat surprisingly, suggest that these regulatory effects are important even in animals fed insufficient methionine.

Based on our data on CBS and MTHFR, two rate-limiting enzymes in their respective pathways, it would seem that this SAM-switch may be optimally active at about 200 μM, or approximately 90 nmol/g liver. Both the activation of CBS and the inhibition of MTHFR are about 50% effective at this concentration.

One interesting aspect of this study is the lack of effect that SAM has on the activity of BHMT. Previous reports by Finkelstein and Mudd (1984) have indicated that increasing concentrations of SAM result in a decreased flux through the BHMT enzyme. This disparity between the two branches of the remethylation pathway creates a situation where one branch, the MTHFR/MS branch, is dependent on SAM for regulation, while the other branch, consisting of betaine metabolism, is SAM independent.

The fact that the betaine branch of remethylation is unaffected by SAM poses an interesting question – why is one branch of remethylation affected by SAM, yet not the other? The explanation for this could simply be that the remethylation pathways have two different functions – the folate remethylation pathway could be responsible for the maintenance of SAM concentrations in the hepatocyte, while the betaine remethylation pathway could be responsible for something completely different, the catabolism of choline and betaine. In this scenario, a lowered SAM level would result in increased flux through the folate remethylation pathway, creating more SAM for the hepatocyte. However, there is no reason why the catabolism of choline and betaine should be regulated by SAM levels.

3.4.4 Conclusion

After examining the key regulatory enzymes in the remethylation and transsulfuration pathway, it is clear that SAM does have profound effects on both these pathways, as CBS activity increases exponentially with the addition of SAM, while MTHFR decreases exponentially. The midpoint of both of these regulatory curves is at approximately 200 μM SAM, indicating that this may be the approximate concentration for the hypothesized "SAM switch" that can change the methionine metabolic pathways from methionine conservation to methionine expenditure. Finally, the fact that BHMT seems to be unaffected by SAM concentrations leads one to believe that the branches of remethylation have two different purposes: the folate remethylation pathway may be used to replenish intracellular SAM requirements, while the betaine remethylation pathway is primarily for the catabolism of choline and betaine.

Bibliography

Amenta, F., S.K. Tayebati (2008) "Pathways of acetylcholine synthesis, transport and release as targets for treatment of adult-onset cognitive dysfunction." Curr Med Chem. **15**: 488-498

Avila, M.A., C. Berasain, L. Torres, A. Martin-Duce, F.J. Corrales, H. Yang, J. Prieto, S.C. Lu, J. Caballeria, J. Rodes and J.M. Mato (2000). "Reduced mRNA abundance of the main enzymes involved in methionine metabolism in human liver cirrhosis and hepatocellular carcinoma." J. Hepatol. **33**: 907-914.

Bader, J.P., N.R. Brown, P.K. Chiang and G.L. Cantoni (1978). "3-deazaadenosine, an inhibitor of adenosylhomocysteine hydrolase, inhibits reproduction of Rous sarcoma virus and transformation of chick embryo cells." Virology **89**: 494-505.

Baker, D.H. (2004). "Animal models of human amino acid responses." J. Nutrition **134 (6 Suppl):**1646S-1650S.

Barak, A.J., D.J. Tuma (1983) "Betaine: metabolic by-product or vital methylating agent?" Life Sci **14**: 771-774

Bell, I.R., J.S. Edman, J. Selhub, F.D. Morrow, D.W. Marby, H.L. Kayne, and J.O. Cole (1992). "Plasma homocysteine in vascular disease and in nonvascular dementia of depressed elderly people." Acta Psychiatr. Scand **86**: 386-390.

Benevenga, N.J. (1974). "Toxicity of methionine and other amino acids." J. Agric. Food Chem. **22**: 2-9.

Berry, M., A. Edwards and G. Barritt (1991). High yield preparation of hepatocytes from rat liver. Oxford, UK, Elsevier.

Bittles A.H., N.A. Carson (1981). "Homocystinuria: studies on cystathionine beta-synthase, S-adenosylmethionine synthase and cystathionase activities in skin fibroblasts." J. Inherit Metab Dis. **4**: 3-6

Bostom, A., J.T. Brosnan, B. Hall, M.R. Nadeau and J. Selhub (1995). "Net uptake of plasma homocysteine by the rat kidney in vivo." Atherosclerosis **116**: 59-62.

Boushey, C.J., S.A. Beresford, G.S. Omenn and A.G. Motulsky (1995). "A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: Probable benefits of increasing folic acid intakes." JAMA **274**: 1049-1057.

Braunstein, A.E. and E.V. Goryachenkova (1984). "The beta-replacement-specific pyridoxal-P-dependent lyases." Adv. Enzymol. Relat. Areas Mol. Biol. **56**: 1-89.

Broer, S., J.A. Cavanaugh, J.E. Rasko (2005). "Neutral amino acid transport in epithelial cells and its malfunction in Hartnup disorder." Biochem Soc Trans **33**: 233-236

Brosnan, J.T., K.C Man, D.E Hall, S.A. Colbourne and M.E. Brosnan (1983). "Interorgan metabolism of amino acids in streptozotocin-diabetic ketoacidotic rat." Am. J. Physiol. (Endocrinol. Metab.) **244**: E151-E158.

Burke, G.T., J.H. Mangum and J.D. Brodie (1971). "Mechanism of mammalian cobalamin-dependent methionine biosynthesis." Biochemistry **10**: 3079-3085.

Butz, L. and V. du Vigneaud (1932). "The formation of a homologue of cystine by the decomposition of methionine with sulfuric acid." J Biol Chem **99**: 135-42.

Cabrerra, C., A. Duce, P. Oriz, S. Alemany and J. Mato (1988). "Specific loss of the high-molecular-weight form of S-adenosyl-l-methionine synthetase in human liver cirrhosis." Hepatology **8**: 1530-1534.

Chen, Z., S. Chakraborty and R. Banerjee (1995). "Demonstration that mammalian methionine synthases are predominantly cobalamin-loaded." J. Biol. Chem. **270**: 19246-19249.

Chiang, P. (1981). "Conversion of 3T3-L1 fibroblasts to fat cells by an inhibitor of methylation: Effect of 3-deazaadenosine." Science **211**: 1164-1166.

Chomczynski, P. and N. Sacchi (1987). "SIngle-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction." Anal. Chem. **162**: 156-159.

Cianciolo G., G. La Manna, L. Coli, G. Donate, F. D'Addio, E. Persici, G. Comai, M. Wratten, A. Dormi, V. Mantovi, G.Grossi, S. Stefoni (2008). "5-methyltetrahydrofolate administration is associated with prolonged survival and reduced inflammation in ESRD patients." Am J Nephrol. **28**: 941-948

Cook, R. and C. Wagner (1984). "Glycine N-methyltransferase is a folate-binding protein of rat liver cytosol." Proc. Natl. Acad. Sci. USA **81**: 3631-3639.

Cook, R., D. Horne and C. Wagner (1989). "Effect of dietary methyl group deficiency on one carbon metabolism in rats." J. Nutr. **119**: 612-617.

Corrales, F., I. Perez-Mato, M. Sanchez del Pino, F. Ruiz, C. Castro, E. Garcia-

Travijano, U. Latasa, M. Martinez-Chanter, A. Martinez-Cruz, M. Avila and J. Mato (2002). "Regulation of mammalian liver methionine adenosyltransferase." J. Nutr. **132**: 2377S-2381S.

Daubner, S. and R. Matthews (1982). "Purification and properties of methylenetetrahydrofolate reductase from pig liver." J. Biol. Chem. **257**: 140-145.

de la Haba, G. and G. Cantoni (1959). "The enzymatic synthesis of S-adenosyl-l-homocysteine from adenosine and homocysteine." J. Biol. Chem. **234**: 603-608.

Di Buono, M., L.J. Wykes, R.O. Ball and P.B. Pencharz (2001). "Total sulfur amino acid requirement in young men as determined by indicator amino acid oxidation with L-[1-13C]phenylalanine." Am. J. Clin. Nutr. **74**: 761-766.

du Vigneaud, V., H. Dyer and J. Harmon (1934). "The growth promoting properties of homocystine when added to a cystine-deficient diet and the proof of structure of homocystine." J. Biol. Chem. **101**: 719-726.

Duce, A., P. Ortiz, C. C and J. Mato (1988). "S-adenosyl-L-methionine synthetase and phospholipid methyltransferase are inhibited in human cirrhosis." Hepatology **8**: 65-68.

Duerre, J., C. Miller and G. Reams (1969). "Metabolism of S-adenosyl-l-homocysteine *in vivo* by the rat." J. Biol. Chem. **244**: 107-111.

Engberson, A., D. Franken, G. Boers, E. Stevens, F. Trijbels and H. Blom (1995). "Thermolabile 5,10-methylenetetrahydrofolate reductase as a cause of mild hyperhomocysteinemia." Am. J. Hum. Genet. **56**: 142-150.

Finkelstein, J.D. (1967). "Methionine metabolism in mammals: effects of age, diet, and hormonal therapy on three enzymes of the pathway in rat tissues." Acta. Biochem. Biophys **122**: 583-590.

Finkelstein, J.D. (1990). "Methionine metabolism in mammals." J. Nutr. Biochem. **1**: 228-237.

Finkelstein, J.D. (2000a). "Homocysteine: a history in progress." Nutr Rev **58**: 193-204.

Finkelstein, J.D. (2000b). "Pathways and regulation of homocysteine metabolism in mammals." Seminars in Thrombosis and Hemostasis **26**: 219-225.

Finkelstein, J.D. and B. Harris (1974). "Methionine metabolism in mammals. Synthesis of S-adenosylhomocysteine in rat tissues." Arch Biochem Biophys **146**: 84-92.

Finkelstein, J.D. and J.J. Martin (1984). "Inactivation of betaine-homocysteine methyltransferase by adenosylmethionine and adenosylethionine." Biochem. Biophys. Res. Commun. **118**: 14-19.

Finkelstein, J.D. and J.J. Martin (1986). "Methionine metabolism in mammals: Adaptation to methionine excess." J. Biol. Chem. **261**: 1582-1587.

Finkelstein, J.D. and S.H. Mudd (1967). "Transsulfuration in mammals - the methionine sparing effect of cystine." J. Biol. Chem. **242**: 873-880.

Finkelstein, J.D., W. Kyle and B.J. Harris (1971). "Methionine metabolism in mammals. Regulation of homocysteine metabolism in rat tissues." Arch Biochem Biophys **146**: 84-92.

Finkelstein, J.D., B.J. Harris and W.E. Kyle (1972). "Methionine metabolism in mammals: Kinetic study of betaine-homocysteine methyltransferase." Arch. Biochem. Biophys. **153**: 320-324.

Finkelstein, J.D., W.E. Kyle, J.J. Martin and A. Pick (1974). "Activation of cystathionine synthase by S-adenosylhomocysteine." Biochem. Biophys. Res. Commun. **165**: 774-779.

Finkelstein, J., W. Kyle, J. Martin and A. Pick (1975). "Activation of cystathionine synthase by adenosylmethionine and adenosylethionine." Biochem. Biophys. Res. Commun. **66**: 81-87.

Finkelstein, J.D., J.J. Martin, W.E. Kyle and B.J. Harris (1978). "Methionine metabolism in memmals. Regulation of methylenetetrahydrofolate reducatase content of rat tissue." Arch Biochem Biophys **191**: 153-160.

Finkelstein, J.D., J.J. Martin and B.J. Harris (1986). "Effect of dietary cysteine on methionine metabolism in rat liver." Journal of Nutrition **116**: 985-990.

Finkelstein, J.D., J.J. Martin and B.J. Harris (1988). "Methionine metabolism in mammals: the methionine-sparing effect of cystine." J. Biol. Chem. **263**: 11750-11754.

Folin, O. and A. Marenzi (1929). "An improved colorimetric method for the determination of cystine in proteins." J. Biol. Chem. **83**: 103-108.

Fontecave, M., M. Atta and E. Mulliez (2004) "S-adenosylmethionine: nothing goes to waste." Trends Biochem Sci **29**: 243-249

Frosst, P., H.J. Blom, R. Milos, P. Goyette, C.A. Sheppard, R.G. Matthews, G.J.

Boers, M. den Heijer, L.A. Kluijtmans and L.P. van den Heuval (1995). "A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase." Nat. Genet. **10**: 111-113.

Garrow, T. (1996). "Purification, kinetic properties, and cDNA cloning of mammalian betaine-homocysteine methyltransferases." J. Biol. Chem. **271**: 22831-22838.

Gaull, G.E., W. von Berg, N.C. Raiha and J.A. Sturman (1973). "Development of methyltransferase activities in human fetal tissues." Pediatric Res. **7**: 527-533.

Gil, B., M. Casado, M.A. Pajares, L. Bosca, J.M. Mato, P. Martin-Sanz and L. Alvarez (1996). "Differential expression pattern of S-adenosylmethionine synthetase isoenzymes during rat liver development." Hepatology **24**: 876-881.

Green, R. and D. Jacobson (1995). Clinical implications of hyperhomocysteinemia. Folate in health and disease. L. Bailey. New York, Dekker: 75-124.

Grossman, E. and S. Hebert (1989). "Renal inner medullary choline dehydrogenase activity: characterization and modulation." Am. J. Physiol. **256**: F107-F112.

Halim, A.B., H.L. LeGros, A. Geller and M. Kotb (1999). "Expression and functional interaction of the catalytic and regulatory subunits of human methionine adenosyltransferase in mammalian cells." J. Biol. Chem. **274**: 29720-29725.

Haubrich, D.R. and N.H. Gerber (1981). "Choline dehydrogenase: Assay, properties and inhibitors." Biochem. Pharmacol. **30**: 2993-3000.

Hoffman, J. (1983). "Fractionation of methionine adenosyltransferase isozymes (rat liver)." Methods Enzymol. **94**: 223-228.

Hong, S., D. Yang and S. Chang (1998). "The relationship between plasma homocysteine and amino acid concentrations in patients with end stage renal disease." J Renal Nutr. **9**: 34-39.

Humphrey L.L., R. Fu, K. Rogers, M. Freeman and M. Helfand (2008). "Homocysteine level and coronary heart disease incidence: a systematic review and meta-analysis." Mayo Clin. Proc. **83**: 1203-1212

Jackson, R. and R. Block (1933). "The metabolism of cystine and methionine: the availability of methionine in supplementing a diet deficient in cystine." J. Biol. Chem. **98**: 465-477.

Jacobowski, H. (2000). "Homocysteine thiolactone: Metabolic origin and protein homocysteinylation in humans." J. Nutr. **130**: 377S-381S.

Jacobs, R.L., J.D. House, M.E. Brosnan and J.T. Brosnan (1998). "Effects of streptozotocin-induced diabetes and of insulin treatment on homocysteine metabolism in the rat." Diabetes **47**: 1967-1970.

Jacobs, R.L., L.M. Stead, M.E. Brosnan and J.T. Brosnan (2001). "Hyperglucagonemia in rats results in decrease plasma homocysteine and increased flux through the transsulfuration pathway in liver." J. Biol. Chem. **276**: 43740-43747.

Jencks, D. and R. Matthews (1987). "Allosteric inhibition of methylenetetrahydrofolate reductase by adenosylmethionine." J. Biol. Chem. **262**: 2485-2493.

Joosten, E., E. Lesaffre and R. Riezler (1997). "Is metabolic evidence for vitamin B12 and folate deficiency more frequent in elderly patients with Alzheimer's disease?" J. Gerontol. A Biol Sci Med **52**: M76-79.

Kato, A., M. Ogura and M. Suda (1966). "Control mechanism in the rat liver enzyme system converting L-methionine to L-cystine. 3. Noncompetitive inhibition of cystathionine synthetase-serine dehydratase by elemental sulfur and competitive inhibition of cystathionase-homoserine dehydratase by L-cysteine and L-cystine." J. Biochem. **59**: 40-48.

Kerr, S.J. and J.E. Heady (1974). "Modulation of tRNA methyltransferase activity by competing enzyme systems." Adv. Enzyme Reg. **12**: 103-111.

Kery, V., L. Poneleit and J.P. Kraus (1998). "Trypsin cleavage of human cystathionine beta-synthase into an evolutionarily conserved active core: structural and functional consequences." Arch Biochem Biophys **355**: 2716-2724.

Kinsell, L.W., H.A. Harper, H.C. Barton, G. Michaels and H. Weiss (1947). "Rate of disappearance from plasma of intravenously administered methionine in patients with liver damage." Science **106**: 589-594.

Koblin, D.D., L. Waskell, J.E. Watson, E.L. Stokstad and E.I. Eager 2nd (1981). "Inactivation of methionine synthetase by nitrous oxide." Anesthesiology **54**: 318-324.

Kotb, M. and A.M. Geller (1993). "Methionine adenosyltransferase: structure and function." Pharmacol. Ther. **89**: 125-143.

Krahenbuhl, S., E.P. Brass and C.L. Hoppel (2000). "Decreased carnitine biosynthesis in rats with secondary biliary cirrhosis." Hepatology **31**: 1217-1223.

Lieber, C.S., S.J. Robins and M.a. Leo (1994). "Hepatic phosphatidylethanolamine methyltransferase activity is decreased by ethanol and increased by phosphatidylcholine." Alcohol Clin. Exp. res. **18**: 592-595.

Li, Z., D.E. Vance (2008) "Phosphatidylcholine and choline homeostasis." J. Lipid Res. **49**: 1187-1194

Lu, S. (1999). "Regulation of hepatic glutathione synthesis: current concepts and controversies." FASEB J **13**: 1169-1183.

Lu, S. (2000). "S-Adenosylmethionine." Int. J. Biochem. Cell Biol. **32**: 391-395.

Mato, J.M., L. Alvarez, P. Ortiz and M.A. Pajares (1997). "S-adenosylmethionine synthesis: molecular mechanisms and clinical implications." Pharmacol. Ther. **73**: 265-180.

Marlatt, M.W., P.J. Lucassen, G. Perry, M.A. Smith, X. Zhu (2008) "Alzheimer's disease: cerebrovascular dysfunction, oxidative stress, and advanced clinical therapies." J Alzheimers Dis. **15**: 199-210

Matthews, R. and C. Sheppard (1998). "Methylenetetrahydrofolate reductase and methionine synthase: biochemistry and molecular biology." Eur. J. Pediatr. **157**: S54-S59.

McCully, K. (1969). "Vascular pathology of homocysteinemia: Implication for the pathogenesis of artherosclerosis." Am J Pathol **56**: 111-128.

Miller, J.W., M.R. Nadeau, J. Smith, D. Smith and J. Selhub (1994). "Folate-deficiency-induced homocysteinaemia in rats: disruption of S-adenosylmethionine's co-ordinate regulation of homocysteine metabolism." Biochem. J. **298**: 415-419.

Mudd, S.H. (1963). "Activation of methionine for transmethylation. Enzyme-based tripolyphosphate as an intermediate in the reaction catalyzed by the methionine-activating enzymes of baker's yeast." J. Biol. Chem. **238**: 2156-2163.

Mudd, S.H. (1979). "Diseases of sulphur metabolism: implications for the methionine-homocysteine cycle, and vitamin responsiveness." Ciba Found Symp **72**: 239-258.

Mudd, S.H. and J. Poole (1975). "Labile methyl balance for normal humans on various dietary regiments." Metabolism **24**: 721-735.

Mudd, S.H., J.D. Finkelstein, F. Irreverre and L. Laster (1965). "Transsulfuration in mammals: Microassays and tissue distribution of three enzymes of the pathway." J. Biol. Chem. **240**: 4382-4392.

Mudd, S.H., H. Levy, R. Abeles and J.P. Jennedy Jr. (1969). "A derangement in B12 metabolism leading to homocystinemia: Cystathioninemia and methylmalonic aciduria." Biochem Biophys Res Commun **35**: 121-126.

Mudd, S.H., F. Slovby, H. Levy, K. Pettigrew, B. Wilcken, R. Pyeritz, G. Andria, G. Boers, I. Bromberg, R. Cerone, B. Fowler, H. Grobe, H. Schmidt and L. Scheweitzer (1985). "The natural history of homocystinuria due to cystathionine B-synthase deficiency." Am. J. Hum. Genet. **37**: 1-31.

Mudd, S.H, H. Levy and F. Skovby (1995). Disorders of transsulfuration. The Metabolic and Molecular Basis of Inherited Disease. C. B. Scriver, AL; Sly, WS; Valle, D. New York, McGraw-Hill: 1279-1327.

Mueller, J. (1923). "A new sulfur-containing amino-acid isolated from the hydrolytic products of protein." J. Biol. Chem. **56**: 157-169.

Mueller, J. (1924). "A new sulfur-containing amino-acid isolated from the hydrolytic products of protein. II. Sulfur excretion after ingestion." J. Biol. Chem. **58**: 373-375.

Newberne, P., A. Rogers, C. Bailey and V. Young (1969). "The induction of liver cirrhosis in rats by purified amino acid diets." Cancer Research **29**: 230-235.

Noga, A.A., L.M. Stead, Y. Zhao, M.E. Brosnan, J.T. Brosnan and D.E Vance (2003). "Plasma homocysteine is regulated by phospholipid methylation." J. Biol. Chem. **278**: 5952-5955.

Ogawa, H. and M. Fujioka (1982). "Purification and properties of glycine N-methyltransferase from rat liver." J. Biol. Chem. **257**(3447-3452).

Ogawa, H., T. Gomi and M. Fujioka (1993). "Mammalian glycine N-methyltransferase. Comparative kinetic and structural properties of the enzymes from human, rat, rabbit, and pig liver." Comp. Biochem. Physiol. **106B**: 601-611.

Ogawa, H., T. Gomi, F. Takusagawa and M. Fujioka (1998). "Structure, function, and physiological role of glycine N-methyltransferase." Int. J. Biochem. Cell Biol. **30**: 13-26.

Pajares, M.A., D. Perez-Sala (2006) "Betaine homocysteine S-methyltransferase: just a regulator of homocysteine metabolism?" Cell Mol Life Sci **63**: 2792-803

Raguso, C.A., A.M. Ajami, R. Gleason and V.R Young (1997). "Effect of cystine intake on methionine kinetics and oxidation determined with oral tracers of methionine and cysteine in healthy adults." Am. J. Clin. Nutr. **66**: 283-292.

Raguso, C.A., M. Regan and V.R. Young (2000). "Cysteine kinetics and oxidation at different intakes of methionine and cystine in young adults." Am. J. Clin. Nutr. **71**: 491-499.

Rao, A.M., M.R. Drake and M.H. Stipanuk (1990). "Role of the transsulfuration pathway and of gamma-cystathionase activity in the formation of cysteine and sulfate from methionine in rat hepatocytes." J. Nutrition **120**: 837-845.

Ratnam, S., K.N. Maclean, R.L. Jacobs, M.E. Brosnan and J.T. Brosnan (2002). "Hormonal regulation of cystathionine beta-synthase expression in liver." J. Biol. Chem. **277**: 42912-42918.

Reeves, P.G., K.L. Rossow and J. Lindlauf (1993). "Development and testing the AIN-93 purified diets for rodents; results on growth, kidney calcification and bone mineralization in rats and mice." J. Nutr. **123**: 1923-1931.

Riggs, K.M., A. Spiro, K. Tucker and D. Rush (1996). "Relations of vitamin B-12, vitamin B-6, folate, and homocysteine to cognitive performance in the Normative Aging Study." Am. J. Clin. Nutr. **63**: 306-314.

Rogers, Q. and A. Harper (1965). "Amino acid diets and maximal growth in the rat." J. Nutrition **87**: 267-273.

Rose, W. and R. Wixom (1955). "The amino acid requirements of man. XIII. The sparing effect of cystine on methionine requirement." J. Biol. Chem. **216**: 763-774.

Rosenburg, I. and J. Miller (1992). "Nutritional factors in physical and cognitive functions of elderly people." Am. J. Clin. Nutr. **55**: 1237S-1243S.

Sanchez del Pino, M., Corrales, E-J, Mato, JM (2000). "Histeretic behavior of methionine adenosyltransferase III. Methionine switches between two conformations of this enzyme with different specific activity." J. Biol. Chem. **275**: 23976-23982.

Shan, X. and W.D. Kruger (1998). "Correction of disease-causing CBS mutations in yeast." Nat. Genet. **19**: 91-93.

Shane, B. and E.L. Stokstad (1985). "Vitamin B12-folate interrelationships." Annu. Rev. Nutr. **5**: 115-141.

Skovby, F., J. Kraus and L. Rosenberg (1984). "Biosynthesis and proteolytic activation of cystathionine beta-synthase in rat liver." J. Biol. Chem. **259**: 588-593.

Spark, J.L., P. Laws and R. Fitridge (2003). "The incidence of hyperhomocysteinemia in vascular patients." Eur J Endovasc Surg **26**: 558-561.

Stead, L.M., K. Au, R.L. Jacobs, M.E. Brosnan and J.T. Brosnan (2001). "Methylation demand and homocysteine metabolism: effects of dietary provision of creatine and guanidinoacetate." Am. J. Physiol. Endocrinol. Metab. **281**: E1095-E1100.

Stipanuk, M.H. (1979). "Effect of excess dietary methionine on the catabolism of cystine in rats." J. Nutr. **109**: 2126-2139.

Stipanuk, M.H. (1986). "Metabolism of sulfur-containing amino acids." Annu. Rev. Nutr. **6**: 179-209.

Stipanuk, M.H. and N.J. Benevenga (1979). "Effect of cystine on the metabolism of methionine in rats." J. Nutr. **107**: 1455-1467.

Storch, K.J., D.A. Wagner, J. Burke and V.R. Young (1990). "[1-13C; methyl-2H3]methionine kinetics in humans: methionine conservation and cysteine sparing." Am. J. Physiol.: E790-E797.

Suliman, M., P. Stenvinkel, P. Barany, O. Heimburger, B. Anderstam and B. Lindholm (2003). "Hyperhomocysteinemia and its relationship to cardiovascular disease in ESRD: influence of hypoalbuminemia, malnutrition, inflammation, and diabetes mellitus." Am J Kidney Dis **41**: S89-S95.

Svardal, A. and P. Ueland (1987). "Compartmentalization of S-adenosylhomocysteine in rat liver. Determination and characterization of the *in vivo* protein binding." J. Biol. Chem. **262**: 15413-15417.

Taoka, S., S. Ohja, X. Shan, W. Kruger and R. Banerjee (1998). "Evidence for heme-mediated redox regulation of human cystathionine beta-synthase activity." J. Biol. Chem. **273**: 25179-25184.

Taoka, S., M. West and R. Banerjee (1999). "Characterization of the heme and pyridoxal phosphate cofactors of human cystathionine beta-synthase revels nonequivalent active sites." Biochemistry **38**: 2738-2744.

Tateishi, N. and Y. Sakamoto (1983). Nutritional significance of glutathione in rat liver. Glutathione: Storage, Transport and Turnover in Mammals. Y. Sakamoto. Tokyo, Scientific Socoetu Press: 13-38.

Tawakol, A., T. Omland, M. Gerhard, J. Wu and M. Creager (1997). "Hyperhomocyst(e)inemia is associated with impaired endothelium-dependent vasodilation in humans." Circulation **95**: 1119-1121.

Torres, L., Avila MA, Carretero, MV, Latasa, MU, Caballeria J, Lopez-Rodas, G, Boulcab, A, Lu, SC, Franco, L, Mato, JM (2000). "Liver-specific methionine adenosyltransferase MAT1A gene expression is associated with a specific pattern of promoter methylation and histone acetylation; implications for MAT1A silencing during transformation." FASEB J **14**: 98-107.

Troen, A.M., E. Lutgens, D.E. Smith, I.H. Rosenburg and J. Selhub (2003). "The atherogenic effect of excess methionine intake." PNAS **100**: 15089-15094.

Tseng, C.K., V.E. Marquez, R.W. Fuller, B.M. Goldstein, D.R. Haines, H. McPherson, J.L. Parsons, W.M. Shannon, G. Arnett, M. Hollingshead and J. Driscoll (1989). "Synthesis of 3-deazaneplanocin A, a powerful inhibitor of S-adenosylhomocysteine hydrolase with potent and selective in vitro and in vivo anitviral activities." J. Med. Chem. **32**: 1442-1446.

Tuite, N., K. Fraser and C. O'Byrne (2005). "Homocysteine toxicity in Escherichia coli is caused by a perturbation of branched-chain amino acid biosynthesis." Journal of Bacteriology **187**: 4362-4371.

Upchurch, G.R., G.N. Welch, A.J. Fabian, A. Pigazzi, J.F. Keeney and J. Loscalzo (1997). "Stimulation of endothelial nitric oxide production by homocyst(e)ine." Atherosclerosis **132**: 177-185.

Varela-Moreiras, G., E. Alonso-Aperte, M. Rubio, M. Glasso, R. Deulufeu, L. Alvarez, J. Caballeria, J. Rodes and J. Mato (1995). "Carbon tetrachloride-induced hepatic injury associated with global DNA hypomethylation and homocysteinemia: effect of S-adenosylmethionine treatment." Hepatology **22**: 1310-1315.

Vester, B. and K. Rasmussen (1991). "High performance liquid chromatography method for rapid and accurate determination of homocysteine in plasma and serum." European Journal of Clinical Chemistry **29**: 549-554.

Wagner, C., W. Briggs and R. Cook (1985). "Inhibition of glycine N-methyltransferase activity by folate derivatives. Implication for regulation of methyl group metabolism." Biochem. Biophys. Res. Commun. **137**: 746-752.

Wagner, C., W. Decha-Umphai and J. Corbin (1989). "Phosphorylation modulates the activity of glycine N-methyltransferase, a folate-binding protein. *In vitro* phosphorylation is inhibited by the natural folate ligand." J. Biol. Chem. **264**:

9638-9642.

Wang, J., N. Dudman, J. Lynch and D. Wilcken (1991). "Betaine:homocysteine methyltransferase - a new assay for the liver enzyme and its absence from human skin fibroblasts and peripheral blood lymphocytes." Clin. Chim. Acta **204**: 239-249.

Whitehead, A., P. Gallagher, J. Mills, P. Kirke, H. Burke, A. Molloy, D. Weir, D. Shields and J. Scott (1995). "A genetic defect in 5,10-methylenetetrahydrofolate reductase in neural tube defects." QMJ **88**: 763-766.

Wilcken, D. and B. Wilcken (1997). "The natural history of vascular disease in homocystinuria and the effects of treatment." J. Inherit. Metab. Dis **20**: 295-300.

Wilcken, D., X. Wang and T. Adachi (2000). "Relationship between homocysteine and superoxide dismutase in homocysteinuria. Possible relevance to cardiovascular risk." Arterioscler Thromb Vasc Biol **20**: 1199-1202.

Womack, M., K. Kemmerer and W. Rose (1938). "The relation of cystine and methionine to growth." J. Biol. Chem. **121**: 403-410.

Wyss, M. and T. Wallimann (1994). "Creatine metabolism and the consequences of creatine depletion in muscle." Mol. Cell. Biochem. **133-134**: 255-261.

Yamamoto, N., T. Tanaka and T. Noguchi (1995). "Effect of cysteine on expression of cystathionine beta-synthase in the rat liver." J. Nutr. Sci. Vitaminol. **41**: 197-205.

Yap, S., G. Boers, B. Wilcken, D. Wilcken and E. Naughten (2004). "Vascular complications of severe hyperhomocysteinemia in patients with homocystinuria due to cystathionine beta-synthase deficiency: effects of homocysteine-lowering therapy." Semin Thromb Hemost **26**: 335-340.

Zou, C. and R. Banerjee (2003). "Tumor necrosis factor-alpha-induced targeted proteolysis of cystathionine beta-synthase modulates redox homeostasis." J. Biol. Chem. **278**: 16802-16808.