INTESTINAL METABOLISM OF SULFUR-CONTAINING AMINO ACIDS IN THE RAT

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by

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Abstract

The portal-drained viscera play a fundamental role in the supply of dietary nutrients to the rest of the body. On a tissue mass basis these organs consume a disproportionately higher quantity of dietary available nutrients. Of the portal-drained viscera, the intestine is unique, being able to utilize nutrients from both luminal and arterial sources. With regard to sulfur-containing amino acids, the small intestine consumes about 30% of dietary available methionine though the gastrointestinal system represents only about 7% of the whole body mass. This organ is known to contain all the necessary enzymes of transmethylation, remethylation and transsulfuration.

Although the A-V balance of sulfur-containing amino acids across portal-drained viscera has been demonstrated by previous studies, the fluxes of sulfur-containing amino acids across the portal-drained viscera in the rat are not known. The fate of exogenous methionine within the enterocytes has not been elucidated. Therefore, in this thesis, the fluxes of major thiols across the portal-drained viscera and the metabolic fate of [1-^{14}C] methionine in isolated rat enterocytes were studied. Total cysteine was found to be exported to the portal blood in rats fed a 60% casein diet, a 20% casein supplemented with 0.6% cystine diet and a 20% casein supplemented with 0.5% methionine diet. The exported cysteine was entirely in the non-protein bound form in the portal plasma. Homocysteine was taken up from the arterial blood in rats fed a 2% methionine-containing diet. Glutathione was taken up by portal venous-drained viscera in all diet groups except the 60% casein fed rats.

Exogenous labelled methionine was metabolized by enterocytes at approximately the same rate as in hepatocytes. However, there was no appreciable flux through the transmethylation pathway. Homocysteine was metabolized through the transsulfuration pathway into cysteine. These studies reveal an active sulphur-containing amino acid metabolism in the intestine.

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LIST OF ABBREVIATIONS

5-MTHF	N^5 methyltetrahydrofolate
AEC	aminoethyl L-cysteine hydrochloride
ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid
ATP	adenosine triphosphate
A-V	arteriovenous
BHMT	betaine:homocysteine methyltransferase
CBS	cystathionine-β-synthase
CDO	cysteine dioxygenase
CGL	cystathionine-γ-lyase
CSDC	cysteinesulfinate decarboxylase
Cys	cysteine
Cys-Gly	cysteinylglycine
D_1	20% casein + 0.3% cystine diet
D_2	20% casein + 0.6% cystine diet
D_3	60% casein + 0.3% cystine diet
D_4	20% casein + 0.3% cystine + 0.5% methionine diet
D_5	20% casein + 0.3% cystine + 1% methionine diet
D_6	20% casein + 0.3% cystine + 2% methionine diet
dSAM	decarboxylated S-adenosylmethionine
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
fCys	non-protein bound cysteine
GCS	γ-glutamyl cysteine synthase
Glu	glutamate
Gly	glycine
GSH	glutathione (reduced)
GSSG	oxidized glutathione
γ-GT	γ-glutamyltranspeptidase
Hcy	homocysteine
HPLC	high performance liquid chromatography
IBD	inflammatory bowel disease
KH	Krebs-Henseleit
LDH	lactate dehydrogenase
Met	methionine
MTA	methylthioadenosine
PBF	portal blood flow
PCA	perchloric acid
PCV	packed cell volume
PI	protease inhibitor cocktail
PLP	pyridoxal-5'-phosphate
SAA	sulfur amino acids

SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SBD	short bowel syndrome
SBDS	7-fluorobenzofuranzan-4-sulfonic acid
Ser	serine
SSA	sulfosalicylic acid
tCys	total cysteine
tGSH	total glutathione
tHcy	total homocysteine
THF	tetrahydrofolate
TNB	tri-normal-butylphosphine

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CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 METABOLISM OF SULFUR CONTAINING AMINO ACIDS

The metabolism of sulfur-containing amino acids is shown in Figure-1. Since the other sulfur-containing amino acids are synthesized from methionine, methionine is the only essential (non-dispensable) sulfur-containing amino acid in mammals. The liver is thought to be the major organ of methionine metabolism and it contains all the enzymes shown in Figure-1. The foremost functions of the methionine cycle are to provide methyl groups for methylation reactions, to conserve the methionine carbon skeleton via remethylation and to catabolise excess methionine via the transsulfuration pathway. In addition, cysteine can be converted to glutathione and taurine. Alternative minor pathways also metabolize methionine. For example, methionine is transaminated to its keto acid, α-keto-γ-methiolbutyrate. The other possible alternative pathways are polyamine synthesis and the cleavage of SAM into methylthioadenosine, which may be converted further, into methylthioribose and homoserine thiolactone.

The methionine metabolic cycle comprises of two major arms: transmethylation and remethylation. In the transmethylation reactions, methionine is first converted into the high-energy sulfonium compound, S-adenosylmethionine (SAM). The next reaction is catalyzed by a large number of transmethylases by which the methyl group of SAM is transferred to any one of the large number of methyl acceptors. These enzymes bring about DNA, RNA and protein methylation, creatine synthesis and phosphotidylcholine synthesis. As a result, SAM is converted to S-adenosylhomocysteine (SAH), which is, then, reversibly hydrolysed to homocysteine (Hcy) and adenosine.

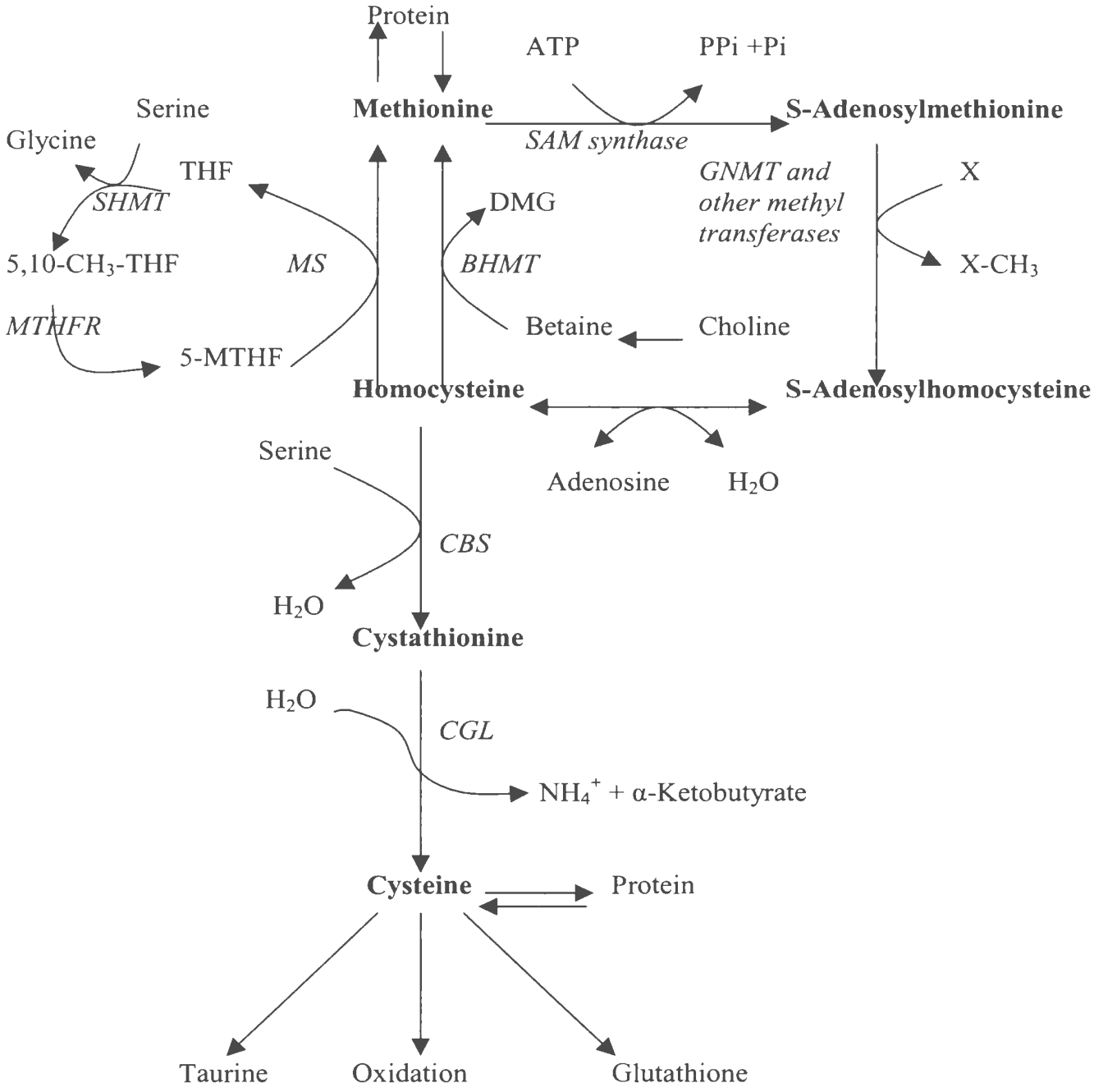

Figure 1.1. The Transmethylation, Remethylation and Transsulfuration pathway.

Hcy at this point can either be remethylated to methionine or irreversibly catabolized to cysteine (Cys) via the transsulfuration pathway. In the transsulfuration pathway, Hcy is first condensed with serine to form cystathionine, which is then cleaved into Cys, α-ketobutyrate and NH_4^+. In the remethylation pathway, a methyl group is added to Hcy moiety, which is thereby reconverted to methionine. The methyl group can arise from two possible sources. One is from N^5-methyltetrahydrofolate (5-MTHF) and the other is from betaine. In the liver, both sources of methyl groups can be utilized for remethylation of Hcy while in most extrahepatic tissues, only 5-MTHF can be utilized due to the absence of betaine: homocysteine methyltransferase (BHMT).

Interest in methionine metabolism has been stimulated with the finding of a number of genetically determined defects as well as by demonstrating that elevated plasma total Hcy is an independent risk factor for cardiovascular disease.

METHIONINE METABOLISM- TRANSMETHYLATION REACTIONS

S-adenosylmethionine, a high-energy sulfonium compound, is the first product of the transmethylation pathway. The conversion of methionine into SAM is catalyzed by S-adenosylmethionine synthase (SAM synthase; EC 2.5.1.6). The adenosyl moiety is transferred from adenosyl triphosphate (ATP) to the sulfur atom of L-methionine by this reaction (Cantoni 1953). In this reaction, ATP is completely dephosphorylated, which results in the formation of enzyme bound tripolyphosphate followed by an asymmetrical cleavage to inorganic phosphate and pyrophosphate. The reaction is physiologically irreversible. Three isozymes of SAM synthase have been found in mammals. The liver

specific and non-liver specific SAM synthases are products of two different genes (Chou 2000). SAM synthase-I (α) is liver specific and shows half-maximal activity (K_m) with an intermediate K_m for methionine of 41 μM (Hoffman *et. al.* 1979). It is slightly inhibited by the product, SAM and represents about 15% of total activity in the liver. SAM synthase-I is a tetramer of four identical subunits with a molecular weight of 208 000 kDa. SAM synthase-II (γ) is found in hepatic as well as extrahepatic tissues. Interestingly, this is the only isoform of SAM synthase found in fetal liver. It has the lowest K_m for methionine and is strongly inhibited by SAM (Hoffman *et. al.* 1979). This kind of inhibition is extremely important, particularly in this enzyme reaction, because rapid synthesis of SAM would be fatal, perhaps as a consequence of ATP depletion (Karwick *et. al.*1970). Five percent of total SAM synthase activity in the liver is brought about by this isozyme, which has a molecular weight of 120 000 kDa (Hoffman *et. al.* 1979). Because of this low K_m and the resulting high affinity of the γ isoform for methionine, the velocity of SAM synthesis would be at its maximal in extrahepatic tissues. SAM synthase–III (β) is also liver specific and has the highest K_m value ranging from 0.3-1.3 mM for methionine (Finkelstein *et. al.* 1978). The high K_m hepatic isozymes increase the production of SAM and thereby facilitate rapid clearance of excess dietary methionine available in the portal blood. Due to this tissue specificity and the different K_m of SAM synthase for methionine, the tissue content of SAM is a function of both the organ concerned and the availability of methionine. For instance, the pool of SAM in the liver can increase to 650 nmol/g from the normal range of 50-100 nmol/g when the diet is supplemented with excess methionine (Finkelstein and Martin 1986).

SAM is at a key junction in methionine metabolism. It can undergo three possible metabolic fates, i.e. (i) transmethylation to SAH by transferring the methyl group to any one of the large number of methyl acceptors. SAM is considered as the methyl group donor for almost all the biological methylation reactions in mammals. There is one notable exception; it cannot act as a methyl group donor in the remethylation of Hcy back into methionine (Stipanuk 1986). SAH is the spent form of transmethylated SAM. (ii) decarboxylation to decarboxylated SAM which is the aminopropyl group-donor for polyamine synthesis (Pegg *et. al.* 1998). (iii) cleavage of the linkage between carbon 4 and the sulfur atom of the amino acid chain leading to the formation of methylthioadenosine and homoserine thiolactone (Swiatek *et. al.* 1973). Approximately two thirds of intravenously administered SAM is utilized by the combination of transmethylation and cleavage into methylthioadenosine plus homoserine in the rat (Giulidori *et. al.* 1984). However, the cleavage reaction uses only a minor quantity of SAM. Thus the bulk of SAM is used in transmethylation reactions. The rest is decarboxylated and used in the polyamine synthesis in rats (Giulidori *et. al.* 1984). Both the synthesis and metabolism of SAM via all of the above-mentioned mechanisms are ubiquitous reactions.

In transmethylation reactions, the methyl group of SAM is transferred to a nitrogen, sulfur or oxygen atom in a wide range of methyl acceptor molecules. Among the products of this biological methylation, creatine, epinephrine, carnitine, phospholipids, proteins, DNA and RNA are prominent. However, among these, the methylation of guanidinoacetate in creatine synthesis accounts for a greater consumption

of methyl groups than all other methylation reactions combined (Mudd and Poole. 1975; Giulidori *et. al.* 1984). In addition, transmethylation reaction may be used as a means of degrading methyl groups (Stipanuk 1986). The methyl group of SAM can be degraded via the formation and oxidation of sarcosine (Mudd *et. al.* 1980; Wagner 1982; Wittwer 1981) (Figure-2). It has been found that the excess methyl groups available by means of methionine or choline are used in sarcosine synthesis, via the glycine methyltransferase reaction or the dimethylglycine dehydrogenase reaction (Mudd *et. al.* 2001). Therefore, production and degradation of sarcosine play a major role in removing excess methyl groups (Mudd *et. al.* 1980).

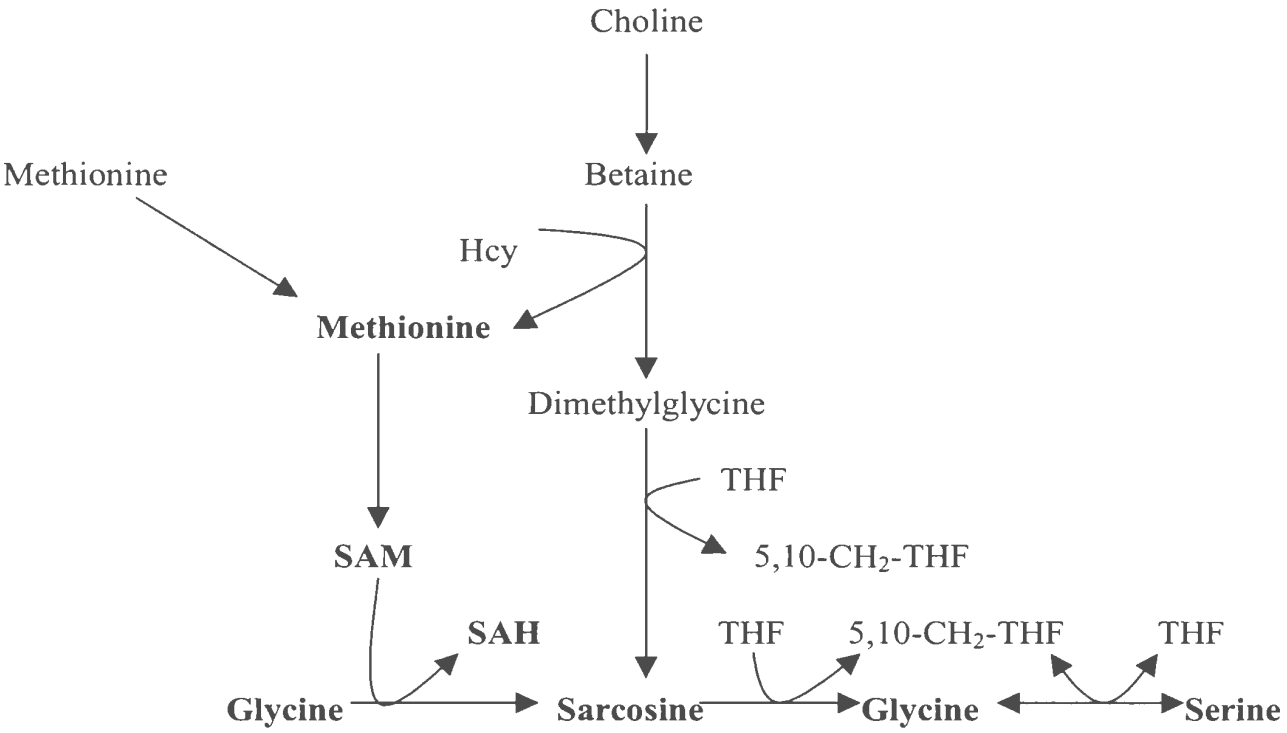

Figure 1.2. Pathway for methyl group oxidation

The co-product of all the SAM-dependent methyltransferase reactions is SAH which is then hydrolysed into adenosine and Hcy (De La Haba and Cantoni 1959). This reaction is catalyzed by S-adenosylhomocysteine hydrolase (EC 3.3.1.1) and is the only physiologically reversible reaction in the pathway. This enzyme has a ubiquitous tissue distribution and contains a tightly bound NAD^+, which is involved in the catalytic process (Elrod *et. al.* 2002; Kloor *et. al.* 2003). The disposal of SAH by this reaction eliminates undue product inhibition by SAH in most of the methyltransferases (Hoffman *et. al.* 1979). Thus, the hydrolysis of SAH serves two purposes: maintaining the flux of methionine sulfur towards cysteine and playing a critical regulatory role in biological methylation (Hoffman, *et. al.* 1979). The molecular weight of the enzyme is 188 000 kDa and it comprises of four identical subunits (Fujiolca and Takata 1981). The K_m value for SAH is in the range of 8-60 μM while for Hcy, it is about 160 μM (Finkelstein *et. al.* 1978). The equilibrium constant of this reaction favours SAH synthesis. Therefore, for the forward reaction to occur, adenosine and Hcy should be removed. Hcy can be removed by two possible ways; either by remethylation to methionine or by transsulfuration to Cys (Eloranta *et. al.* 1984; 1982). Adenosine is removed by adenosine deaminase. Accumulation of SAH could occur when either Hcy or adenosine accumulates, obstructing the hydrolytic reaction by mass action (Fox *et. al.* 1982). People with inherited defects in transsulfuration and remethylation pathways accumulate extremely high concentrations of SAH and SAM leading to interference with many of the biological methylation reactions. However, it appears that accumulation of intracellular SAH, which is present in free as well as protein-bound pools inside the cell, can be

reduced via a method independent of the hydrolase (Svardal and Ueland 1987). SAH is exported to the extracellular space when the cellular concentration is high (Hoffman *et. al.* 1980).

1.2 TRANSSULFURATION

Metabolism of Hcy (2-amino-4-mercaptobutyric acid) is at a key junction of the methionine metabolic pathway and it links the methionine metabolic cycle with the folate metabolism. The metabolic fate of methionine depends on the route that Hcy takes at this juncture, because transsulfuration is the only means of irreversibly dispensing methionine. Two pyridoxal-5'-phosphate (PLP) dependent enzymes, cystathionine-β-synthase (CBS; EC 4.2.1.22) and cystathionine-γ-lyase (CGL; EC.4.4.1.1) catalyze the transsulfuration of Hcy to Cys. The final product of the transsulfuration pathway is Cys. Only very few organs, the liver, kidney, pancreas and small intestine, contain the capability of synthesizing Cys from methionine or Hcy (Mudd *et. al.* 1965). Therefore, the synthesis of Cys from methionine and Hcy is restricted to these tissues. Other tissues require an exogenous supply of Cys either from the diet or from any of those four organs. Thus, transsulfuration is essential not only as a way of elimination of excess Hcy or methionine, but also, as a source of Cys and GSH. GSH may serve as a reservoir and a transport form of Cys. The initial and rate-limiting step of the transsulfuration pathway is catalyzed by CBS (Banerjee *et. al.* 2003). The irreversible nature of this reaction as well as the absence of an adequate source of Hcy in the mammalian diet explains why methionine is a dietary essential amino acid (Sakamoto *et. al.* 2002). In this reaction, Hcy

condenses with serine to form cystathionine. Cystathionine is an amino acid without a known function, other than as an intermediate in the biosynthesis of cysteine. CBS is unique in being dependent on two cofactors, PLP and heme. The presence of heme is required for PLP binding. Thus the amount of PLP bound is limited by the heme content (Kery *et. al.* 1994). Human CBS has a molecular weight of 63 kDa and exists as aggregates of tetramer of identical subunits (Taoka *et. al.* 1998).

The distribution of Hcy between the transsulfuration and remethylation pathways is nutritionally regulated. For example, when the diet contains a basal methionine level, Hcy moieties are found to cycle through the methylation pathway about 1.5-2.0 times before they are catabolized through the transsulfuration pathway. When dietary methionine content is reduced by half, this cycling of Hcy moiety increases twofold. Conversely, when the dietary methionine level is high, Hcy cycling falls below basal levels (Eloranta *et. al.* 1990). This coordination is achieved by at least two mechanisms. One is the effect of SAM as an allosteric modulator of two enzymes in the remethylation and transsulfuration pathways. SAM allosterically inhibits methyltetrahydrofolate reductase, which catalyzes the formation of 5-MTHF, one of the two methyl donors for the remethylation of Hcy to methionine (Finkelstein and Martin 1984a). SAM also activates SAM synthase. In patients with inherited SAM synthase deficiency a decreased activity of CBS is seen, possibly due to the lack of SAM mediated allosteric activation of CBS (Stabler *et. al.* 2002). The C-terminal domain of CBS is responsible for the SAM-mediated allosteric activation of the enzyme. Human CBS contains an N-terminal heme (protoporphyrin IX) - containing domain and a catalytic domain in addition to the C-

terminal domain. The catalytic domain binds to PLP (Taoka *et. al.* 1998). At the transcriptional level, hepatic CBS gene expression is hormonally regulated. Insulin is found to decrease the expression of the gene while glucagon and glucocorticoid hormones increase it (Jacobs *et. al.* 1998; Goss 1996; Jacobs *et. al.* 2001; Ratnam *et. al.* 2002). The overexpression of CBS in children with Down's syndrome results in significantly reduced plasma levels of Hcy, Met, SAH and SAM. Plasma cystathionine and cyst(e)ine are significantly increased in these patients (Finkelstein 2000).

The intracellular concentration of SAM itself co-ordinates remethylation and transmethylation. Of the two SAM synthases in the liver, SAM synthase -I (α) has a higher affinity for methionine and is believed to function at normal physiological conditions (Hoffman *et. al.* 1979). SAM synthase –III (β) has the lowest affinity among all three SAM synthase isozymes for methionine, and is thought to function under conditions of high intake of methionine (Finkelstein *et. al.* 1978). Therefore, the changes in dietary methionine availability and the resulting intracellular methionine level, affects the rate of SAM synthesis, which in turn will determine the route the Hcy moiety takes at the remethylation and the transsulfuration junction (Selhub 1999).

Since Hcy can be remethylated to methionine, CBS has to compete for the available Hcy with the remethylation enzymes. A study using liver extracts and substrates at their *in vivo* concentrations has revealed that CBS has the lowest affinity for Hcy when compared with methionine synthase and BHMT. On adaptation from a high protein to a low protein diet, the activity of CBS is reduced by 83%, which is equivalent to a 53% decrease in the Hcy flux through transsulfuration. Therefore, this hierarchal access for

available Hcy by the 3 enzymes is another way of regulation of the transsulfuration pathway (Finkelstein and Martin 1984a). In contrast to the diminished activity of CBS upon adaptation to a low protein diet, methionine supplementation in the diet increases the activity of CBS with a concomitant decrease in the hepatic levels of methionine synthase. An increase in the dietary methionine level from 0.3 to 3.0 % causes the hepatic activity of CBS to increase by about 12-fold in rats. Simultaneously, the hepatic content of serine is decreased when the dietary methionine level is increased above 0.3% (Finkelstein and Martin 1986). This is due to the higher demand for serine to be condensed with Hcy in the transsulfuration pathway.

Although Cys is not a precursor of methionine, the supplementation of Cys in the diet can replace the dietary requirement in humans for methionine by more than half (Di Buono *et. al.* 2003). In the rat, the supplementation of 0.8% of Cys in the diet results in a significant decrease in SAM and a two-fold increase in the serine content. The outcome is a 70% sparing of the dietary requirement for methionine (Finkelstein *et. al.* 1988). The enzymatic basis for this phenomenon is the marked decrease in hepatic CBS due to the low SAM level and the resulting absence of a potent allosteric stimulus on the CBS enzyme (Di Buono *et. al.* 2001). This is consistent with the early findings of *in vivo* studies in rats fed a low methionine diet with added Cys to meet the sulfur amino acid requirement. The liver CBS activity was diminished in these animals (Finkelstein and Mudd 1967).

CGL catalyzes the final step of the transsulfuration pathway where cystathionine is converted to Cys. In addition, homoserine deamination and the desulfhydration of Cys

are also catalyzed by this enzyme (Pascal *et. al.* 1972; Yao *et. al.* 1979). CGL is a PLP-dependent enzyme which catalyses the β-cleavage of cystathionine. Its molecular weight is 160 000-210 000 kDa for the rat liver enzyme and 190 000 kDa for the human lymphoid enzyme (Matsuo and Greenberg 1959). The rat liver enzyme has a K_m value for cystathionine of 3 mM (Finkelstein 2000). The products of this reaction are Cys, α-ketobutyrate and NH_3. Cysteine has a number of different metabolic fates, depending on the tissue. These include incorporation into proteins as well as glutathione (GSH) and synthesis of taurine. These fates are discussed under the section of cysteine metabolism.

1.3 REMETHYLATION

Hcy can be remethylated to methionine via two different means. By remethylation, the carbon skeleton of an essential amino acid is conserved. Remethylation may occur via methionine synthase (E.C.2.1.1.13), which has a ubiquitous distribution in mammalian tissues (Finkelstein *et. al.* 1971 and Xue 1985). This enzyme is the focus that connects methionine, vitamin B_{12} and folate metabolism. The mammalian enzyme contains tightly bound vitamin B_{12} as the prosthetic group. It is methylated by the folate substrate. Methionine is generated as a result of the methyl group transfer from methylated cobalamin to Hcy. A catalytic amount of SAM is needed for the initial priming of enzyme bound cobalamin. Methionine synthase is one of the two enzymes, identified so far, to require cobalamin as a cofactor, the other being methylmalonyl –CoA mutase that catalyzes the conversion of methylmalonyl-CoA into succinyl-CoA (Burke *et. al.* 1971; Cantoni *et. al.* 1982). Methionine synthase has a K_m value of 60 μM for Hcy (Finkelstein

1974). The methyl group donor in this reaction is 5-MTHF. This is the only reaction known in mammalian tissues involving 5-MTHF. Thus, the folate cycle is highly dependent upon the methionine synthase reaction to generate free tetrahydrofolate (THF) from its methylated form.

Deficiency of vitamin B_{12} possibly results in an accumulation of intracellular 5-MTHF at the expense of other forms of folate such as pteroic acid, dihydrofolic acid etc (Vidal and Stokstad1974). This is known as the methyl trap. 5-MTHF cannot be reoxidized to methylenetetrahydrofolate, because this reaction is essentially irreversible under physiological conditions (Kutzbach *et. al.* 1971). Under physiological conditions, it appears that the action of SAM inhibits MTHFR (EC. 1.1.99.15) which tends to minimize the trapping of 5-MTHF (Daubner *et. al.* 1982).

Methionine synthase is not the only means for the remethylation of Hcy into methionine (Finkelstein *et. al.* 1982a). A second route is via the reaction catalyzed by BHMT (E.C. 2.1.1.5). This is a zinc metalloenzyme (Breksa *et. al.* 1999; Evans *et. al.* 2002). Unlike methionine synthase, BHMT has more limited tissue distribution with a significant activity found only in the rat liver (Finkelstein *et. al.* 1971). In humans a significant BHMT activity is found in the liver, kidney and eye lens. In sheep, this enzyme is active in a number of tissues such as kidney, liver, adrenal and pancreas, which, surprisingly, accounts for the highest activity (Xue G.P *et. al.* 1985).

The reported K_m values of the rat liver BHMT are 15-21 μM and 49-56 μM for Hcy and betaine, respectively (Finkelstein *et. al.* 1971, Skiba *et. al.* 1982). Betaine, an obligatory intermediate in the choline catabolism, is the methyl group donor in this

reaction. BHMT activity is inhibited in vitro by SAM (Finkelstein and Martin1984b). In rats fed a high protein diet, liver BHMT activity is increased (Finkelstein *et. al.* 1971). The response of BHMT to methionine in the diet is biphasic; the supplementation of a low (0.3%) level of methionine to a methionine-free diet in rats causes a significant decrease in the enzyme activity. In contrast, the addition of 1% methionine to the diet results in a marked increase in rat liver BHMT activity. Conversely, when the diet is deficient in methionine, BHMT activity is increased and at this situation BHMT takes part in methionine conservation (Finkelstein 1982b).

1.4 POLYAMINE SYNTHETIC PATHWAY

Polyamines are ubiquitous constituents of mammalian tissues and hence, the biosynthesis of polyamines is an important pathway of SAM utilization particularly in organs with rapid proliferating cells. *In vivo* studies in rats intravenously injected with differentially labelled SAM, reveal that 10-30% of exogenous SAM is metabolized by the polyamine synthetic pathway (Giulidori *et. al.* 1984). Polyamines are aliphatic organic cations with important physiological roles in cell growth and proliferation. Decarboxylated S-adenosylmethionine (dSAM) is the source of the aminopropyl group for polyamine synthesis (Pegg *et. al.* 1998). No other reaction that utilizes dSAM at physiologically significant rates is known. Adenosylmethionine decarboxylase is the enzyme responsible for the decarboxylation of the carboxyl carbon of SAM in mammals (Pegg *et. al.* 1969).

The tissue availability of SAM is believed to be one of the regulators of the rate of polyamine synthesis. The tissue content of dSAM ranges from 0.9 to 2.5 nmol/g wet weight which is 2-5% of that of SAM (Hibasami *et. al.* 1980b). In addition to methionine, arginine is also required for the *de novo* synthesis of polyamines. Arginase converts arginine to ornithine. Then, ornithine is decarboxylated to putrescine in a reaction catalyzed by ornithine decarboxylase. The active aminopropyl group of dSAM that contains the carbon atoms 3 and 4 of the original methionine molecule is then transferred to putrescine which results in the formation of polyamines and 5-methylthioadenosine (MTA) by a reaction catalyzed by spermidine/spermine synthase (Heby and Persson 1990). As illustrated in Figure 1.3, methionine channelled into this pathway is not wasted; instead it is salvaged by the conversion of MTA through methylthioribose into methionine. The methionine salvage pathway has two physiologically important functions; as a method of conservation of adenosine and methionine and most importantly, it removes MTA which is a strong inhibitor of aminopropyl transferase of polyamine biosynthesis (Hibasami *et. al.* 1980a; Pegg *et. al.* 1981a and 1981b).

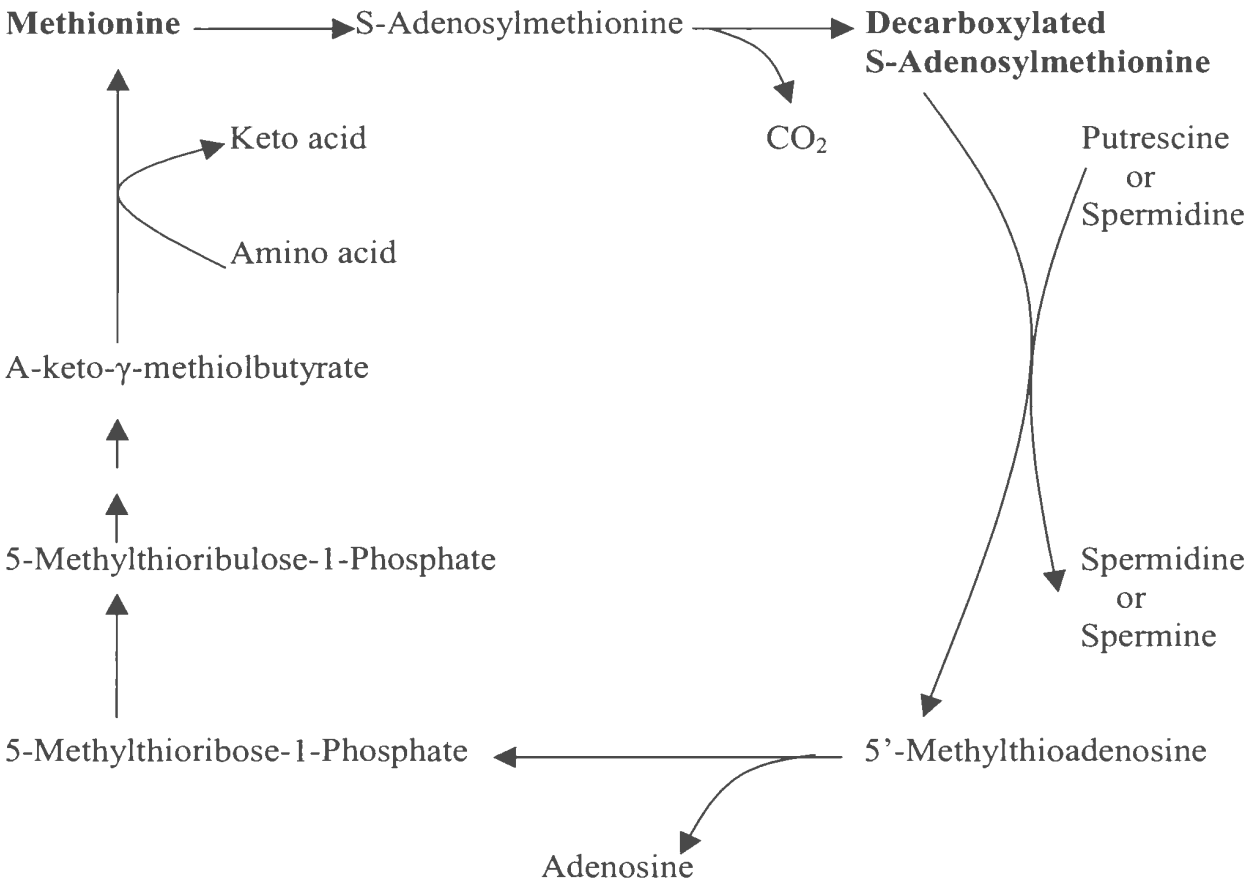

Figure 1.3. Polyamine pathway

1.5 TRANSAMINATION

Benevenga and coworkers have suggested an alternative pathway for methionine metabolism (Figure 1.4). This involves transamination of methionine to α-keto-γ-methiolbutyrate as the initial step (Case and Benevenga 1976; Steele and Benevenga 1978). The presence of the amino-accepting keto acid, α-keto-γ-methiobutyrate, has resulted in an increase in CO_2 production from the carboxyl carbon of methionine during *in vitro* studies (Benevenga and Eagen 1983). This reaction has been detected in liver, kidney, heart, brain, spleen, skeletal muscles and small intestinal homogenates of rats. The keto acid is decarboxylated within the mitochondria resulting in the formation of 3-methylthiopropionate (Dixon and Benevenga 1980; Livesey and Lund 1980). Further metabolism of this product gives rise to methenethiol, H_2S, sulfate, CO_2, formaldehyde and formate (Cohen and Benevenga 1985). The methyl carbon of 3-methylthiopropionate has been observed to be oxidized to CO_2 in liver and kidney homogenates (Steele and Benevenga 1979). However, the occurrence of this pathway in intact animals is not well established. SAM is neither a substrate nor an inhibitor of methionine transamination in liver homogenates (Case and Benevenga 1976).

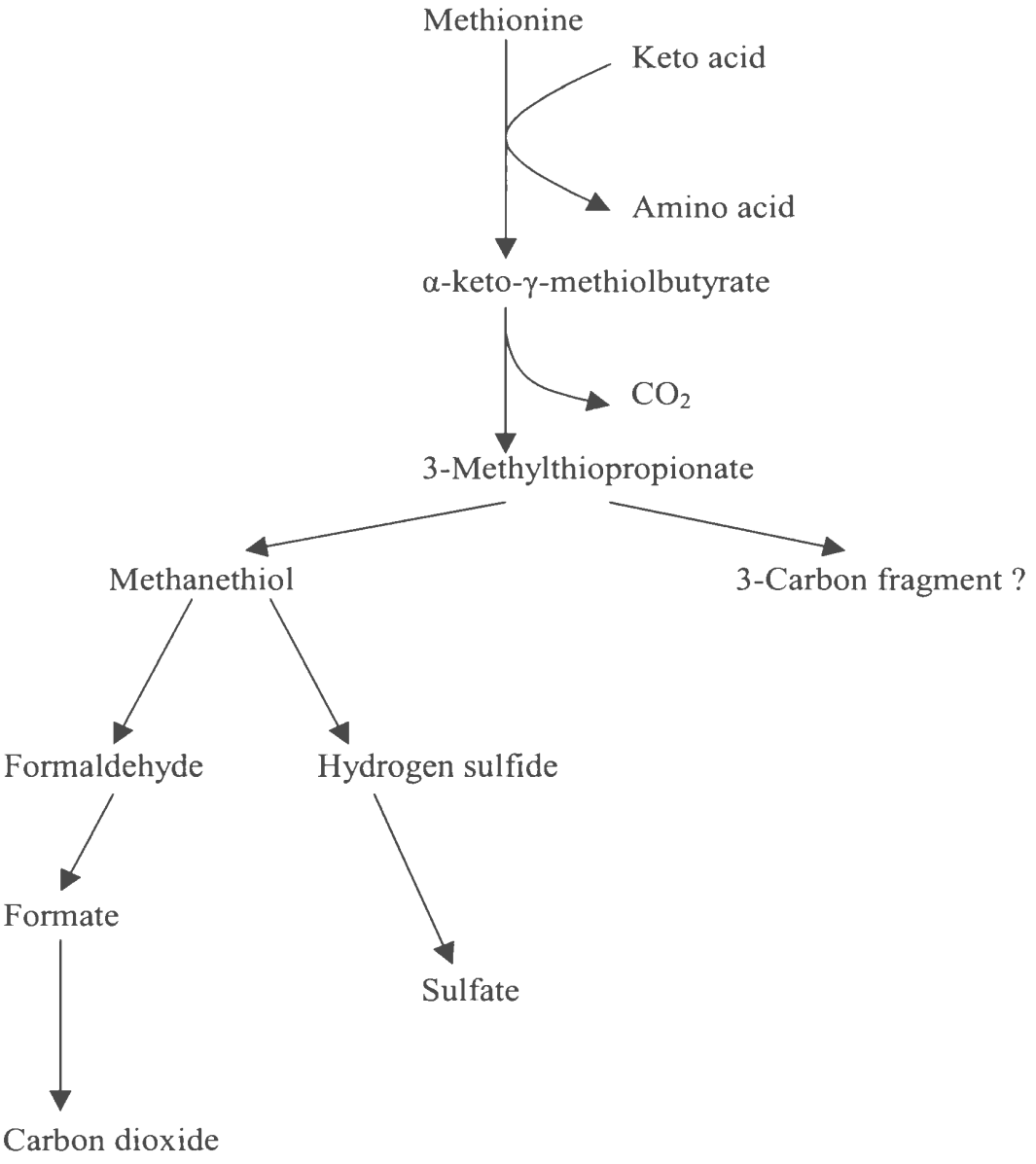

Figure 1.4. Methionine transamination pathway

1.6 CLEAVAGE OF SAM INTO METHYLTHIOADENOSINE

Cleavage of SAM into methylthioadenosine and homoserine thiolactone is another fate of this nucleotide. SAM is cleaved at the linkage between carbon 4 and the sulfur atom in the amino acid chain, leading to the formation of methylthioadenosine and homoserine thiolactone (Swiatek *et. al.* 1973). The utilization of SAM in this reaction was first identified in cell-free extracts of *Aerobacter aerogenes* and in *Escherichia coli* infected with bacteriophage T_3 (Shapiro and Mather 1958; Gefter *et. al.* 1965). Later, it was found to occur in mammalian tissues as well (Swiatek *et. al.* 1973). In rats, this pathway together with transmethylation pathway is responsible for the utilization of two thirds of the intravenously administered labelled SAM (Giulidori *et. al.* 1984). However, it does not necessarily mean that cleavage of SAM to homoserine is responsible for the utilization of a significant amount of SAM. The physiologic significance of this pathway is still not clear as there are no strong data to show the synthesis of homoserine from SAM under normal physiological conditions. The ability of cells to take up intravenously administered SAM is not clear. Hoffman *et. al.* (1980) claimed that the perfused liver is impermeable to SAM. Nevertheless, experiments cited above show the metabolism of intravenously administered SAM. As shown in Figure 1.5, methylthioadenosine can be reconverted to methionine, while homoserine thiolactone is catabolized via α-keto-butyrate to propionyl CoA. The carboxyl carbon atom of homoserine, which comes from methionine, is decarboxylated at this step. Further degradation of propionate releases the methyl carbon atom 3 as CO_2. α-keto-butyrate produced from the γ cleavage of

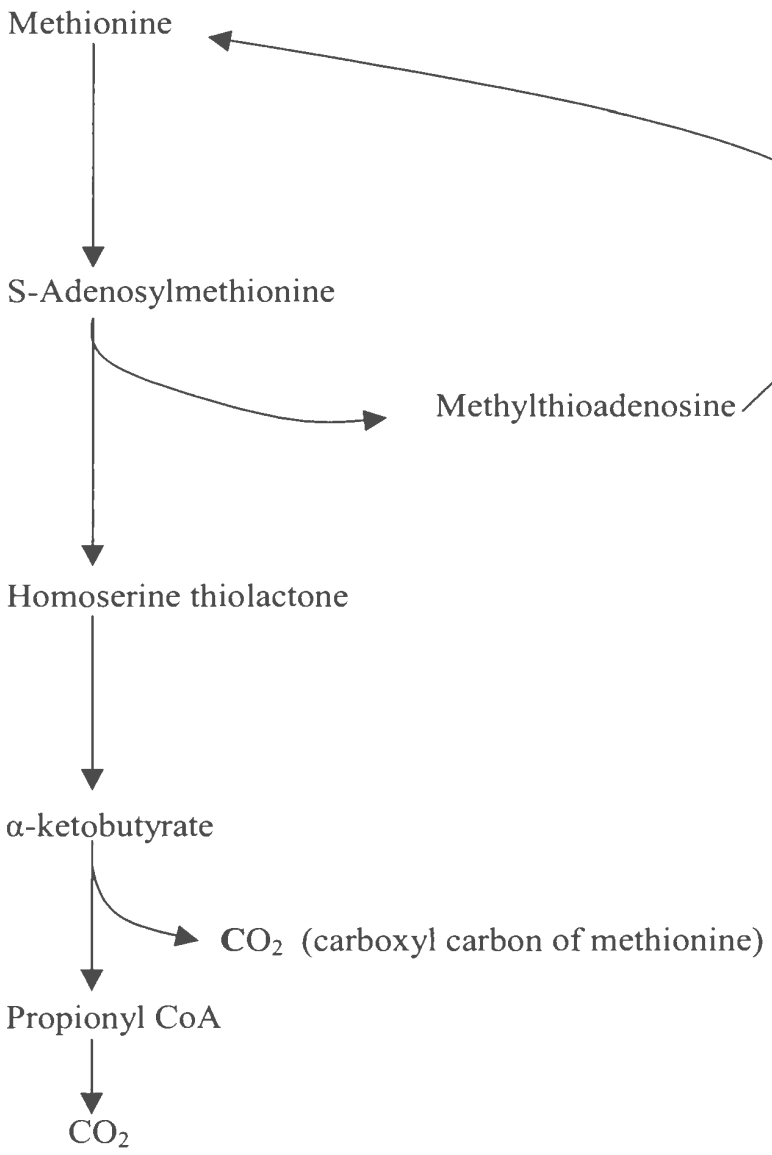

Figure 1.5. Cleavage of SAM at the linkage between carbon 4 and the sulfur atom in the amino acid chain, leading to the formation of methylthioadenosine and homoserine thiolactone

cystathionine in the transsulfuration pathway also shares the same catabolic pathway with homoserine (Giulidori *et. al.* 1984).

1.7 METABOLISM OF CYSTEINE

The major fates of Cys, which is derived from the transsulfuration pathway or provided from dietary sources, include incorporation into proteins and utilization as a precursor in the synthesis of the tripeptide GSH and the synthesis and catabolism of taurine. Among the critical functions of these metabolic products of Cys, synthetic reactions, osmotic regulation, antioxidative function, detoxification and involvement in nervous system functions are prominent. Cys present in excess is associated with some cytotoxic and neurotoxic effects (Montine *et. al.* 1997 and Reis *et. al.* 2000). Elevated plasma Cys was recently suggested to be an independent risk factor for cardiovascular disease (El-Khairy *et. al.* 2001).

As shown in the Figure 1.6 Cys is mainly catabolized via cysteinesulfinate-dependent pathways (Yamaguchi *et. al.* 1973; Griffith 1983). Cys in this pathway, is utilized either as a precursor for taurine synthesis or as a substrate for pyruvate and inorganic sulfate production (Stipanuk and Rotter 1984). Intake of a high protein diet is associated with metabolic acidosis, which is largely attributed to the degradation of the sulfur amino acids to yield sulfonic acid (Lemann *et. al.*, 1959 and Trilok *et. al.*, 1989). Therefore, in the cysteinesulfinate-dependent pathway, the distribution of Cys catabolism between taurine synthesis and the complete catabolism into sulphate products has implications for the acid-base homeostasis (Bella and Stipanuk 1995).

Cysteine dioxygenase (CDO; EC.1.13.11.20) catalyzes a key regulatory step in the conversion of Cys into cysteinesulfinate. This is an Fe^{2+} metalloenzyme and has a K_m of 0.45 mM for Cys. In this reaction, CDO adds molecular oxygen to the thiol group of cysteine to synthesize cysteinesulfinate. Thus, the sulfhydryl group is oxidized (Yamaguchi *et. al.* 1978). The hepatic activity of CDO in the rat liver is highly sensitive to the dietary protein and SAA level. The increase in dietary casein or SAA causes a rapid rise in rat liver CDO activity (Bella *et. al.* 1999b; Lee *et. al.* 2004). The rat liver CDO activity is found to increase 7-fold when the protein content of the diet is increased from 10% to 60% casein. Further, the supplementation of 0.3% and 1% L-methionine in the diet results in 2.8 and 13-fold increase in the CDO activity in the rat liver, respectively (Stipanuk *et. al.* 1994). The activity of CDO does not change in response to supplemental non-sulfur amino acids alone (Bella *et. al.* 1999a).

γ-Glutamylcysteine Synthase (GCS; EC. 6.3.2.2) which catalyzes the key reaction in GSH synthetic pathway competes with CDO for the available cysteine. GCS also responds to the sulfur amino acid and protein availability in the diet. Rat liver GCS activity, GCS-HS (catalytic/ heavy subunit) protein level and GCS mRNA concentration are lowered as a result of high casein or methionine supplementation (Bagley and Stipanuk 1994 and 1995; Lea *et. al.* 2004). There are also alternative, cysteinesulfinate-independent and cyst(e)ine desulfhydration, pathways which make some contribution to Cys catabolism (Drake *et. al.* 1987). The enzymatic catabolism by the latter two pathways include the cleavage of cyst(e)ine by γ-cystathionase and the transamination of cysteine to form 3-mercaptopyruvate (Stipanuk and Beck 1982; Drake *et. al.* 1987).

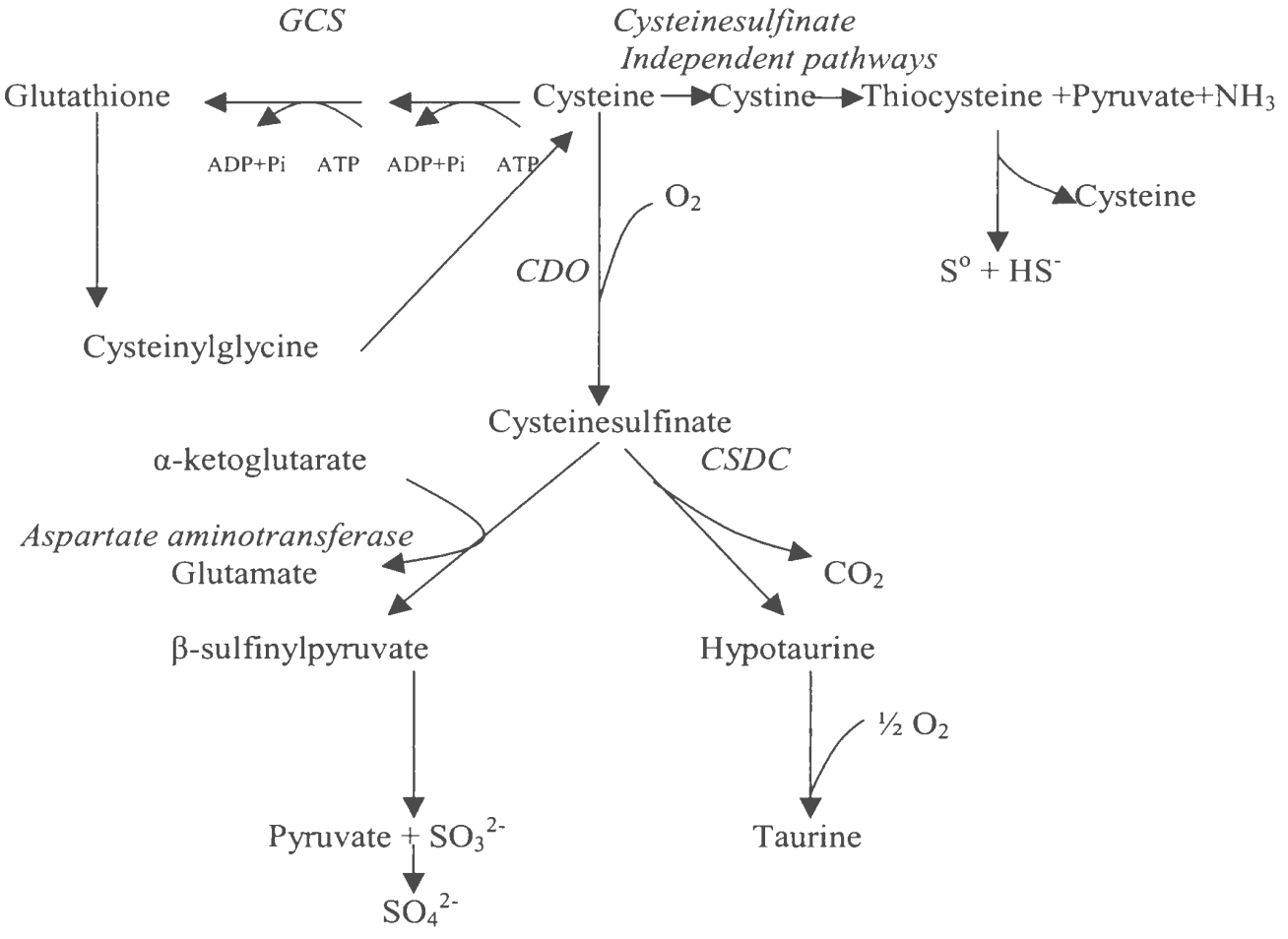

Figure 1.6. Pathways of cysteine metabolism

1.8 INTESTINAL METABOLISM OF SAA

The liver is thought to be the major site of methionine metabolism; hence most of the previous studies have focused on this tissue. Most studies of SAA metabolism in intestinal tissues are related to the metabolism of Cys. Studies on intestinal metabolism of methionine are lacking. Even though the liver is considered to be the major organ responsible for methionine metabolism, about 30% of dietary available methionine is sequestered in the portal-drained viscera (PDV) in the first pass metabolism in piglets (Reeds *et. al.* 1996). This is all the more remarkable, since the PDV represents only about 7% of the total body mass. However, among the very few organs which contain all the enzymes of methionine metabolic pathway except BHMT, the rat small intestine has the least capacity for transsulfuration. (Mudd *et. al.* 1965).

Methionine and Cys are consumed in dietary proteins. The estimated requirement of these two sulfur amino acids for an adult human is 15 mg/kg/day (Di Buono *et. al.* 2001; Storch *et. al.* 1988). The average intakes of methionine and cysteine in the United States are 2.3 and 1.3 g/day for men and 1.6 and 0.9 g/day for women (Stipanuk 2004). Thus, the daily intake is well above the requirement. The transport of dietary methionine into the intestinal mucosa is carried out by neutral amino acid transport systems such as $B^{o,+}$, ASC and L (Segawa *et. al.* 1999). Also, a fraction of dietary methionine is transported in peptide form by peptide transport systems (Matthews *et. al.* 1969). The intestinal absorption of dietary Cys is Na^+ dependent (Segawa *et. al.* 1999). Neutral amino acid transporters, including system B and ASC, absorbs Cys in this way (Stipanuk 2004).

In vitro studies reveal that Cys is catabolized into pyruvate and sulfate products within the enterocytes (Coloso *et. al.* 1989). However, Cys or cysteinesulfinate is not utilized for taurine synthesis in these cells (Coloso *et. al.* 1989). Half of Cys metabolism occurs via cysteinesulfinate-dependent pathway, which includes a transamination reaction catalyzed by aspartate aminotransferase. The other half is oxidized via cysteinesulfinate-independent desulfhydration pathways, mainly one that involves the β-cleavage of cystine by γ-cystathionase. The metabolic product, pyruvate, is further catabolized to CO_2 within the enterocytes. The catabolism of Cys to CO_2 within the enterocytes is substantial when compared with that of hepatocytes (Coloso *et. al.* 1989).

1.9 REDOX STATUS AND PROTEIN BINDING OF THIOLS IN THE PLASMA

The major aminothiols in plasma include Hcy, Cys, Cys-Gly and GSH. They exist in different forms in plasma. The sulfhydryl group has an ability to oxidize in the presence of an electron acceptor such as molecular oxygen. This kind of autooxidation results in the formation of disulfide bonds between the same type of thiol molecules or with another thiol. Thus, Hcy, Cys and GSH will autooxidize into their oxidized forms; homocystine, cystine and oxidized GSH (GSSG), respectively. Thiols oxidized with another thiol results in the formation of mixed disulfides such as the homocysteine-cysteine mixed disulfide and the homocysteine-glutathione mixed disulfide. The rate of autooxidation of thiols depends on several factors. Among them the copper-binding plasma protein, ceruloplasmin, and albumin are important (Fedman *et. al.* 1982; Sengupta *et. al.* 2001a). The copper ion attached to ceruloplasmin is responsible for the

autooxidation of L-Cysteine, Cys-Gly and GSH. However, neither ceruloplasmin nor copper ion bound to ceruloplasmin is a significant physiological catalyst for the autooxidation of L-Homocysteine. The autooxidation of Hcy in plasma is mainly mediated by albumin by thiol:disulfide exchange reaction. Copper attached to albumin is responsible for only about 20% of the total autooxidation of Hcy (Sengupta *et. al.* 2001a).

In spite of the free reduced and free oxidized thiols, the bulk of Hcy and Cys present in human plasma is bound to plasma proteins (Peters 1996). In healthy individuals, the free reduced Hcy in the circulation is less than 1% of the total value which is about 10 μmol/L. The free oxidized fraction is about 30% and the predominant form, the plasma protein-bound fraction is almost 70%. Cys is the most abundant type of all aminothiols at a total concentration of about 250 μmol/L in human plasma. About 65% of this is in the albumin-bound form and of the rest, 3-4% is in the free reduced form (Mansoor *et. al.* 1993). However, in rat plasma, about 60% of plasma total Cys is in the free form; not bound to albumin (Maloy *et. al.* 1981). The plasma total Cys-Gly concentration in humans is about 30 μmol/L and of it 17 μmol/L is bound to plasma proteins (Mansoor *et. al.* 1993). The free reduced fraction is about 10% (Mansoor *et. al.* 1993). GSH is the least abundant thiol in the human plasma, with a concentration of about 6 μmol/L. More than 80% of GSH in human plasma is in the free reduced form. The protein-bound fraction represents only about 25% of the total (Mansoor *et. al.* 1993).

Albumin is the most abundant protein in plasma. In humans total plasma albumin concentration ranges from 0.6 to 0.75 mM (40-45 g/L). This represents more than 50% of total plasma protein level in humans (Curry *et. al.* 1998). In young rats, the plasma

albumin concentration is reported to be 33 g/L (Peters 1996). Albumin is the predominant Hcy-binding protein in human plasma. More than 90% of total bound Hcy in the plasma is bound to albumin (Sengupta *et. al.* 2001b). The fraction of protein-bound Hcy increases as a function of total plasma Hcy up to a maximal binding of 140 μmol/L in humans. This may represent the saturation of all the available Hcy-binding sites in plasma albumin. Cys is displaced and thereby the albumin-bound Cys is decreased in these situations, equivalent to the increase in Hcy binding (Ueland *et. al.* 1996).

Albumin is a nonglycosylated, single-chain polypeptide that is synthesized exclusively by the liver. Formation of 17 intra-chain disulfide bonds between 34 of the total of 35 Cys residues defines the final structure of the tightly folded three domain structure of albumin. One Cys residue at Cys^{34} is left unbound as a result of this intra-chain disulfide bonding. This free Cys^{34} residue accounts for the bulk of bound thiols (-SH) in plasma (Peters 1996). Thus, one third of the albumin molecules in the plasma are disulfide-bonded with plasma thiols (Carter *et. al.* 1994). Once synthesized in the liver, albumin is secreted into the circulation in the free thiolate anion form (Peters 1996); this highly reactive thiolate anion is able to make disulfide bonds with plasma thiols at physiological pH (Carter *et. al.* 1994).

According to a model proposed by Christoduolou *et. al.* (1994 and 1995) Cys^{34} exists in two forms; one in exposed and the other in the buried form. In the buried form Cys^{34} is in close proximity to His_{39}, making a stable salt bridge. The exposed form is stabilized with the formation of a disulfide bond with Hcy or Cys. *In vitro* studies using human serum albumin reveal that Hcy displaces Cys from albumin. Nevertheless,

stoichiometrically, the formation of new albumin-bound Hcy does not equal the Cys released from albumin. Instead, a higher amount of Cys is released in response to a formation of a lesser amount of albumin-bound Hcy (Togawa *et. al.* 2000). The reason for this difference is believed to be the formation of albumin thiolactone anion (Sengupta *et. al.* 2001b). The bulk of the Hcy added and as well as the Cys which is released from albumin-bound form then undergo autooxidation; homocystine, cystine and homocysteine-cysteine mixed disulfide are formed (Sengupta *et. al.* 2001b).

According to a model proposed recently by Sengupta *et. al.* (2001a), L-cysteine in human plasma is oxidized into cystine by ceruloplasmin. Nascent albumin thiolate anion released from the liver into the circulation attacks L-cystine to form albumin-Cys^{34}-S-S-cysteine. L-homocysteine then reacts with this Cys-bound albumin, which results in the formation of the homocysteine-cysteine mixed disulfide. Albumin then returns to its thiolate anion form, which can then attack the homocysteine-cysteine mixed disulfide. As a result, albumin-Cys^{34}-S-S-homocysteine and the cysteine thiolate anion are formed. The autooxidation of L-homocysteine is catalyzed by copper co-ordinated to His^3 of albumin but not by ceruloplasmin. Albumin thiolate anion can react with this homocystine to make some additional albumin-Cys^{34}-S-S-homocysteine (Sengupta *et. al.* 2001a). In the measurement of total plasma Hcy or Cys, the protein-bound fraction is reduced to the free form by a suitable reducing agent. However, it does not include thiols bound to protein by an amide linkage, such as compounds formed by the reaction of homocysteine thiolactone with the lysine residues of protein (Jakubowski 1999).

1.10 HYPERHOMOCYSTEINEMIA

1.10.1 Hyperhomocysteinemia and cardiovascular disease risk

Thirty years after the discovery of homocysteine by du Vigneaud, the first case of a congenital anomaly associated with homocysteine was found. It was observed in mentally challenged children who had a high level of Hcy in blood as well as urine (Carson and Neil 1962; Gerritsen *et. al.* 1964). From then, numerous vascular lesions were found in patients with hyperhomocysteinemia (McCully 1969; Kanware *et. al.* 1976), which led McCully and Wilson (1975) to postulate that hyperhomocysteinemia predisposes to atherosclerosis. Hyperhomocysteinemia can result from either increased production or decreased removal of Hcy. A number of factors such as inborn errors in methionine metabolic enzymes, deficiencies of various B vitamins which play important roles as cofactors in this cycle, chronic renal failure and certain chemotherapeutic agents are responsible for decreased rate of removal of plasma Hcy (Kang 1996; Selhub 1999).

The most common cause of severe homocysteinemia and consequential homocystinuria in humans is inborn errors in CBS. Inadequate CBS activity is known to cause Hcy accumulation and its consequential export from the cell, leading to hyperhomocysteinemia (Pogribna *et. al.* 2001). This was shown in CBS knockout mice; plasma Hcy levels were about 40 times higher than normal in the homozygous mice and 2 times higher than normal in heterozygous mutants (Watanabe *et. al.* 1995). Vitamin B_6 deficiency is another common cause of hyperhomocysteinemia since both CBS and CGL are PLP- dependent enzymes (Cuskelly *et. al.* 2001; Guttormsen *et. al.* 1996). The second most common cause of hyperhomocysteinemia is decreased remethylation due to inborn

defects in $N^{5,10}$ methylene-tetrahydrofolate reductase activity (Poole *et. al.* 1975). Inborn errors affecting methionine synthase and various steps in the synthesis of methylcobalamin, which is an essential cofactor for this enzyme, also are among the causes for hyperhomocysteinemia. In addition, vitamin B_{12} and folate deficiency result in hyperhomocysteinemia (Guttormsen *et. al.* 1996; Kluijtmans *et. al.* 2003).

Urinary excretion is not a significant route of removing Hcy, as only a very limited amount of extracellular Hcy is excreted via this route. About 1-2% (3.5 – 9.8 μmol/day) of Hcy in the glomerular filtrate is excreted (Refsum *et. al.* 1985; Svardal *et. al.* 1986). The bulk is reabsorbed by renal cortical tubules via a high affinity system shared with cystine and dibasic amino acids (Foreman *et. al.* 1982). However, plasma total Hcy is quite elevated in patients with renal failure. This level rises steeply when the patient develops terminal uremia (Chauveau *et. al.* 1996).

Hcy metabolism can be affected by certain chemotherapeutic agents such as theophylline, carbamazepine and valproate. Theophylline is a vitamin B_6 antagonist whereas valproate has antifolate effects. Therefore, hyperhomocysteinemia resulting from these drugs is secondary to vitamin deficiencies (Karabiber *et. al.* 2003).

10.1.2 Hyperhomocysteinemia associated with intestinal diseases

Hyperhomocysteinemia and venous thrombosis are found in patients with Inflammatory Bowel Disease (IBD) and Short Bowel Syndrome (SBS). Since these patients require long term indwelling catheters because of the requirement for parenteral feeding, it was earlier believed that the venous thrombosis is developed secondary to the

usage of intravenous catheters. However, it is now established that venous thrombosis in these patients is due to hyperhomocysteinemia (Cattaneo *et. al.* 1998; Compher *et. al.* 2000; Oldenburg *et. al.* 2000).

Hyperhomocysteinemia associated with SBS is usually related to vitamin B_{12} deficiency (Compher *et. al.* 2000). However, in IBD patients, it is not necessarily related to a deficiency of vitamin B_{12} though these patients have low levels of vitamin B_{12} (Romagnuolo *et. al.* 2001; Koutroubakis *et. al.* 2000). The elevation of tHcy in IBD and SBS patients is minor (from 10.5 μmol/L to 12.2 μmol/L) when compared that with severe hyperhomocysteinemia in patients with inherited defects in methionine metabolic enzymes.

Bakker *et. al.* (1997) reported on a patient with ulcerations of the stomach and the small intestine who had a substantially higher plasma tHcy level than normal. In addition, this patient had atherosclerotic lesions in the mesenteric artery (Bakker *et. al.* 1997). According to another case report, acute thrombosis was found in the superior mesenteric artery of a patient who had previously undergone bowel resection surgery. Further, the same patient had a markedly elevated level of plasma tHcy (Gradman *et. al.* 2001). A cross-sectional study carried out by Romagnuolo *et. al.* (2001), showed that hyperhomocysteinemia is significantly more common in patients with IBD than in the normal population. Though these patients have some lower values for vitamin B_{12}, they are not necessarily deficient in it (Romagnuolo *et. al.* 2001).

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

Animals and Diets

Male Sprague-Dawley rats weighing 200-300 g were used for all experiments. Rats were obtained from Memorial University's breeding colony and were housed and treated in accordance with the guidelines of the Canadian Council on Animal Care (1993). The university's Institutional Animal Care Committee approved all procedures. The rats were housed, two in a cage, at 22 ± 2 °C, with free access to tap water and commercial rat chow (Prolab® RHM 3000). The feeding conditions during the diet experiments will be described in the section of experimental protocol.

Chemicals

All chemicals used in these experiments were of analytical grade. Sodium pentabarbitol was obtained from MTC Pharmaceuticals (Cambridge, Ontario, Canada). Ammonium-7-fluorobenzo-2-oxo-1,3-diazole-4-sulphonate was purchased from Molecular Probes (Eugene, Oregon, USA). Sodium heparin was from Solopak Laboratories Inc. (Elk Grove Village, Illinois, USA). All the diet constituents were purchased from ICN Biomedicals Inc. (Aurora, Ohio, USA). Tri-n-butylphosphine was obtained from Acros (New Jersey, USA). All other chemicals and amino acids used in this study were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Radioisotopes

All radioisotopes were purchased from American Radiolabelled Chemicals Ltd., (St. Louis, MO, USA)

METHODS

I). Net Fluxes of Thiols and Amino Acids across the Portal-Drained Viscera in Rats Fed Different Diets

Feed Ingredients

A modified AIN 93G diet, designed to meet the nutritional requirements for the growth of laboratory rats, was used. The six diet groups were different from each other in the content of casein, cystine and methionine. A control group of 20% casein with 0.3% cystine (D_1) was tested against the rest of the diet groups: 20% Casein + 0.6% cystine (D_2), 60% Casein + 0.3% cystine (D_3), 20% Casein + 0.3% cystine + 0.5% methionine (D_4), 20% Casein + 0.3% cystine + 1% methionine (D_5) and 20% Casein + 0.3% cystine + 2% methionine (D_6). The composition of the diet is given in Table 2.1.

Table 2.1. Composition of purified diets

Ingredient	20% + Casein +0.3% cystine diet **(D_1)**	20% Casein+ 0.6% cystine diet **(D_2)**	60% Casein +0.3% cystine diet **(D_3)**	20% Casein+ 0.3% cystine +0.5% Met **(D_4)**	20% Casein+ 0.3% cystine +1% Met **(D_5)**	20% Casein+ 0.3% cystine +2% Met **(D_6)**
Cornstarch	397.486	394.486	144.486	392.486	387.486	377.486
Casein	200.00	200.00	600.00	200.00	200.00	200.00
Dextrinized cornstarch	132.00	132.00	48.00	132.00	132.00	132.00
Sucrose	100.00	100.00	37.00	100.00	100.00	100.00
Soybean oil	70.00	70.00	70.00	70.00	70.00	70.00
Alphacel	50.00	50.00	50.00	50.00	50.00	50.00
AIN-93G mineral mix	35.00	35.00	35.00	35.00	35.00	35.00
AIN-93G Vitamin mix	10.00	10.00	10.00	10.00	10.00	10.00
L-Cystine	3.00	6.00	3.00	3.00	3.00	3.00
Choline bitartrate	2.50	2.50	2.50	2.50	2.50	2.50
Tert-butylhydroquinone	0.014	0.014	0.014	0.014	0.014	0.014
L-Methionine	0.00	0.00	0.00	5.00	10.00	20.00

The diet constituents are given in grams per kg diet

Experimental Protocol

For the study of arteriovenous difference of thiols and other amino acids across the intestine, rats were maintained on the above diets for one week prior to experimentation. All animals had free access to water and diet. Rats were housed in plastic cages, two to a cage, with a stainless steel mesh lid. The room was maintained at 22 ± 2 °C and 60-70% humidity with a 12-hour light, 12-hour dark cycle which was light from 8.00 to 20.00 hours. Initial body weights were recorded. After a 7-day period of *ad libitum* feeding, rats were anaesthetized with sodium pentabarbitol (65 $mg.kg^{-1}$, ip) in the morning (9.00 hrs). Then, each rat was kept on a dorsal recumbent position on a heating pad at around 37°C. The abdomen was opened by a mid-line incision and the gut was deflected to the rat's left to expose the hepatic portal vein which was then dissected free of surrounding connective tissues. A 2RB perivascular blood flow probe with an L type bracket (Transonic® Small Animal Flowmeter T206, Transonic systems Inc., Ithaca, NY, USA) was placed around the portal vein as close as possible to the liver. The dead space between the portal vein and the flow probe was filled with KY jelly, which improved the acoustic coupling. Blood flow was recorded in ml/min, 5 minutes after the probe was placed. During this period, blood flow attained a steady value.

After recording the blood flow of the portal vein, the flow probe was cautiously removed. Blunt tearing of the adipose tissues around the vessel exposed the abdominal aorta. Blood samples of 1.5 ml from each vessel were collected from the portal vein and the abdominal aorta simultaneously, into heparinized syringes. Samples were kept on ice for a few minutes. The time from the laparotomy to blood sampling was less than 10

minutes. All the rats were in the absorptive state as evidenced by the presence of food in the intestine.

A microcapillary tube was filled with arterial blood to determine the hematocrit (Packed Cell Volume, PCV). These tubes were then centrifuged for 15 minutes (Adams Autocrit^{TM} Centrifuge, NY, USA) and the PCV was read as a percentage. Blood samples collected in heparinized syringes were then transferred to microcentrifuge (Eppendorf) tubes and were kept on ice for few minutes. They were centrifuged within 15 minutes of sample collection to separate plasma. The immediate removal of blood cells from the plasma is important to prevent the release of Hcy from erythrocytes into plasma (Rasmussen and Moller 2000). Plasma was separated by centrifugation in a clinical centrifuge at 3 700 x g for 5 minutes and stored at –20°C until required for HPLC analysis of total thiols. However, in the case of samples for the analysis of free thiols, plasma was deproteinized and the entire analytical procedure was performed on the same day as described below.

Sample Preparation

Total and free values of plasma Hcy, Cys, Cys-Gly and GSH were measured by a method described by Vester and Rasmussen (1991).

Total Thiol Assay

150 µl of plasma was mixed with 20 µl mixture of 10% (v/v) tri-normal-butylphosphine (TNB) (Acros Organics, NJ, USA) in N,N dimethyl-formamide. After mixing thoroughly, the reduction was allowed to proceed for 30 minutes at 4°C. To each sample then added 50 µl of the sodium salt of 0.2 mM 8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS) (Molecular Probes, Oregon, USA) prepared in 0.1 M potassium borate buffer at pH 9.5 containing 2 mM EDTA. ANTS is used as an internal standard. Plasma proteins were precipitated by adding 125 µl of 0.6% perchloric acid (PCA) and left at room temperature for 10 minutes. They were then centrifuged at 3 700 x g for 5 minutes after which 100 µl of the supernatant was transferred into a separate set of microcentrifuge tubes. 200 µl of 2 M potassium borate at pH 10.5 containing 5 mM EDTA and 100 µl of the ammonium salt of 7-Fluorobenzofurazan-4-sulfonic acid (SBDS) were added. SBDS was used as the derivatization agent and it was prepared by dissolving 1 g of SBDS in 1L of 2 M potassium borate at pH 10.5. Sample mixtures were then incubated at 60 °C for 1 hour in a light-protected water bath. The samples were cooled in ice for 5 minutes and centrifuged at 3 700 x g followed by filtering through a 0.45 µm Millipore filter. At this stage the samples were ready for HPLC analysis and kept on ice until they were loaded into the HPLC autosampler.

Free Thiol Assay

For this assay it was necessary to precipitate plasma proteins (with bound thiols) before the reduction procedure. 150 µl of fresh plasma was mixed with 85 µl of 0.6 M

PCA and kept at room temperature for 10 minutes. It was then centrifuged at 3 700 x g for 5 minutes and 150 µl of the supernatant was transferred into a new microcentrifuge tube. 20 µl mixture of 10% (v/v) TNB in N,N dimethyl-formamide was added and mixed and the tube was kept at 4° C for 30 minutes. The internal standard, 32 µl of 0.2 mM ANTS in 0.1 M potassium borate buffer at pH 9.5 with 2 mM EDTA, was then added and, finally, 18 µl of 0.1 M potassium borate buffer at pH 9.5 containing 2 mM EDTA was added. The rest of the procedure was same as for free thiol assay except that 0.6 M PCA was not added after ANTS because it had been added at the beginning of the procedure.

HPLC Analysis of Total and Free Thiols

Plasma free and total thiols were determined by reverse-phase HPLC and fluorescence detection of ammonium SBDS thiol adducts, using the method of Vester and Rasmussen (1991). Samples prepared as described above, were run through a Hypersil ODS 5 µm, 150 mm x 4.6 mm column for the separation of thiols. For both free and total thiol assay, 0.2 mM Hcy, Cys, Cys-Gly and GSH standards in 0.1 M potassium borate buffer, pH 9.5, containing 2 mM EDTA were prepared. 50 µl aliquots of these 0.2 mM standard thiol solution were diluted with 100 µl of 0.1 mM potassium borate buffer, pH 9.5, containing 2 mM EDTA to bring the concentrations approximately equal to those in the plasma.

Samples, 20 µl, were injected from an autosampler. Elution was brought about by means of a gradient of two buffers prepared as described by Vester and Rasmussen

(1991). Both buffers were filtered through a 0.45 μm Millipore filter. Two different HPLC machines; Shimadzu (Shimadzu Scientific Instruments Inc, USA) and Waters (Millipore corporation, Milford, MA, USA) were used during these assays. Both machines deployed a similar method in which a linear gradient was run from buffer A to buffer B over 12.5 minutes at a flow rate of 1 ml/minute. Then buffer B was run for 2.5 minutes followed by returning to buffer A for 3 minutes. Finally, buffer A was run for a further 2 minutes before the next sample was injected.

Analysis of Amino Acids

150 μl of plasma was deproteinized with 25 μl of 10% sulfosalicylic acid (SSA). After thorough mixing the samples were kept on ice for 15 minutes. The internal standard, 25 μl of 0.25 mM amino ethyl L-cysteine hydrochloride (AEC), was added, followed by 162.5 μl of Lithium Citrate at pH 2.2. The samples were then mixed and centrifuged at 3 700 x g for 5 minutes. The supernatant, which was about 250 μl in volume, was collected and analyzed for amino acids on a Beckman 121 MB amino acid analyzer by Mr. Craig Skinner. This system employed Benson D-X, 0.25 Cation Xchange Resin and a single-column, three-buffer lithium method as per Beckman 121 MB-TB-017 application notes. A Hewlett Packard Computing Integrator Model 3395A was used for quantification.

II). Intestinal Homogenate for Enzyme Assay

Rats, fed normal chow, were anaesthetized with sodium pentabarbitol (65mg.kg^{-1},ip) during the fed state. A midline laparotomy was performed to expose the intestinal tract. The whole intestine was removed by severing at the proximal duodenal region and at the ileo-caecal junction. It was then put in ice-cold 0.9% saline solution. Squeezing gently along the intestine, from the proximal to the distal end, emptied the luminal contents. Then, using a small piece of rubber tubing attached to a 20 cc syringe, ice-cold normal saline was driven through the intestine 3-4 times. Excess saline was swabbed with gauze, followed by sectioning the whole length of the intestine into 10 approximately equal segments. They were divided into two groups each having alternate segments. Both groups were weighed separately and were placed in cold homogenization medium (0.05 M potassium phosphate buffer at pH 7.0). One group was placed in a centrifuge tube containing 4.5 ml of homogenization medium + 0.5 ml of protease inhibitor cocktail and the other group was put in 5 ml of homogenization medium alone. The final concentration of protease inhibitor in the incubation medium was 5 μg/ml each of Phenylmethylsulfonyl fluoride (PMSF), Pepstatin, Chymostatin and Aprotinin and 1 mM EDTA. The intestinal tissue segments were cut into small pieces with a pair of scissors, while the tissue segments were immersed in the homogenization medium. The tissues were homogenized by two 10-second bursts with a Polytron (Brinkman instruments, Toronto, Canada). Between these two bursts, the tubes were kept on ice. The homogenate was then centrifuged at 18 000 x g for 30 minutes at 4 °C. Supernatants were saved and kept on ice until used for protein and enzyme assays.

Measurement of Protein Content in the Intestinal Homogenate

Protein was assayed by the Biuret method (Gornall, 1949). The biuret reaction is based on the complex formation of cupric ions with proteins. In this reaction, copper sulfate is added to a protein solution in strong alkaline solution. A purplish-violet colour is produced, resulting from complex formation between the cupric ions and the peptide bond. Bovine serum albumin was used as the standard protein solution. 5% deoxycholic acid was used to solubilize any membranous material.

Enzyme Assays of Intestinal Homogenates

A). Cystathionine-β-synthase

The assay was based on a method developed by Mudd *et. al.* (1965) and modified by Taoka *et. al.* (1998). The assay involved the incorporation of labelled serine into cystathionine, which was isolated by anion exchange chromatography. Since the enzyme activity was thought to be very low in intestinal tissues, a higher quantity (1 to 9 mg protein of intestinal homogenate) was incubated at 37 ^{o}C for 1 hour with a cocktail containing the following ingredients. 125 mM Tris HCl at pH 8.3 and 2.1 mM EDTA comprised the cocktail medium which contained the final concentration of 0.146 mM L (+) Cystathionine, 41.7 mM DL-Hcy, 0.316 mM SAM, 2.1 mM Propargylglycine and 0.42 mM PLP. 500 µl of the supernatant of the intestinal homogenate was mixed with 1.33 ml of cocktail. The reaction was initiated by adding 167 µl of 300 mM 1-^{14}C serine (2 µCi/ml) to the incubation medium. 1 ml of ice-cold 15% PCA was added to each tube

at the end of a one-hour incubation period to stop reactions. The reaction mixture was then centrifuged at 3 700 x g for 5 minutes and the supernatant was saved.

An aliquot of 1.67 ml of the supernatant was passed through a Bio-Rad AG 50W-X4 column containing 200-400 mesh hydrogen-form resin. The column was washed in the following order; 2 times with 4 ml of water, 6 times with 4 ml of 1 N HCL and 4 times with 4 ml of water. Then, labelled cystathionine was eluted once with 5 ml of 3 N NH_4OH. Eluates were collected and 1 ml aliquots of the collected eluate were transferred into 20 cc scintillation vials. 10 ml of ScintiVerse Xylene solution (Fisher Scientific, NJ, USA) was added into each vial. The radioactivity was counted the next day, in a LKG WALLAC 1214 RACKBETA liquid scintillation counter. The radioactivity of 10 µl of 300 mM stock 1-^{14}C serine was also determined in 10 ml of ScintiVerse. Each determination was corrected by blank values obtained by assays, both with labelled substrate-free reaction media and acid denatured homogenates.

B). S-Adenosylmethionine synthase

The assay followed a method described by Mudd *et. al.* (1965). The assay cocktail contained 100 mM Tris HCl at pH 7.8, 200 mM KCl, 10 mM $MgCl_2$, 1 mM DL-Dithiothreitol (DTT) and 5 mM ATP. Intestinal homogenates prepared with and without protease inhibitors were used as enzyme source. The volume of the intestinal homogenate was adjusted so that 60 µl volume was equivalent to 0.27 – 1.62 mg protein. The intestinal homogenate was incubated with the assay cocktail of 400 µl at 37 °C for 30 minutes in a shaking water bath at 60-70 oscillations per minute. The reaction was

initiated with the addition of 40 µl of 62.5 mM [1-^{14}C] methionine (2 µCi/ml). After 30 minutes of incubation, addition of 1.5 ml of ice-cold water followed by immediate transfer of all the reaction tubes into an ice water bath stopped the reactions.

For separation of labelled product from the substrate, Bio-Rad 50W-X4 200-400 mesh, NH_4^+ form resin was used. The counter ion of the resin was changed from H^+ to NH_4^+ by the following procedure. The resin was suspended in de-ionized water for 15 minutes. Once the resin was settled to the bottom of the beaker, water was discarded. The resin was then resuspended in 3 N NH_4OH twice and washed 3 times with water to remove excess NH_4OH. Finally, the columns were prepared by adding approximately 2 ml of the resin.

Once the resin was set in the column, 1.4 ml of stopped sample was added. The resin was then washed 5 times with 4 ml of water. Finally, the labelled SAM was eluted with 2, 3 ml aliquots of 3 N NH_4OH. The eluate was collected in 20 cc scintillation vials which were then mixed with 10 ml of ScintiVerse. Radioactivity was counted on the following day in a LKG WALLAC 1214 RACKBETA liquid scintillation counter. The Radioactivity of 10 µl of 62.5 mM stock [1-^{14}C] methionine was also determined in 10 ml of ScintiVerse. Each determination was corrected by blank values obtained by assays both with labelled substrate-free reaction media and acid denatured homogenates.

III). Isolation of Enterocytes

In preliminary studies, we obtained the best yield of enterocytes from rats that weighed between 150 and 250 g. Therefore, the rats used in the isolation of cells were approximately 200 g in body weight. They had ingested a chow diet. Enterocytes were isolated by a method described by Watford *et. al.* (1978). Three different modified Krebs-Henseleit saline solutions were prepared fresh daily; (Saline-1) the medium of Krebs and Henseleit (144 mM Na^+/6 mM K^+/1.3 mM Ca^{2+}/1.2 mM Mg^{2+}/126 mM Cl^-/1.2 mM $H_2PO_4^-$/1.2 mM SO_4^{2-}/25 mM HCO_3^-) [see appendix –II (Krebs and Henseleit, 1932)] from which $CaCl_2$ was omitted, (Saline-2) the same to which 0.25% (w/v) dialysed serum albumin and 5 mM EDTA were added, (Saline-3) the medium of Krebs-Henseleit (1932) with 2.5% (w/v) dialysed serum albumin (Table 2.2).

Rats were killed by cervical dislocation in order to avoid the use of anaesthetics, which may increase the secretion of mucus into the intestinal lumen. Immediately after the rats were killed, the abdominal cavity was incised open along the linea alba. The small intestine was cut proximally, about 5 cm below the pyloric sphincter and distally, about 10 cm above the colon. After emptying the luminal contents, the intestine was flushed 2-3 times from the proximal end of the intestine with ice-cold saline-1 gassed with O_2/CO_2 (19:1)

Table 2.2. The composition of Krebs-Henseleit saline solutions

Constituents	Saline Solution type		
	1	2	3
1). 0.9% Nacl (0.154M)	103 (ml)	103 (ml)	100 (ml)
2). 1.15% KCl (0.154M)	4 (ml)	4 (ml)	4 (ml)
3). 1.22% $CaCl_2$ (0.110M)	0	0	3 (ml)
4). 2.11% KH_2PO_4 (0.154M)	1 (ml)	1 (ml)	1 (ml)
5). 3.8% $MgSO_4.7H_2O$ (0.154M)	1 (ml)	1 (ml)	1 (ml)
6). 1.3% $NaHCO_3$ (0.154M)	21 (ml)	21 (ml)	21 (ml)
7). Dialyzed serum albumin	0	0.325 (g)	3.25 (g)
8). EDTA	0	242.2 (mg)	0

A 40 cc syringe connected with a piece of rubber tubing equipped with a stop-valve was connected to the proximal end of the intestine. This rubber tubing was used for rinsing and filling the lumen with different saline solutions. The distal end of the intestine was ligated and the lumen was filled with about 18 ml of gassed saline-2. Once the lumen was slightly distended with filled saline-2, the stop-valve was closed and the intestine was incubated at 37°C for 15 minutes in a 250 ml conical flask containing 100 ml of saline-1. The flask was gassed with O_2/CO_2 (19:1) throughout and shaken at 60-70 oscillations/min. Then, the intestine was opened from the distal end and fluid was drained off. The lumen was rinsed two times with about 20 ml of gassed saline-3 injected through the rubber tubing attached to the proximal end to remove mucus and loose cells from the tips of the villi. The distal end of the intestine was ligated once again and the lumen was filled through the proximal end with gassed saline-3 making sure the volume of saline injected was sufficient to distend the intestine slightly. The stop-valve was then closed. The intestine filled with saline-3, was patted with fingertips for 1 min on an ice-block covered by a polythene bag and with three layers of gauze pads on the surface. This was

performed to release the enterocytes into the saline-3 medium, which filled the lumen. The fluid was drained and collected into polystyrene tubes. These last two steps were repeated 4 times and all the drained fluid was collected and centrifuged at 500 x g for 3 minutes. The supernatant was discarded and the packed cells were washed once with approximately 4 volumes of saline-3. Cells were then resuspended in about 4 volumes of saline-3. The cells were dispersed in the medium by drawing them up several times into a wide-mouthed plastic pipette. 1 ml aliquot of the cell suspension was then transferred to microcentrifuge tubes. This was done in order to get an approximately equal number of enterocytes in each millilitre because, with time, the enterocytes tend to adhere, forming cell clumps. Every time, when the cells were transferred from one container to another as well as in the later described incubations, pipette tips that were cut obliquely to provide a large orifice were used.

We also isolated enterocytes in a saline-2 medium containing collagenase (150 mg/ 200 ml) in an attempt to improve the separation and viability. Apart from the addition of collagenase to saline-2, the rest of the procedure was the same as described above.

Dry Weight of Enterocytes

2 ml of the cell suspension and 2 ml of saline-3 in weighing boats were dried in an oven at 60 °C (Fisher isotemp® oven 100 series, Model 106G, USA) for 3 days so as to determine the dry weight of the isolated enterocytes. The mean dry weight was 3.1 ± 0.5 mg/ml.

Enterocytes Viability Studies

Lactate dehydrogenase (LDH) activity was measured to determine the viability of isolated enterocytes. Enterocytes were incubated under our experimental conditions for 0, 15, 30 and 45 minutes. After each incubation, the entire content of cells and the incubation medium was centrifuged at 3 700 x g for 5 minutes. The supernatant was saved and the cell fraction was resuspended in 2 ml of Krebs Ringers phosphate medium. 0.9 ml of the cell suspension was taken into a microcentrifuge tube containing 0.1 ml of 10% Triton X-100 (final concentration was 1%). We had previously determined that 1% Triton X-100 is sufficient to obtain 100% lysis of enterocytes. The microcentrifuge tube containing enterocytes and Triton X-100 was kept in ice for 15 minutes. It was then frozen in liquid nitrogen followed by thawing back to the room temperature. 20 µl volume of supernatant and lysed cell fractions were placed separately in a 2 ml cuvette which contained 1.8 ml of Krebs Ringer phosphate medium, 100 µl of NADH (3 mg dissolved in 1 ml of de-ionized water) and 100 µl of pyruvate (3 mg in 1ml of de-ionized water). The change in absorbance at 340 µm over 5 minutes was recorded and LDH activity of both the supernatant and cell fraction was calculated using the molar extinction coefficient 6.22 X 10^6 (Bergmeyer 1974). The viability of cells was expressed as a percentage.

Transsulfuration Flux of Isolated Enterocytes

Polyethylene Erlenmeyer flasks of 25 cc were used for all of the incubations with enterocytes. This enabled us to avoid glass surfaces that cause enterocytes to adhere.

Also, all assays were carried out in triplicate. Incubations were only for 30 minutes because the enterocytes had a satisfactory (over 85%) viability for only 45 minutes. The incubation medium of 1 ml, comprised the final concentration of 1 mM (1-^{14}C) methionine, 1 mM Serine, 1 mM Glutamate, 1 mM Glycine. The volume was made up to 1 ml with Krebs-Henseleit medium fortified with 10.5 mM Glucose. The final glucose concentration in the incubation medium plus enterocytes was 5 mM. The incubation medium with all or selected combination of the above mentioned substrates was added to Erlenmeyer flasks. The flasks were gassed with 19:1 O_2/CO_2 for 30 seconds and stopped until the enterocytes were added.

In the second reaction of transsulfuration, α-ketobutyrate is produced; the carboxyl carbon of this substance derives from the number 1 carbon of methionine. [1-^{14}C] α-ketobutyrate is converted to propionyl CoA by pyruvate dehydrogenase. As a result, the carboxyl carbon is liberated as $^{14}CO_2$. However, some [1-^{14}C] α-ketobutyrate may remain un-metabolized. The label of this fraction can be cleaved chemically as $^{14}CO_2$ by adding 30% (w/v) H_2O_2. The total production of $^{14}CO_2$ by both of these methods will be a measure of transsulfuration flux. $^{14}CO_2$ was trapped in plastic centre wells containing NCS-II tissue solubilizer (Amersham International, Oakville, Ontario, Canada).

All flasks were incubated at 37°C for 30 minutes in a shaking water bath at 60 oscillations per minute. The transsulfuration reactions were started with the addition of 1 ml enterocytes. The flasks had been gassed for 15 seconds before the cells were added. A further 15 second gassing was carried out after enterocytes were added. Flasks were then

sealed with rubber septae equipped with plastic centre wells, containing a ribbed piece of blotting paper. The collection of $^{14}CO_2$ for the measurement of transsulfuration flux was as described by Stead.*et. al.* (2000)

Transsulfuration Inhibition Studies

In a separate series of experiments, the same incubations were carried out in the presence and absence of 2 mM propargylglycine and 0.5 mM α-cyanocinnamate, which are inhibitors of cystathionine-γ-lyase and the mitochondrial transport of α-ketobutyrate, respectively.

Production / Removal of Thiols in Isolated Enterocytes

Enterocytes also were incubated in the presence and absence of 1 mM methionine and 1 mM Hcy. After 30 minutes of incubation, the reaction was stopped by adding 0.3 ml of 30% (w/v) perchloric acid. The production/removal of Hcy, Cys and GSH were measured by the reverse phase HPLC method (Vester and Rasmussen, 1991).

DATA ANALYSIS AND PRESENTATION

Results are expressed as means ± SD (number of rats used). Significant difference between arterial and portal concentration (A-V differences) of thiols and other amino acids were determined by paired *t*-test, as appropriate. Body weight gain, portal blood flow differences in hematocrit values between diet groups and differences in thiol concentration in arterial and portal venous blood in different diet groups were compared by one way ANOVA and Newman-Keuls Multiple Comparison Test. The Student t test was used to differentiate the significance of free and bound fractions of thiols. Statistical analyses were conducted using GraphPad Prism 3.02 32 Bit Executable. A *P* value less than 0.05 was taken as statistically significant.

RESULTS AND DISCUSSION

CHAPTER 3

INTRODUCTION

The portal-drained viscera play a fundamental role in the supply of dietary nutrients to the rest of the body. These organs consume a disproportionately higher quantity of dietary available nutrients compared with their relative tissue mass. There are numerous experimental data on the fluxes of nutrients and other substrates across the portal-drained viscera of pigs. However, according to our knowledge, there is only one publication on the A-V balance of SAA across this organ bed in rats (Garcia and Stipanuk 1992). These authors did not measure blood-flow. Therefore, no study has been carried out to determine the fluxes of all major thiols. Therefore, we decided to study the net balances and fluxes of SAA across the portal-drained viscera in rats fed diets with different amounts of casein, cystine and methionine.

WEIGHT GAIN OF RATS DURING THE DIETARY PROTOCOL

The initial weight of the rats was 246.6 ± 29.7 g. The mean weight gain of rats during the 7-day period on diets is reported in Table 3.1. Rats fed the control diet and the 0.6% cystine-enriched diet grew at an average rate of about 10 g/day. Rats fed a 2% methionine diet, showed no weight gain during the 7-day dietary period. The high protein and 1% methionine-supplemented groups had a slightly, but significantly lower ($P < 0.05$) weight gain when compared with the controls as well as the rats on the 0.6% cystine-supplemented diets. No significant difference in the weight gain was observed between rats on the high protein diet, the 0.5% and 1% methionine-supplemented diets.

Table 3.1. Effects of diet on weight gain of rats fed for a 7-day period

Diet group	Mean weight gain g/day
20% Casein + 0.3% cystine	10.5 ± 1.7^{a}
20% Casein + 0.6% cystine	10.7 ± 2.6^{a}
60% Casein + 0.3% cystine	8.5 ± 1.8^{c}
20% Casein + 0.3% cystine + 0.5% methionine	9.4 ± 1.9^{bc}
20% Casein + 0.3% cystine + 1% methionine	7.9 ± 1.0^{bc}
20% Casein + 0.3% cystine + 2% methionine	0.3 ± 0.9^{e}

Values are means ± SD for n = 6-18 rats. Values without a common letter superscript are significantly different ($P < 0.05$) by ANOVA and Newman-Keuls Multiple Comparison Test.

Portal Blood Flow and Hematocrit Values

Table 3.2 reports the mean values for the portal blood flow and hematocrit of rats in each diet group. No significant difference was observed for portal blood flow. However, the hematocrit or PCV value varied significantly between diet groups. Except 0.6% cystine-supplemented group, all other diet groups showed a significantly elevated PCV than the control group.

Table 3.2. Effect of diet on Portal Blood Flow (PBF) and Packed Cell Volume (PCV; hematocrit) of rats after a 7-day period on each diet

Diet group	Mean PBF ml/min	Mean PCV %
20% Casein + 0.3% cystine	23.9±5.0	40.5±2.2[a]
20% Casein + 0.6% cystine	23.4±4.8	41.3±2.0[abc]
60% Casein + 0.3% cystine	24.0±3.0	42.4±1.8[cd]
20% Casein + 0.3% cystine + 0.5% methionine	23.0±3.9	43.3±1.2[bde]
20% Casein + 0.3% cystine + 1% methionine	23.7±7.6	45.3±2.5[e]

Values are means ± SD for n = 6-18 rats. Within each column, values without a common letter superscript are significantly different ($P < 0.05$) by ANOVA and Newman-Keuls Multiple Comparison Test.

A-V Differences for Thiols across the Portal Drained Viscera

For reasons, which will be addressed in the discussion, we measured the arterio-venous (A-V) differences of the thiols in plasma rather than in whole blood. Cys, Hcy, Cys-Gly and GSH were measured. The portal vein contains blood draining the stomach, intestines, spleen and pancreas. Therefore, the arteriovenous difference in this study represents the absorptive and metabolic activity of all the portal-drained viscera, not necessarily by the gastrointestinal tract. The concentration of thiols in the portal vein is the balance of a number of effects such as the products of SAA metabolism within these

organs, inputs from diet, turnover of epithelial cells, secretions and products of bacterial actions.

Arterial and portal venous concentrations of each thiol across the portal-drained viscera of rats, fed different diets, were analysed. A-V differences were calculated by subtracting the concentration of a compound in the plasma of portal venous sample from that of arterial sample drawn simultaneously from the same rat. The results were expressed as output (-) or uptake (+) by the portal-drained viscera (Table 3.3). The rates of thiol uptake and output were expressed using portal blood flow and the percentage of plasma volume in whole blood (Table 3.4).

As shown in Figure 3.1, a net output of tCys into the portal vein was observed in rats fed the 0.6% cystine-supplemented diet, the 60% casein diet and the 0.5% methionine-supplemented diet.

Table 3.4 shows the net flux of the different thiols across the portal-drained viscera. There was significant output of total Cys in the portal-drained viscera in rats fed the 0.6% cystine-supplemented diet and 60% casein diet.

Table. 3.3. The arterial and portal venous concentrations & A-V difference of plasma total thiols across the portal-drained viscera of rats fed 6 different diets for 7 days.

Diet	Cysteine (μmoles/L)			Homocysteine(μmoles/L)		
	A	V	A-V	A	V	A-V
D_1	277±36.0	311±28.0	-34.0±34.0	11.0±2.0	11.7±1.8	-0.4±1.2
D_2	262±29.0	294±44.0	-31.0±47.0*	10.0±2.0	9.8±2.1	-0.08±0.7
D_3	298±37.0	343±45.0	-45.0±31.0*	12.0±2.0	12.9±2.6	-0.54±0.8
D_4	282±57.0	299±66.0	-17.0±20.0*	35.0±24.0	34.5±23.9	0.78±3.2
D_5	233±29.0	250±51.0	-16.0±30.0	122±53.0	118±49.0	3.0±12.5
D_6	295±20.0	293±24.0	1.5±19.0	296±139	267±130	29.3±20.4*
Diet	Cysteinylglycine (μmoles/L)			Glutathione (μmoles/L)		
	A	V	A-V	A	V	A-V
D_1	0.64±0.19	0.71±0.17	-0.06±0.1	25.9±5.0	21.9±4.7	3.8±2.2*
D_2	0.68±0.2	0.73±0.2	-0.04±0.1	23.8±2.0	19.9±2.6	3.8±1.1*
D_3	0.80±0.2	0.81±0.1	-0.02±0.1	22.2±3.0	20.6±3.0	1.6±2.4
D_4	0.78±0.1	0.80±0.2	-0.02±0.04	26.3±4.0	21.9±4.1	4.4±3.2*
D_5	0.72±0.1	0.71±0.1	0.00±0.1	21.1±3.0	17.0±3.6	4.1±1.5*
D_6	N/M	N/M	N/M	27.5±6.0	23.0±5.6	4.4±3.8*

The values are means ± SD for 6-14 rats. * Significantly different from zero ($P < 0.05$) by paired *t* test. The results were expressed as output/export (-) or uptake/removal (+) by the portal-drained viscera. D_1, 20% casein + 0.3% cystine diet; D_2, 20% casein + 0.6% cystine diet; D_3, 60% casein + 0.3% cystine diet; D_4, 20% casein + 0.3% cystine +0.5% methionine diet; D_5, 20% casein + 0.3% cystine + 1% methionine diet; D_6, 20% casein + 0.3% cystine + 2% methionine diet. N/M, not measured.

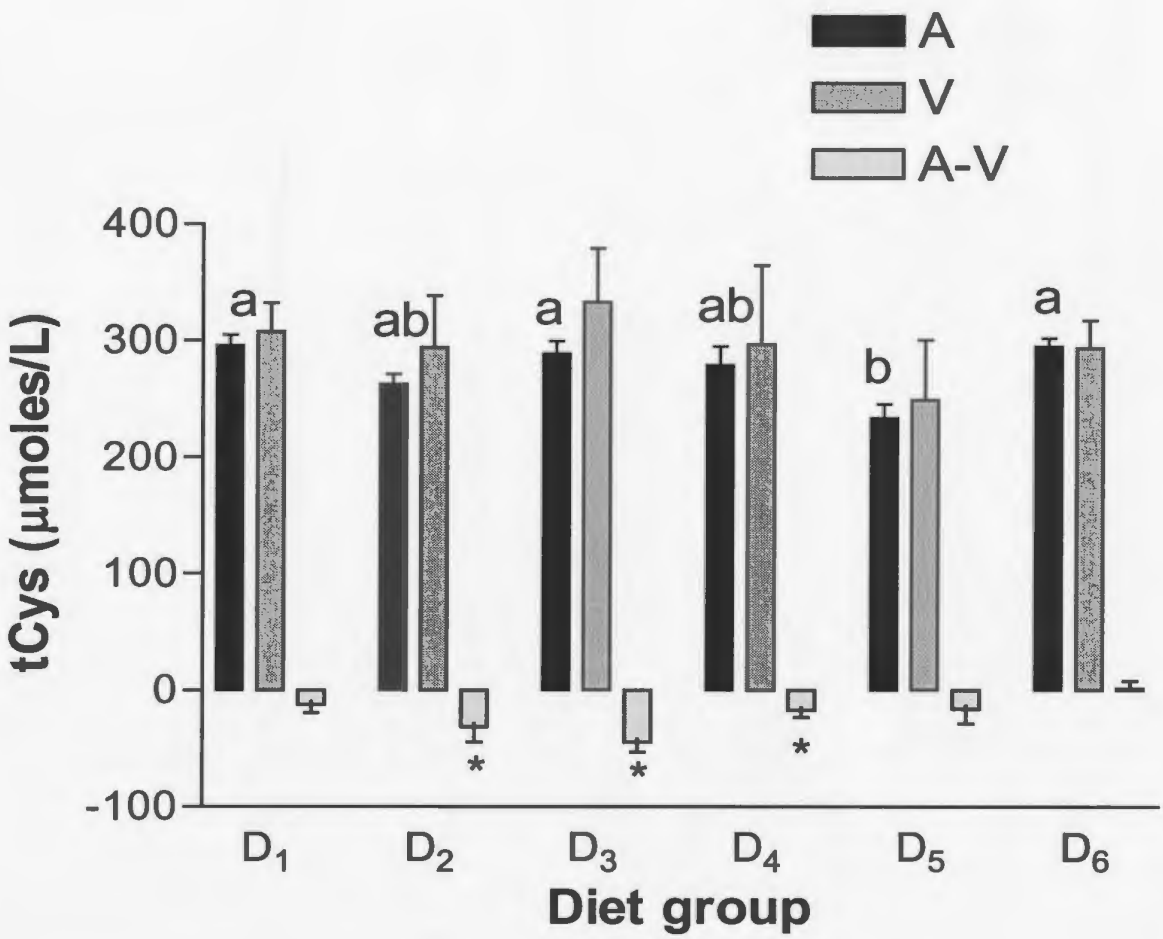

Figure 3.1. Total Cys levels in arterial & portal plasma & A-V difference across the portal-drained viscera for rats on different diets for 7 days.

Values are for means ± SD for n = 6-14 rats. * A-V difference is significantly different from zero ($P < 0.05$) by the paired t test. Values without common letter superscripts are significantly different for arterial tCys concentrations between diet groups ($P < 0.05$) by ANOVA and Newman-Keuls Multiple Comparison Test. D_1, 20% casein + 0.3% cystine diet; D_2, 20% casein + 0.6% cystine diet; D_3, 60% casein + 0.3% cystine diet; D_4, 20% casein + 0.3% cystine +0.5% methionine diet; D_5, 20% casein + 0.3% cystine + 1% methionine diet; D_6, 20% casein + 0.3% cystine + 2% methionine diet.

Table 3.4. Net fluxes of thiols across the portal-drained viscera in rats fed different diets for 7 days.

Diet	Net Flux (μmol/min/100g)			
	tCys	tHcy	tGSH	tCys-Gly
D_1	-0.104±0.124	-0.001±0.007	0.023±0.012*	0
D_2	-0.131±0.204*	-0.000±0.004	0.017± 0.006*	0
D_3	-0.219±0.079*	-0.004±0.002	0.006±0.012	0
D_4	-0.071±0.100	0.001± 0.010	0.020± 0.013*	0
D_5	-0.047±0.135	0.020± 0.055	0.019± 0.012*	0

Values are means ± SD for determinations on 6-12 rats. A positive sign indicates an uptake or removal by the organ bed, and a negative sign indicates a release or export by the organ bed. * A-V difference is significantly different from zero ($P < 0.05$) by the paired t test. Values were analyzed for statistically significant difference by ANOVA and Newman-Keuls Multiple Comparison Test. No differences were found. D_1, 20% casein + 0.3% cystine diet; D_2, 20% casein + 0.6% cystine diet; D_3, 60% casein + 0.3% cystine diet; D_4, 20% casein + 0.3% cystine +0.5% methionine diet; D_5, 20% casein + 0.3% cystine + 1% methionine diet.

In contrast to the output of Cys, we observed a net intestinal uptake of tHcy from the arterial blood in rats fed a 2% methionine-supplemented diet. The tHcy concentrations in the arterial, portal blood and A-V differences are illustrated in Figure 3.2. The tHcy A-V difference in the 2% methionine fed rats was 29.3 ± 20.4 μmol/L. However, we did not measure the blood flow in this diet group. Both arterial and portal tHcy concentrations are significantly higher in rats fed methionine-supplemented diets than in the rest of the diet groups. The arterial tHcy concentration in this group was 296.4± 139.5 μmol/L. It was approximately 25-fold higher than the control value. The 0.5% methionine-supplemented group had about an 11-fold increase in arterial tHcy concentration compared to the control value (Figure 3.2).

When the A-V differences for tHcy in all the diet groups were plotted as a function of arterial tHcy concentration, a positive and significant relationship was observed (Figure 3.3).

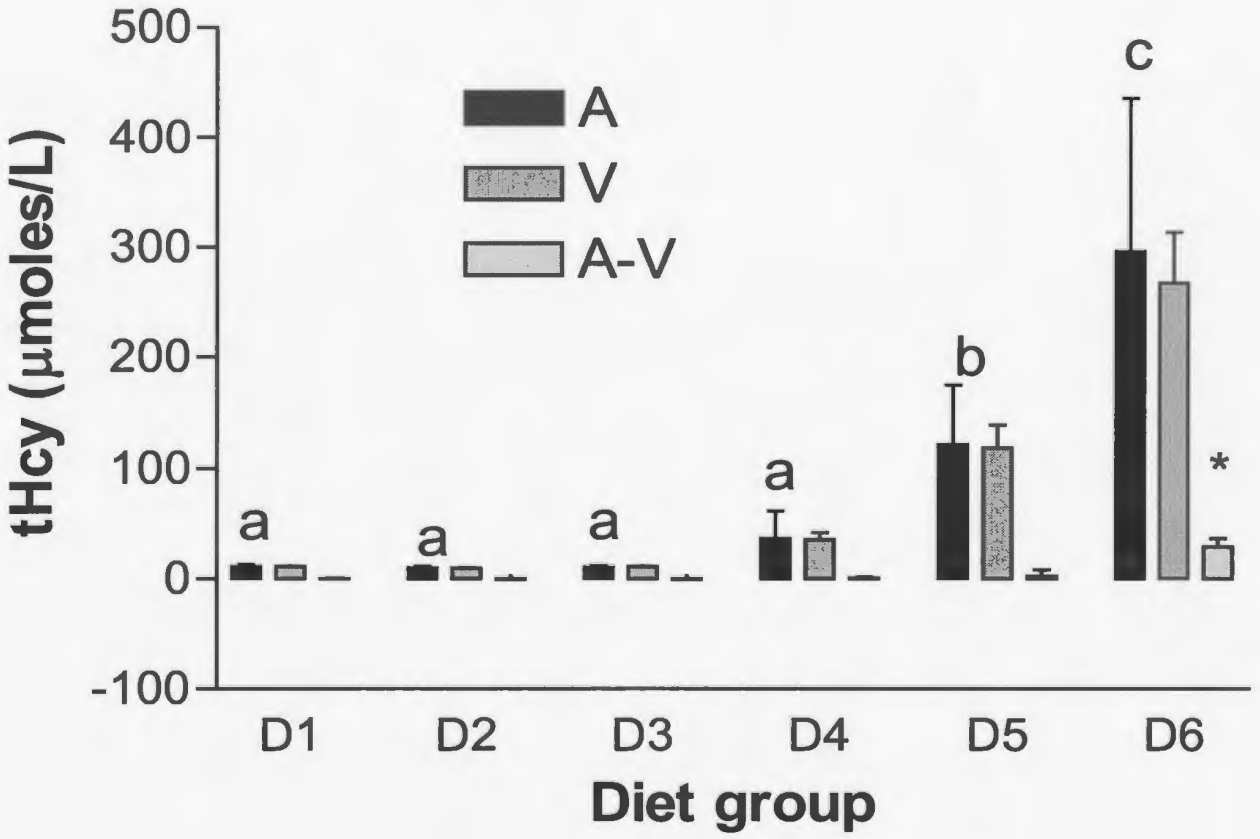

Figure 3.2. Total Hcy levels in arterial & portal plasma & A-V difference across the portal-drained viscera for rats on different diets for 7 days.

Values are for means ± SD for n = 6-14 rats. * A-V difference is significantly different from zero ($P < 0.05$) by the paired *t* test. Values without common letter superscript are significantly different for arterial tHcy concentrations between diet groups ($P < 0.05$) by ANOVA and Newman-Keuls Multiple Comparison Test. D_1, 20% casein + 0.3% cystine diet; D_2, 20% casein + 0.6% cystine diet; D_3, 60% casein + 0.3% cystine diet; D_4, 20% casein + 0.3% cystine +0.5% methionine diet; D_5, 20% casein + 0.3% cystine + 1% methionine diet; D_6, 20% casein + 0.3% cystine + 2% methionine diet.

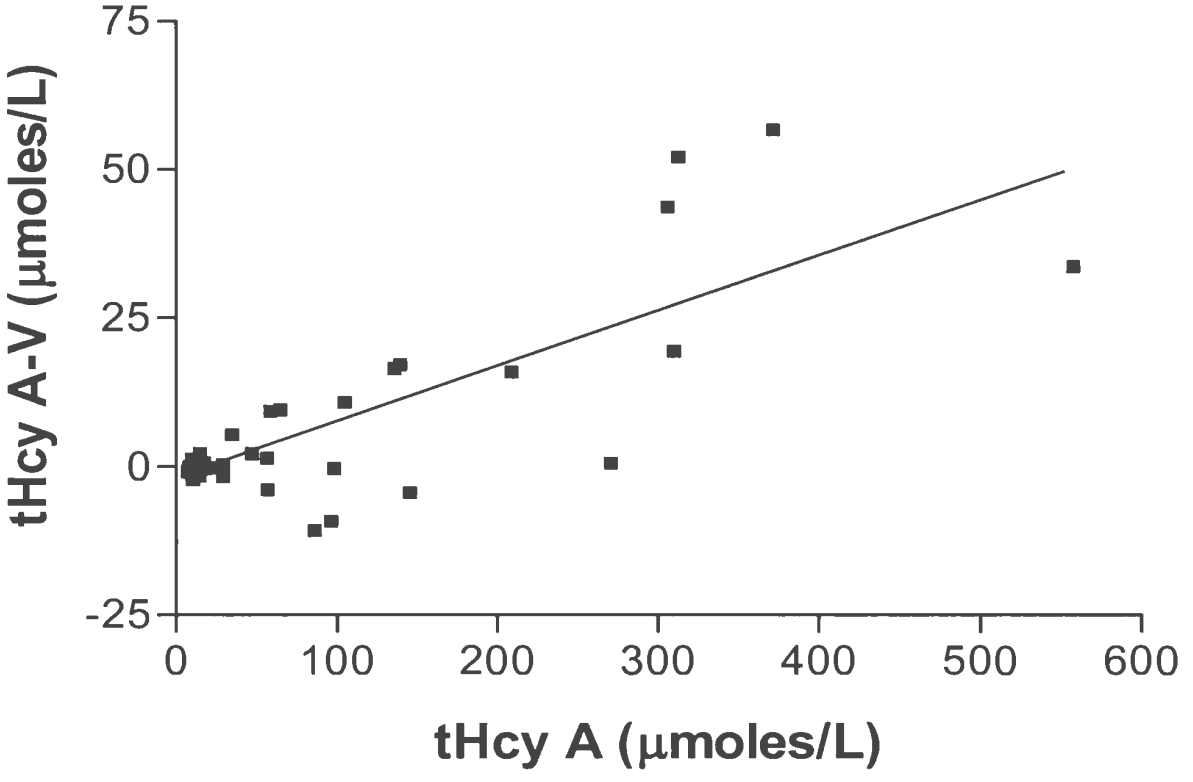

Figure 3.3. A-V of tHcy across the portal-drained viscera as a function of arterial tHcy for rats in all the diet groups

n = 68 rats. Linear regression analysis showed an $r^2 = 0.6462$ and P = <0.0001

As illustrated in Figure 3.4, the portal-drained viscera removed substantial amounts of plasma GSH from arterial blood in all diet groups except the 60% casein group. The rate of arterial GSH uptake was approximately 0.02 μmol/min/100g for the diet groups that showed a net uptake (Table 3.4).

We also measured the balance of Cys-Gly across the portal drained viscera as an indicator of the fate of GSH in the organ bed. Cys-Gly is an intermediate product of GSH catabolism. As shown in Figure 3.5, there was no significant A-V difference across the portal-drained viscera in any of the diet groups. Moreover, no diet group showed a significant difference in their arterial and portal concentrations of Cys-Gly. However, we did not measure Cys-Gly balances in rats fed a 2% methionine diet.

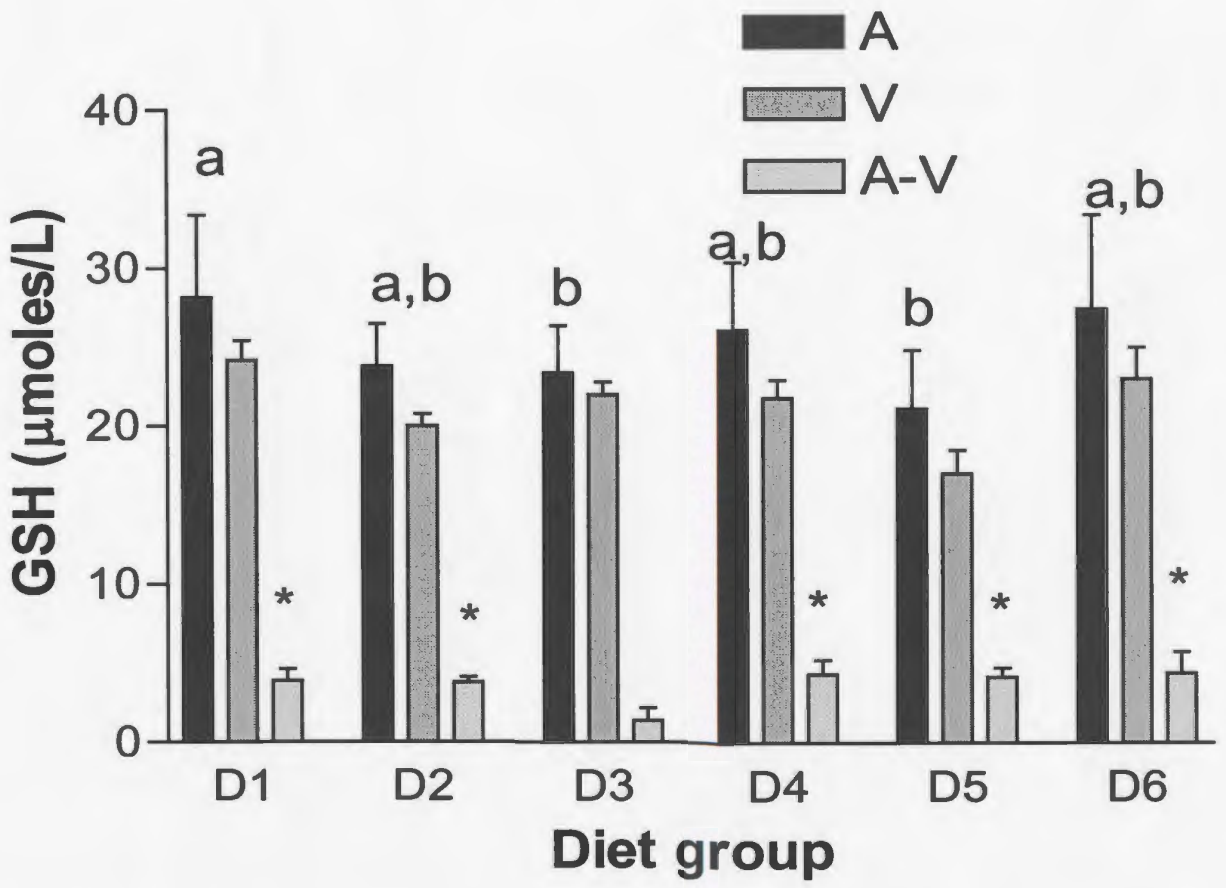

Figure 3.4. Total GSH levels in arterial & portal plasma & A-V difference across the portal-drained viscera for rats on different diets for 7 days.

Values are for means ± SD for n = 6-14 rats. * A-V difference is significantly different from zero ($P < 0.05$) by the paired *t* test. Values without common letter superscript are significantly different for arterial tGSH concentrations between diet groups ($P < 0.05$) by ANOVA and Newman-Keuls Multiple Comparison Test. D_1, 20% casein + 0.3% cystine diet; D_2, 20% casein + 0.6% cystine diet; D_3, 60% casein + 0.3% cystine diet; D_4, 20% casein + 0.3% cystine +0.5% methionine diet; D_5, 20% casein + 0.3% cystine + 1% methionine diet; D_6, 20% casein + 0.3% cystine + 2% methionine diet.

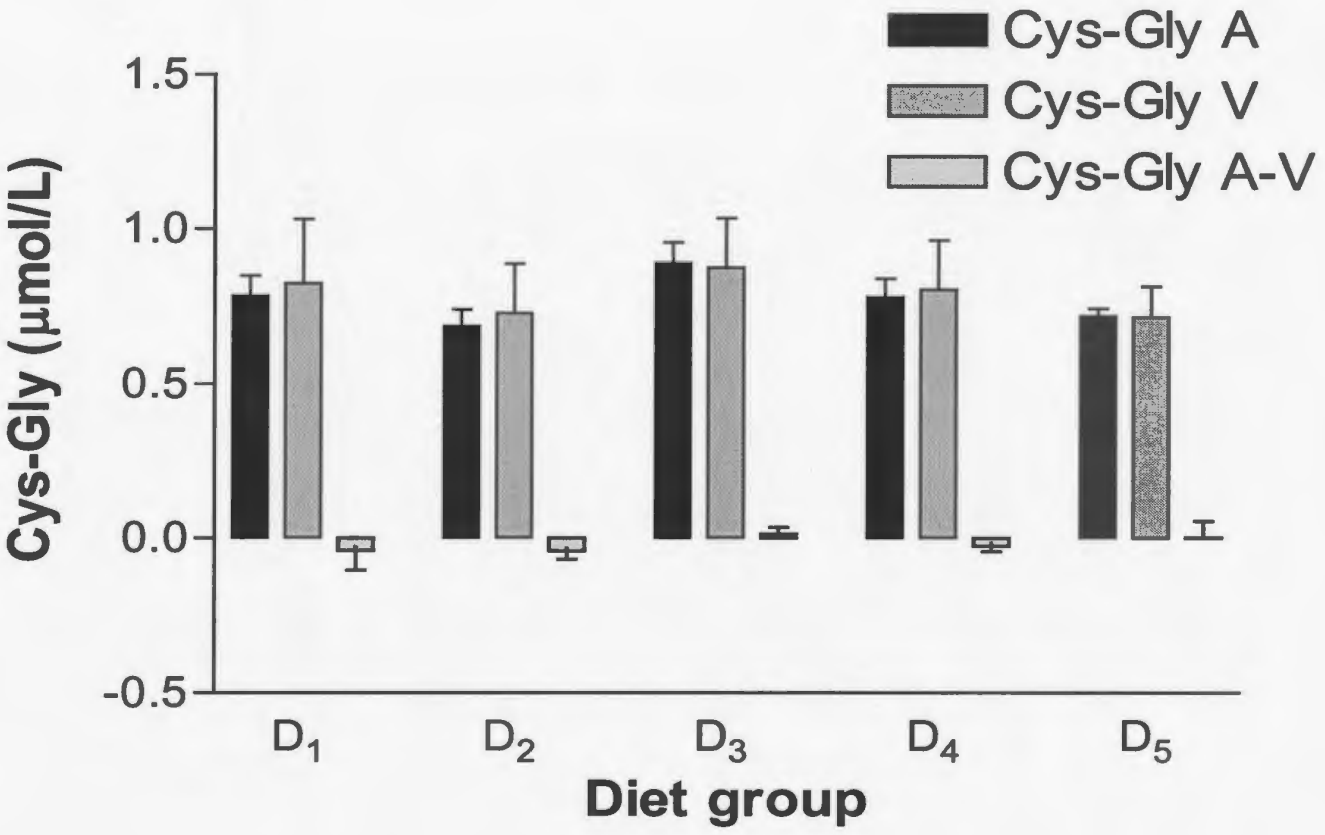

Figure 3.5. Total Cys-Gly levels in arterial & portal plasma & A-V difference across the portal-drained viscera for rats on different diets for 7 days.

Values are for means ± SD for n = 6-12 rats. Values were analyzed for statistically significant difference by ANOVA and Newman-Keuls Multiple Comparison Test. No differences were found. D_1, 20% casein + 0.3% cystine diet; D_2, 20% casein + 0.6% cystine diet; D_3, 60% casein + 0.3% cystine diet; D_4, 20% casein + 0.3% cystine +0.5% methionine diet; D_5, 20% casein + 0.3% cystine + 1% methionine diet; D_6, 20% casein + 0.3% cystine + 2% methionine diet.

Plasma Free and Protein-Bound Thiols across the Portal-Drained Viscera

The free and protein-bound fractions of all 4 thiols were measured so as to determine, in particular, the chemical form by which dietary Cys is added to the portal plasma (Table 3.5). All rats in this experiment were fed the 60% casein diet for 7 days since this diet group was responsible for the highest output of tCys into the portal blood (Table 3.4). The data in Table 3.5 confirm our earlier observation of an appreciable appearance of cysteine in the portal plasma. 64% of plasma Cys was in the free form. Almost all the Cys exported to the portal vein was in the non-protein bound form (Figure 3.6). The percentage of free Hcy and GSH in the arterial blood was 44% and 71%, respectively. Cys-Gly in the arterial blood was absolutely in the free form.

Table 3.5. Plasma total and non-protein bound concentration of thiols across the portal drained viscera in rats fed 60% casein diet for 7 days.

Thiol	Total (μmoles/L)			Free (μmoles/L)		
	A	V	A-V	A	V	A-V
Cys	309.2±30.2	354.0±44.2	-44.7±33.0	199.5±27.9	248.5±30.4	-49.0±25.4
Hcy	14.3±2.3	14.9±2.4	-0.6±0.8	6.3±1.5	7.9±1.6	-1.7±0.8
Cys-Gly	0.7±0.2	0.8±0.1	-0.03±0.05	0.9±0.2	1.03±0.1	- 0.2±0.1
GSH	20.8±2.3	18.9±2.1	1.9±1.5	15.0±2.2	13.1±1.4	1.9±1.8

Values are means ± SD for n = 12 rats.

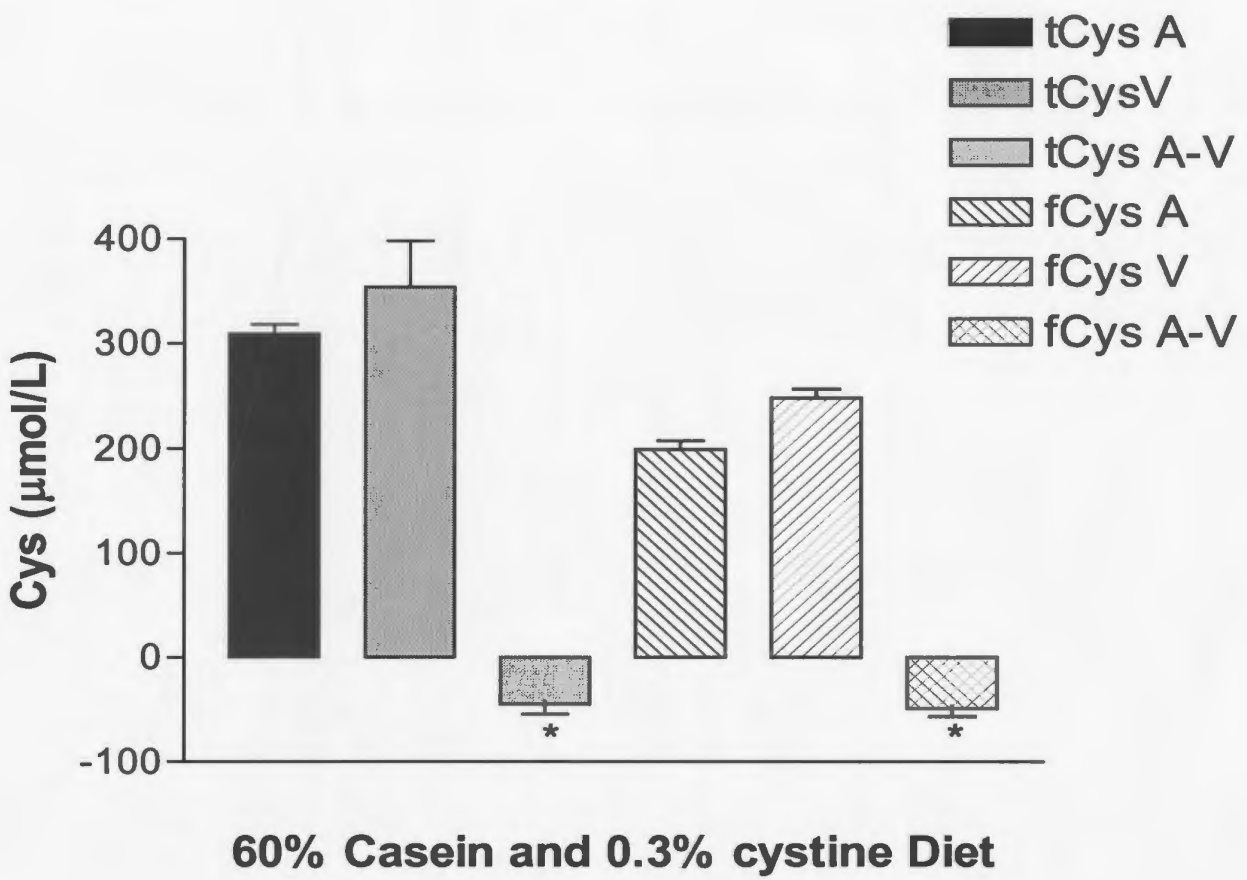

Figure 3.6. Plasma total and non-protein bound Cys in the arterial and portal blood and A-V differences for rats fed on 60% casein diet for 7 days

Values are means ± SD for n = 12 rats. A-V values with asterisks are significantly different from zero ($P < 0.05$) by paired t test.

A-V Differences for Amino Acids across the Portal Drained Viscera

In addition to thiols, we measured the A-V differences and the fluxes of methionine and other amino acids (See the appendix). A significant portal output of methionine was observed only in rats fed 0.5% methionine supplemented diets. The A-V difference was -6.99 ± 4.31 μmol/L. A striking finding was the observation of taurine production by the portal-drained viscera when animals were supplemented with methionine but not with cystine.

DISCUSSION

A-V differences are often measured in whole blood samples rather than in the plasma. Whole blood could give a complete picture independent of certain constraints such as the movement of compounds between plasma and cells during transit through the organ bed. However, in our case, use of whole blood values would result in very high baseline concentrations in contrast to our ability to detect small changes in A-V differences. For instance, the bulk of GSH in whole blood is sequestered in the red blood cells. These cells are impermeable to plasma GSH (Mortenson *et. al.*1956). Conversely, Cys is present primarily in the plasma and its concentration in the blood cells is relatively very low (Levy and Berkin 1971). Therefore, plasma concentrations were used to determine the A-V values for all thiols in this study.

The observation of tCys output (Figure 3.1) into the portal blood does not agree with the observation of Stoll *et. al.* in piglets (1998) who found no additional cystine in the portal vein. Stoll *et. al.* concluded that dietary Cys was quantitatively metabolized within the intestine. Our data do, however, agree with that of Garcia and Stipanuk (1992) who also found a net output of tCys into the portal vein. At first, we thought that these differences might be accounted for by analytical methodology. We and Garcia and Stipanuk used a reduction step in the assay and, therefore, measured total Cys. Stoll *et. al.* only measured non-protein bound cyste(i)ne. For this reason, we postulated that dietary Cys appears in the portal blood in the albumin-bound form and that this form is, therefore, metabolically active (Prathapasinghe *et. al.* 2004). To test this assumption, we measured the arteriovenous difference of total and non-protein bound Cys across the

intestine. Since 60% casein + 0.3% cystine diet gave a highest output of tCys, we fed a group of rats with the same diet in this assay.

Almost all the Cys exported into the portal vein was in the free form. However, we have not defined the relative importance of the different free forms. The simplest hypothesis would be that Cys added to the portal vein is in the form of cysteine and or cystine. However, Cys readily forms mixed disulfides with other thiols. Therefore, it is certainly possible that some of the absorbed Cys is in the mixed disulfide form. It is well known that Cys-Hcy occurs in plasma. In addition, Dahm and Jones (1994) have shown that vascularly perfused rat intestine cleared 45% of oxidized GSH (GSSG) in a single pass. This resulted in an output of the mixed disulfide of cysteine and GSH (CysSG). Therefore, it is possible that some of the absorbed Cys occurs in the form of this mixed disulfide. However, additional experimentation is required to delineate the specific forms in which Cys is added to portal blood.

The 60% casein fed rats exported the highest amount of tCys into the portal blood (Figure 3.1). This may be due to the higher level of Cys that was available in this diet. Each 100 g of casein provided 3.2 g of methionine equivalents (2.8 g methionine + 0.4 g Cys). Thus, the Cys available through casein in this diet group was 0.24%. Given that 1 g of methionine, if completely metabolized to Cys, is equivalent to 0.8 g of Cys, the total availability of Cys in the 60% casein diet group goes even higher (Stipanuk *et. al.* 2002). Therefore, 60% casein group may have had the highest supply of Cys among the rest of the diet groups.

We report, for the first time, that tHcy, at very high concentrations in the arterial blood, is removed by the portal drained viscera. Although this increased plasma tHcy concentration is not observable in any normal dietary situation, human patients with severe hyperhomocysteinemia do have extremely high levels of plasma tHcy. Therefore, it can be suggested that the intestine may play a role in lowering plasma tHcy under certain pathological situations in which the plasma tHcy level is extremely high. This may be important in patients with diseases such as inflammatory bowel disease, Crohn's disease, and short bowel syndrome. All these patients show significantly elevated plasma tHcy level which is not necessarily related to low vitamin B cofactors that are required for methionine remethylation and transsulfuration (Romagnuolo *et. al.* 2001; Koutroubakis *et. al.* 2000). Perhaps the increased Hcy is a result of the bowel diseases.

GSH is needed as a reducing agent to reduce many toxic oxidizing agents. The intestine, in particular, is exposed to severe oxidative stress, especially during the fed state. GSH is the preferred reducing agent to counteract those oxidative stresses in this organ (Harward *et. al.*1994). GSH is used in the small intestine to detoxify peroxidized lipids in the mucosal epithelium and to detoxify reactive electrophils in mucus and mucosal epithelium (Aw and Williams 1992). Bile is thought to be the source of GSH for the functions within the intestinal lumen (Aw *et. al.*1992; Aw and Williams 1992). In addition, GSH is also released to the lumen from the intestinal epithelium (Wien and van Campen 1991). The uptake of GSH by the portal-drained organ bed observed in this thesis, is consistent with previous findings in rats (Garcia and Stipanuk 1992). However, the quantity of GSH taken up by the rats in our experiment is somewhat higher than that

of Garcia and Stipanuk's. Even though the intestinal tissues have the enzymatic capability to synthesize GSH and the rate of its synthesis in the mucosa is very high (Jahoor *et. al.* 1995), a further uptake from arterial source may well explain the extent of oxidative stress that this organ faces during the absorptive state in particular (Jahoor *et. al.* 1996).

We also measured the A-V difference of Cys-Gly in an attempt to study the fate of the GSH that was taken up by the organ bed. Cys-Gly is a metabolic product of GSSG/GSH degradation produced by γ-GT (Grafstrom *et. al.* 1980). Consistent with previous research findings, we also did not observe any intake or output of Cys-Gly across the portal drained viscera (Dahm and John 1994).

CHAPTER 4

INTRODUCTION

From the metabolic prospective, intestinal cells are unique. First, the nutrients for the mucosal epithelial cells of the small intestine can be derived from both the luminal and arterial sides. Second, enterocytes are specialized for the unidirectional movement of dietary absorbed nutrients from the lumen to the portal vein through the basal lamina. Third, enterocytes display substantial metabolic plasticity and a metabolic favouritism towards certain substrates. With regard to sulfur amino acids, the small intestine consumes 30% of dietary available methionine though the gastrointestinal system represents only about 7% of the whole body mass (Reeds *et. al.* 1996). The small intestine is one of the very few organs that is equipped with all of the enzymes required for the transmethylation, remethylation and transsulfuration pathways. A few studies have been carried out on sulfur-containing amino acid (especially Cys) metabolism in enterocytes. However, no data are available on the fate of exogenous methionine in these cells. Since methionine is an essential amino acid as well as the source for all the other sulfur-containing amino acids in the body, we decided to study the metabolic fate of exogenous methionine in isolated rat enterocytes, together with enzyme activities in intestinal tissue homogenates.

ATTEMPTS TO IDENTIFY A CELL ISOLATION MEDIUM THAT WOULD IMPROVE ENTEROCYTES VIABILITY

Initial experiments were conducted to determine a suitable cell isolation medium. We used two different cell isolation media as described in the Materials and Methods; one with saline-2 with added collagenase and the other with saline-2 alone. Table 4.1 reports the viability of cells isolated with these media. Viability was assessed for different times of incubation. Enterocytes isolated without collagenase gave better viability compared with those isolated with collagenase. Therefore, we did not employ collagenase for enterocytes isolation and we restricted our incubation time to no more than 30 minutes.

Table 4.1. Viability percentages of isolated enterocytes by two different cell isolation media

Method	Incubation time (min) and viability (%)			
	0	15	30	45
No collagenase	97, 98	87, 88	79, 82	45, 51
Collagenase	87, 88	83, 84	67, 76	53, 61

n = 2 rats.

DETERMINATION OF A SUITABLE INCUBATION MEDIUM FOR TRANSSULFURATION FLUX STUDIES

Isolated enterocytes were suspended in Krebs-Henseleit–3 medium (see Materials and Methods). This medium contained 2.5% bovine serum albumin. For the studies of transsulfuration flux, we incubated enterocytes in a medium devoid of exogenous sulfur-containing amino acids other than the labelled methionine and the other amino acids such as serine, glutamate and glycine that are involved in transsulfuration and in the synthesis of GSH. In preliminary studies we found that enterocytes incubated in either Krebs-Henseleit-3 or normal Krebs-Henseleit medium, had approximately the same viability (as judged by LDH leakage) for 45 minutes of incubation (Figure 4.1). Therefore, the normal Krebs-Henseleit medium was used as our incubation medium in all the enterocyte experiments.

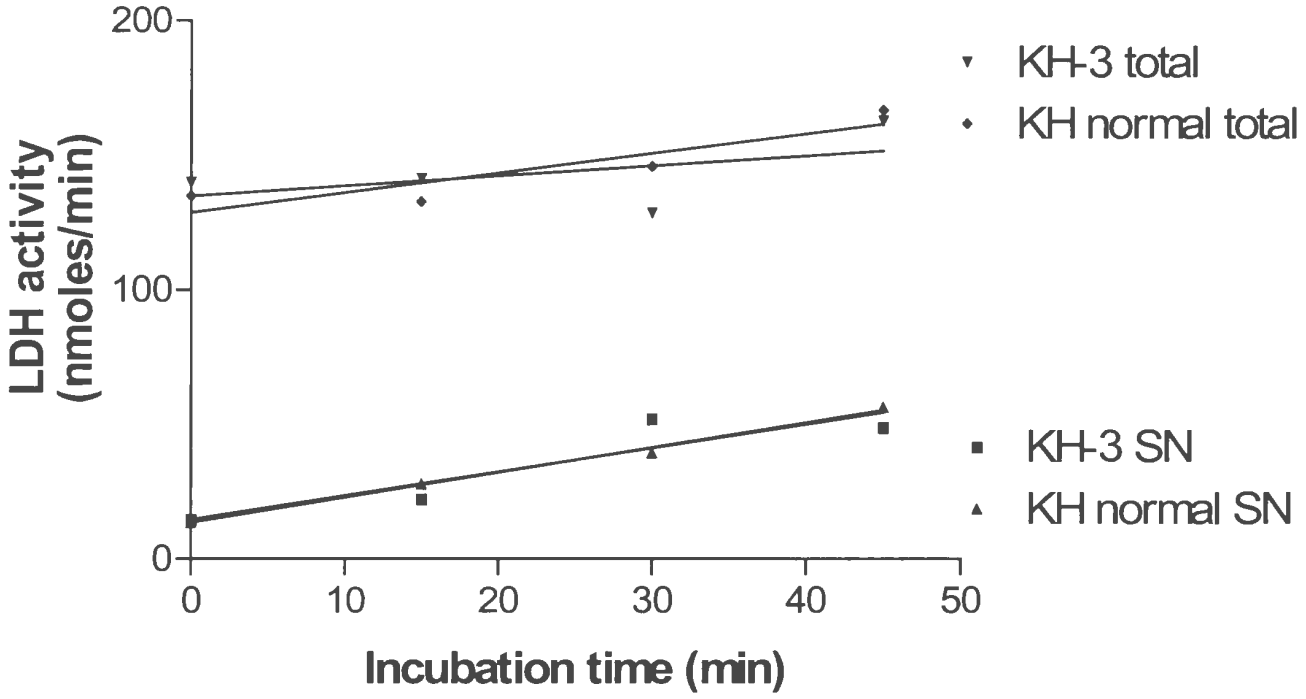

Figure 4.1. Leakage of LDH from enterocytes incubated in Krebs-Henseleit-3 medium vs. normal Krebs-Henseleit. Both media contained 1 mM methionine, 1 mM serine, 1 mM glutamate and 1 mM glycine

KH-3 SN, supernatant from the cells incubated in Krebs-Henseleit-3 medium; KH normal SN, supernatant from the cells incubated in Krebs-Henseleit medium; KH-3 total, LDH activity remaining in enterocytes incubated in Krebs-Henseleit-3 medium and lysed by Triton X-100; KH normal total, LDH activity remaining in enterocytes incubated in Krebs-Henseleit medium and lysed by Triton X-100

TRANSSULFURATION FLUX IN ISOLATED RAT ENTEROCYTES

Transsulfuration of [1-^{14}C] methionine was determined by the total production of $^{14}CO_2$ from enterocytes with 1 mM [1-^{14}C] methionine, 1 mM serine, 1 mM glutamate and 1 mM glycine. When enterocytes are incubated with L-[1-^{14}C] methionine some $^{14}CO_2$ is produced as a result of the mitochondrial metabolism of α-ketobutyrate to propionyl Co-A (Figure 4.2). This fraction of $^{14}CO_2$ is readily collected and counted. However, some α-ketobutyrate may remain unmetabolized. Label in unmetabolized α-[1-^{14}C] ketobutyrate can be released with H_2O_2. Such $^{14}CO_2$, released from α-ketobutyrate, must also be included in measures of flux through the transsulfuration pathway.

Table 4.2 reports the values for transsulfuration flux in isolated enterocytes that were incubated in the presence of [1-^{14}C] methionine, serine, glutamate and glycine. Serine was supplied as it is a substrate for cystathionine β-synthase and glycine and glutamate are necessary for GSH synthesis. The production of $^{14}CO_2$ from 1 mM [1-^{14}C] methionine was unaffected by the presence of 1 mM each of serine, glutamate or glycine. No statistically significant difference was observed in the rate of transsulfuration by isolated enterocytes in the presence and absence of these amino acids.

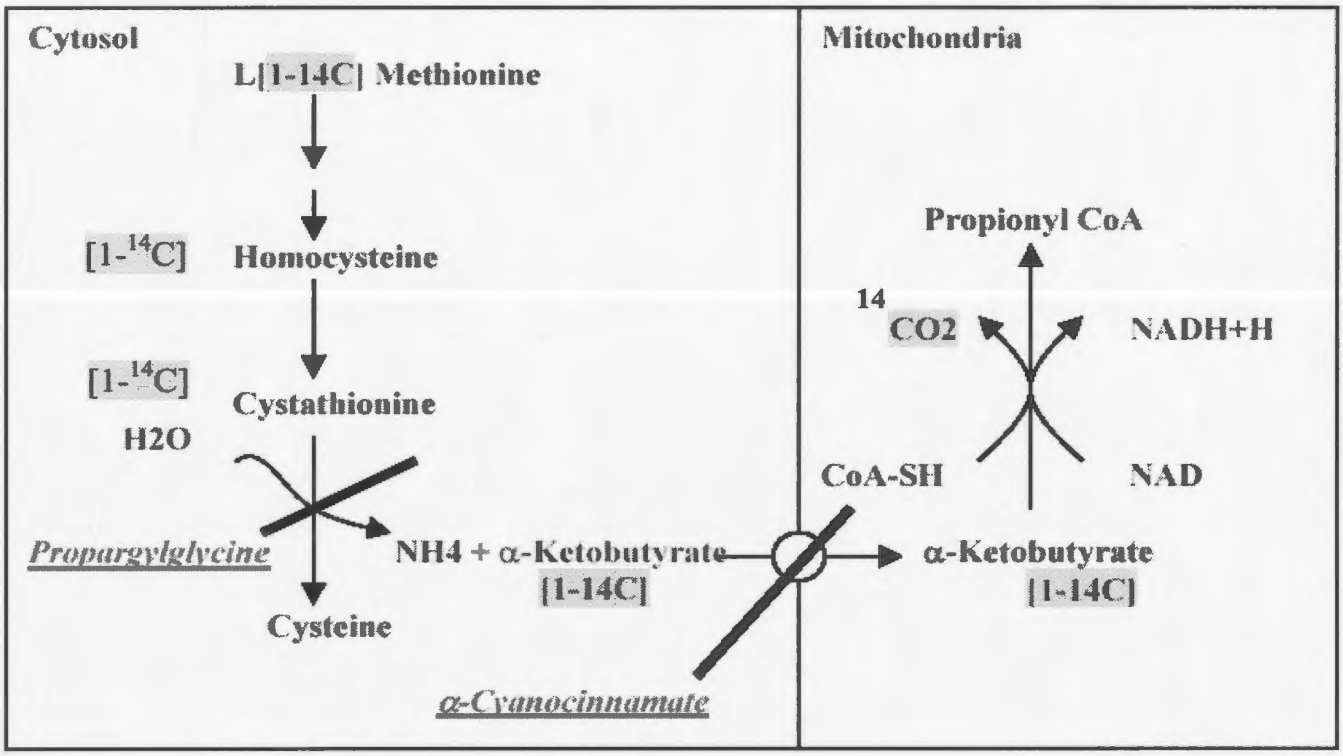

Figure 4.2. The metabolic fate of [1-^{14}C] of methionine in the transsulfuration pathway.

Propargylglycine, an irreversible inhibitor of cystathionine-γ-lyase; α-cyanocinnamate, an inhibitor of mitochondrial transporter of α-ketobutyrate.

Table 4.2. Transsulfuration flux in isolated enterocytes.

Contents of the incubation medium (1 mM)	Total $^{14}CO_2$ production (nmol/mg/30min)
Met*	1.7 ± 0.8
Met*+Ser	1.4 ± 0.9
Met*+Glu+Gly	1.7 ± 1.3
Met*+Glu	1.9 ± 0.6
Met*+Gly	1.5 ± 0.9

Values are means ± SD for n = 5 experiments. Values were analyzed for statistically significant differences by ANOVA and Newman-Keuls Multiple Comparison Test. No difference was found in the rate of transsulfuration between incubation media with different amino acids added. Met*, [1-^{14}C] methionine; Ser, serine; Glu, glutamate; Gly, glycine.

The relative contributions of $^{14}CO_2$ and α-ketobutyrate to the total transsulfuration flux reported in Table 4.2 are illustrated in Figure 4.3. The accumulation of α-ketobutyrate contributed most to the total rate of transsulfuration.

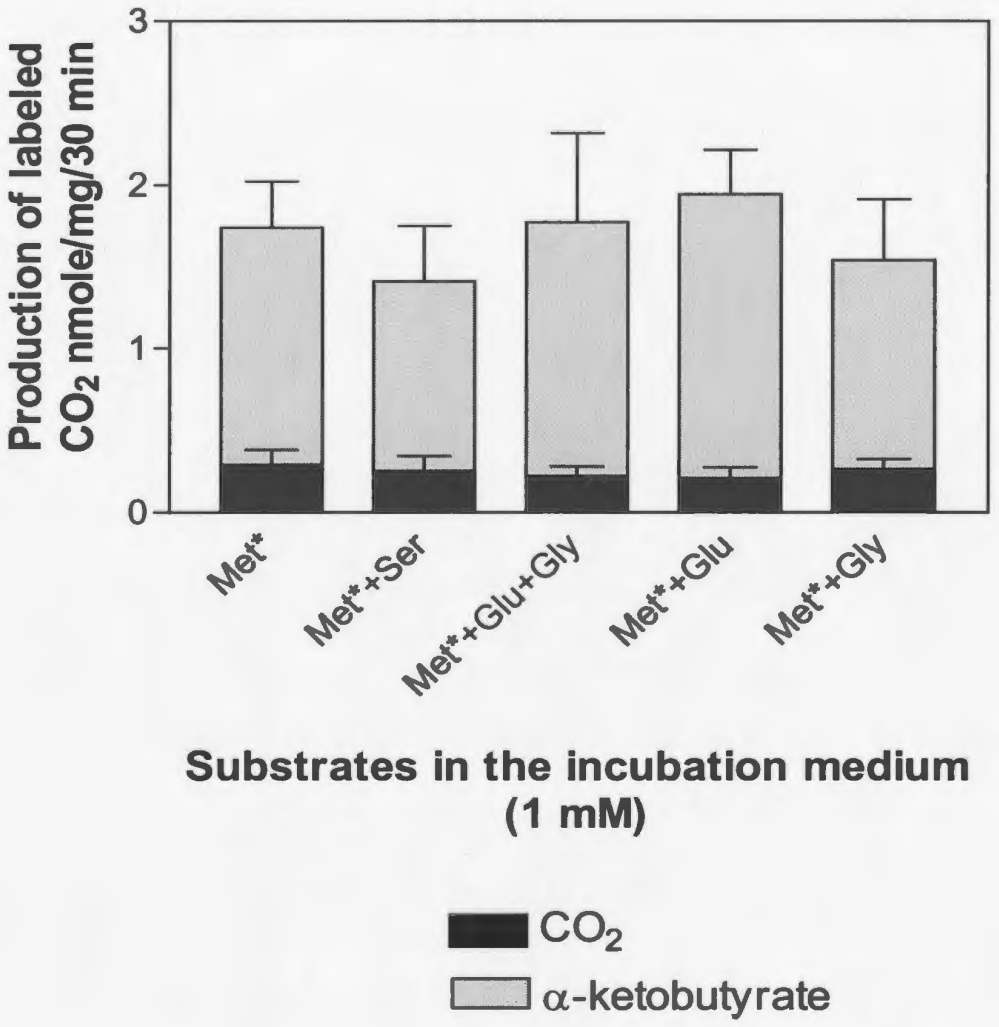

Figure 4.3. Flux of [1-^{14}C] methionine through the transsulfuration pathway in isolated rat enterocytes: production of $^{14}CO_2$ and [1-^{14}C] α-ketobutyrate

Values are means ± SD for n = 5 experiments. Values were analyzed for statistically significant differences by ANOVA and Newman-Keuls Multiple Comparison Test. No difference was found in the rate of transsulfuration between incubation media with

different amino acids added. Met*, [1-^{14}C] methionine; Ser, serine; Glu, glutamate; Gly, glycine.

Transsulfuration is not the sole metabolic pathway by which $^{14}CO_2$ can be released from [1-^{14}C] of methionine. There are three other possible ways: decarboxylation of SAM in the polyamine synthetic pathway, transamination followed by oxidation of the keto acid of methionine, and the cleavage of SAM between carbon atom 3 and 4. To determine whether the $^{14}CO_2$ produced was actually a product of the transsulfuration pathway, propargylglycine, an irreversible inhibitor of cystathionine-γ-lyase, was included in the incubations (see Figure 4.2). As shown in the Figure 4.4, blocking cystathionine-γ-lyase did not result in any significant reduction in total $^{14}CO_2$ production from enterocytes. However, the production of the $^{14}CO_2$ component but not of α-ketobutyrate was significantly lowered. Further, when enterocytes were incubated with α-cyanocinnamate, an inhibitor of the mitochondrial α-keto acid transporter, there was no decline in $^{14}CO_2$ production. These results contrast with those of *Stead et. al.* (2000) with hepatocytes. These investigators found that propargylglycine decreased the total transsulfuration flux and that α-cyanocinnamate decreased the $^{14}CO_2$ component without affecting the total flux.

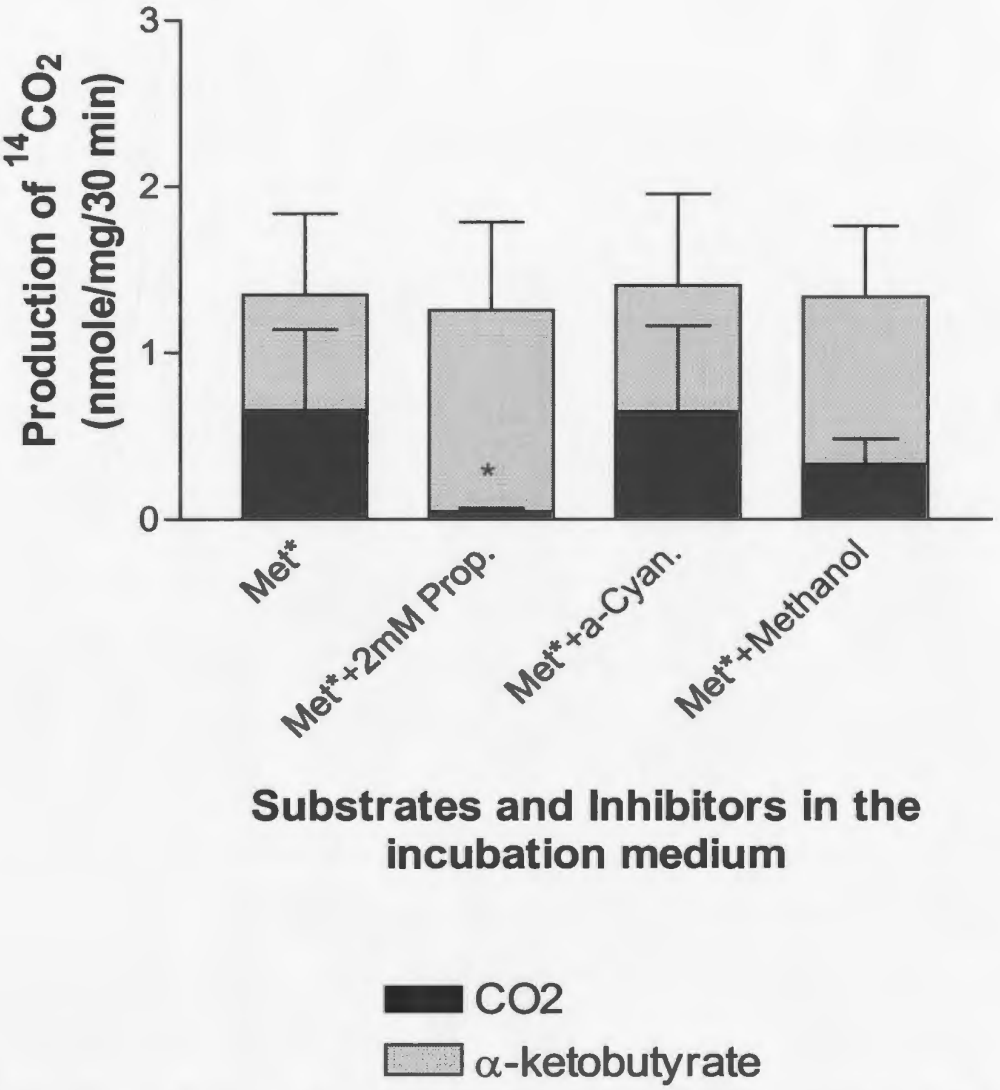

Figure 4.4. Flux of [1-^{14}C] methionine through the transsulfuration pathway in isolated rat enterocytes: Effects of propargylglycine and α-cyanocinnamate

Values are means ± SD for n = 3 experiments. Values were analyzed for statistically significant difference by ANOVA and Newman-Keuls Multiple Comparison Test. There was no difference of total $^{14}CO_2$ production in the presence of propargylglycine or α-cyanocinnamate. Met*, [1-^{14}C] methionine; 2 mM Prop., Propargylglycine; a-Cyan., α-Cyanocinnamate; methanol, the solvent in which the inhibitors were dissolved.

PRODUCTION/REMOVAL OF THIOLS BY INCUBATED ENTEROCYTES

To further examine the contribution of the transsulfuration pathway in the methionine metabolism of isolated enterocytes, we also examined the production and removal of Hcy, Cys and GSH (Table 4.3). As illustrated in Figure 4.5, when enterocytes were incubated in the presence of 1 mM methionine, there was a production of both Hcy and Cys whereas GSH was removed from the cells when compared with their initial concentrations. However, the values were extremely low. In addition, there was no difference between the results with no added amino acids.

Table 4.3 Concentration of thiols in the isolated enterocytes before and after incubation in the presence and absence of 1 mM methionine.

Incubation medium	Hcy (nmoles/mg dry weight)		Cys (nmoles/mg dry weight)		GSH (nmoles/mg dry weight)	
	0 time	30min	0 time	30min	0 time	30min
1 mM each of Met+Ser+Glu+Gly	0	0.48±0.14	5.1±2.8	17.9±6.9	12.5±6.1	6.8±6.6
1 mM each of Ser+Glu+Gly	0	0.48±0.16	5.1±2.8	20.7±5.4	12.5±6.1	6.5±6.6

Values are means ± SD for n =3 experiments. Values were analyzed by Student's t test. There was no difference in the concentration of thiols after 30 minutes of incubation, in the presence or absence of methionine.

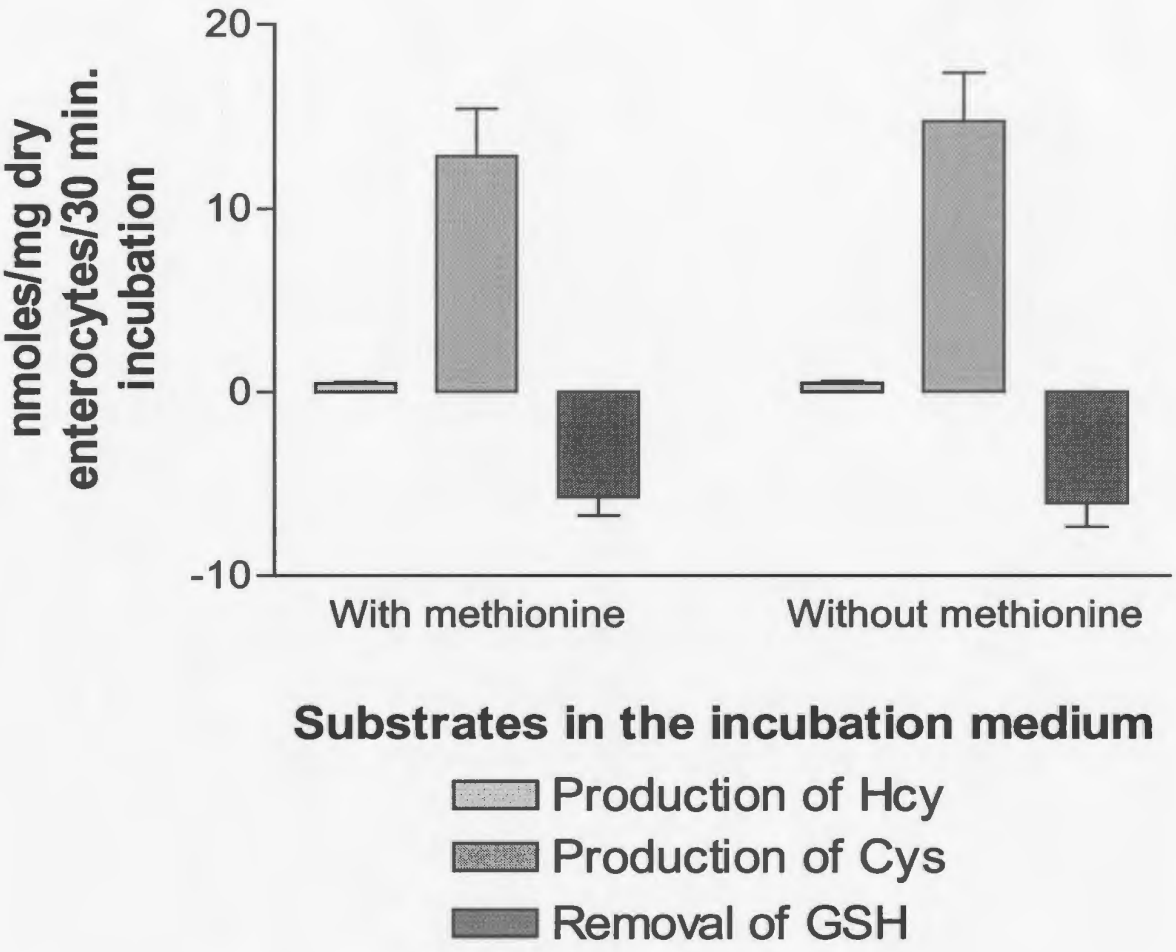

Figure 4.5 Production/Removal of Hcy,Cys & GSH by the enterocytes incubated in the presence and absence of 1 mM methionine, Serine, Glutamate and Glycine.

Values are means ± SD for n =3 experiments. Values were analyzed by Student's t test. There was no difference in the production/removal of thiols in the presence or absence of methionine. Met, methionine; Ser, serine; Glu, glutamate; Gly, glycine. With methionine, 1 mM each of Met+Ser+Glu+Gly; Without methionine, 1 mM each of Ser+Glu+Gly

We then examined whether Hcy is metabolized via the transsulfuration pathway in isolated rat enterocytes. As shown in Figure 4.6, the production of Cys was significantly increased from 10.8 nmoles/mg dry enterocytes/30 min to 41.8 nmoles/mg dry enterocytes/30 min, when enterocytes were incubated with added 1 mM Hcy. However, the removal of Hcy (70.3 nmoles/mg dry enterocytes/30 min) was not stoichiometrically equal to the production of Cys. Relatively low amounts of GSH were removed with or without Hcy in the incubation medium.

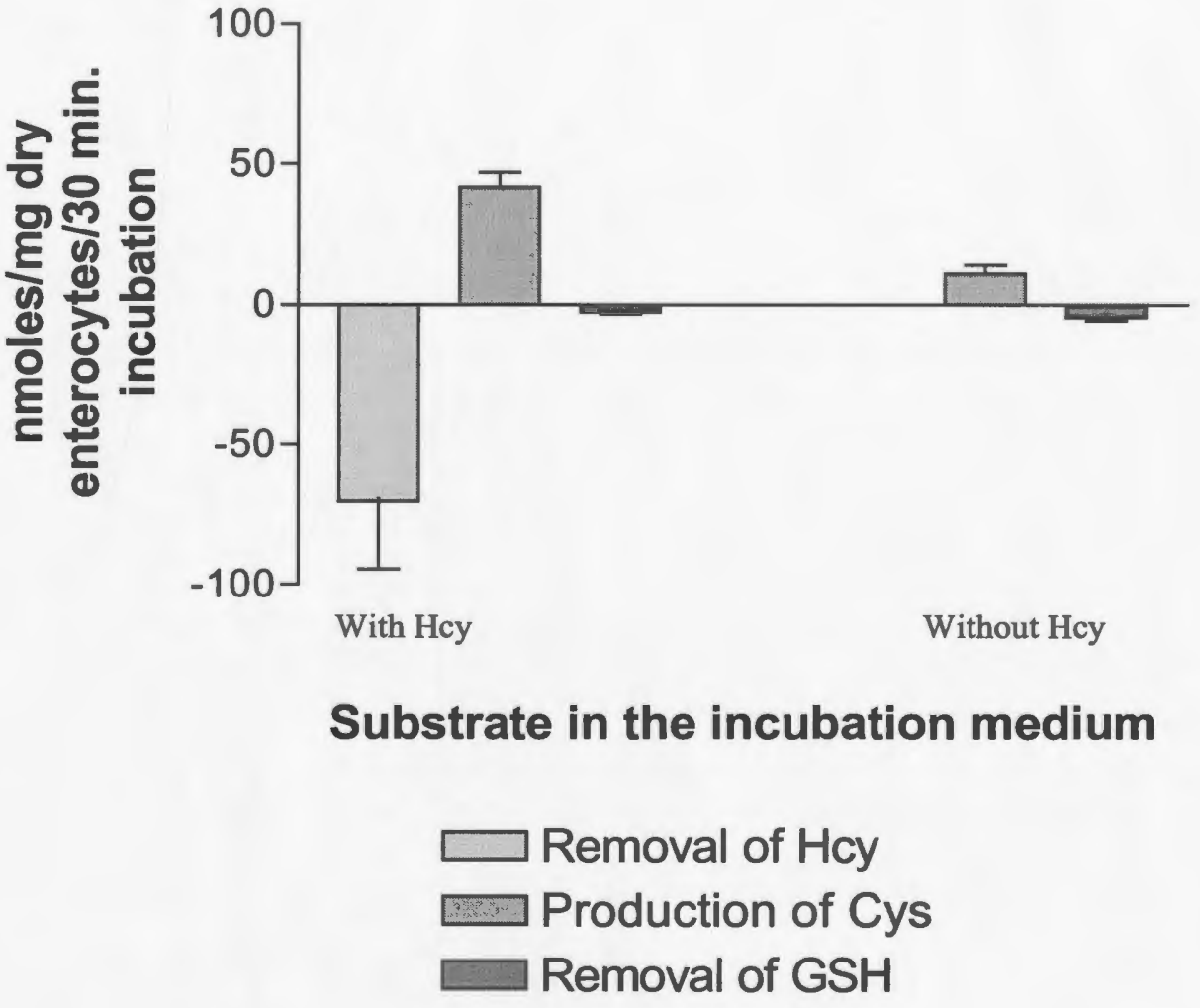

Figure 4.6. Production/Removal of Hcy,Cys &GSH by the enterocytes incubated in the presence and absence of 1 mM each of Homocysteine, Serine, Glutamate and Glycine.

Values are means for n = 2 experiment. Hcy, homocysteine; Ser, serine; Glu, glutamate; Gly, glycine. With Hcy, 1 mM each of Hcy+Ser+Glu+Gly; Without Hcy, 1 mM each of Ser+Glu+Gly

THE ACTIVITY OF ENZYMES OF METHIONINE METABOLISM IN INTESTINAL HOMOGENATES

In a separate set of experiments, we evaluated the activities of two key enzymes of the transmethylation and remethylation pathways in the rat small intestine. Tissue homogenates of rat small intestine were prepared either with or without a cocktail of protease inhibitors in the homogenization medium. The protease inhibitor cocktail contained 5 μg/ml each of Phenylmethylsulfonyl fluoride (PMSF), Pepstatin, Chymostatin and Aprotinin and 1 mM EDTA. The activity of CBS in the intestinal homogenate is illustrated in Figure 4.6. The mean activity of CBS was found to be 6.13 ± 5.40 nmoles/min/g intestine and 5.87± 6.86 nmoles/min/g intestine of homogenate in the presence and absence of protease inhibitor cocktail, respectively. The values were not significantly different from each other. Interestingly, we found no measurable enzyme activity for SAM synthase in the tissue homogenate both in the presence and absence of protease inhibitors. However, we must note that Finkelstein *et al.* (1971) found 2.2 nmoles/mg protein/60 min of SAM synthase activity in intestinal homogenate.

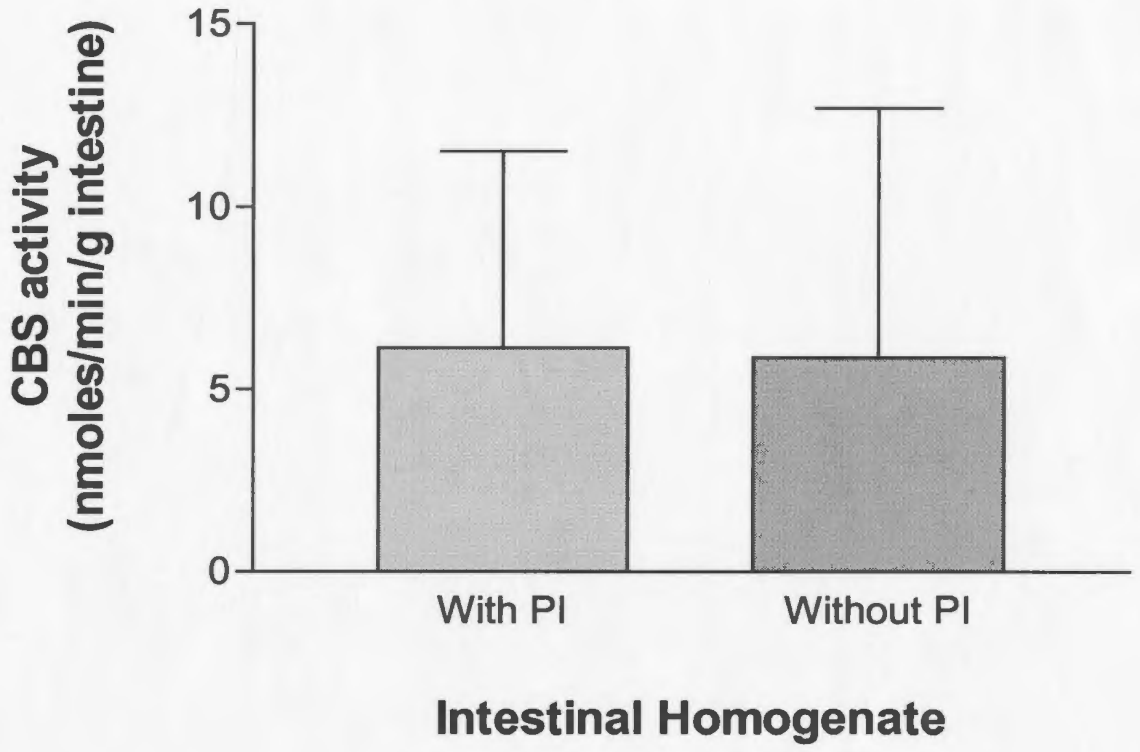

Figure 4.7. The tissue activity of cystathionine-β-synthase in the rat small intestinal homogenate .

Values are means ± SD for n = 3 experiments. Results were analyzed by Student's t test. No differences were found between the two groups. PI, Protease Inhibitor cocktail.

DISCUSSION

We found that methionine is appreciably metabolized by enterocytes. The total $^{14}CO_2$ production is comparable to rates found in isolated hepatocytes. In rat hepatocytes, the total production of $^{14}CO_2$ from 1 mM L-[1-^{14}C] methionine was reported to be 2.79 nmols/mg dry weight/h (Stead *et. al.* 2000). In the present work, enterocytes showed a similar rate; however, the production of the $^{14}CO_2$ component was quite low, compared with the [1-^{14}C] α- ketobutyrate. Conversely, in rat hepatocytes, the major contributor to the total $^{14}CO_2$ production was the production of $^{14}CO_2$ rather than [1-^{14}C] α-ketobutyrate. Therefore, it may be suggested that α- ketobutyrate is not as readily utilized for mitochondrial energy generation in enterocytes, as it is in hepatocytes. In addition, the lack of effect of added 1 mM serine on the rate of $^{14}CO_2$ or [1-^{14}C] α- ketobutyrate production provides another striking difference between enterocytes and hepatocytes. This difference is further emphasized by the finding that the irreversible inhibitor of CGL, propargylglycine, did not decrease the production of [1-^{14}C] α-ketobutyrate and the resulting total production of $^{14}CO_2$. The simplest explanation for this is that propargylglycine may not be an effective inhibitor of CGL in enterocytes possibly because of reduced permeability to the cell. Otherwise, it may be argued that [1-^{14}C] α-ketobutyrate produced from [1-^{14}C] methionine was not from the transsulfuration pathway. The deamination of SAM in the polyamine synthetic pathway, the cleavage of SAM into homoserine and subsequent catabolism into α-ketobutyrate and the transamination of methionine into its keto acid, keto-methionine, are the other possible

ways that uninterrupted production of [1-^{14}C] keto acid could occur in the presence of propargylglycine.

Experiments reported in Figure 4.5 and Figure 4.6 show a considerable production of Cys from Hcy but not from methionine. These results may suggest that SAM synthase and transmethylation may be a significant limitation in the intestinal catabolism of methionine. The data in figure 4.6 give a strong indication that exogenous Hcy is metabolized into Cys. Thus, the transsulfuration pathway from Hcy seems to be functioning in the enterocytes. The presence of CBS activity in intestinal homogenates (Figure 4.7) further supports the possible occurrence of the transsulfuration pathway in the isolated enterocytes. Furthermore, we found no activity of SAM synthase activity in the intestinal homogenate. Mudd *et. al.* (1965) also found very low activity in the intestine. This is consistent with a very low rate of methionine utilization by enterocytes.

The observations that propargylglycine did not inhibit the production of [1-^{14}C] α-ketobutyrate (Figure 4.4) and no SAM synthase activity was found in intestinal homogenate may rule out that the transsulfuration, the polyamine synthesis and the SAM cleavage pathways were responsible for the production of [1-^{14}C] α-ketobutyrate from [1-^{14}C] methionine in enterocytes. Thus, the only remaining known pathway that is left behind is the transamination of [1-^{14}C] methionine. We must consider that, in enterocytes, the $^{14}CO_2$ release by H_2O_2 does not come from α-ketobutyrate. H_2O_2 is known to decarboxylate all α-keto acids. The transaminated product of methionine, α-keto-γ-methiolbutyrate carries the labelled carbon at the carboxyl terminus. Chemical treatment with H_2O_2 may have liberated it as $^{14}CO_2$. Benevenga and Eagen (1983) have

shown the presence of the amino-accepting keto acid, α-keto-γ-methiolbutyrate, in the intestinal homogenate of rats. Therefore, it should be considered, in future studies, that enterocyte methionine metabolism may occur, in part, via pathways other than transsulfuration.

CHAPTER 5

SUMMARY

Summary

1. Cys is exported to the portal vein in rats fed diets supplemented with high casein, cystine or a moderate level (0.5%) of methionine.
2. The exported fraction of Cys is in the non-protein bound form.
3. The portal-drained viscera remove Hcy from the arterial plasma when the arterial tHcy concentration is very high.
4. GSH is removed from arterial plasma in all the dietary conditions except in 60% casein fed rats.
5. Enterocytes metabolize methionine at an appreciable rate. However, this may not occur via transmethylation. However, the transsulfuration pathway is active in isolated enterocytes as evidenced by the catabolism of Hcy to Cys in these cells.
6. The CGL inhibitor, propargylglycine, did not inhibit the "transsulfuration flux" of [1-^{14}C] methionine by enterocytes. It is possible that this inhibition is not effective in enterocytes, possibly because of reduced cellular permeability. Alternatively, it should be considered that $^{14}CO_2$ released by H_2O_2 may not be in α-ketobutyrate. It could, conceivably, arise from the α-keto acid of methionine, α-keto-γ-methythiolbutyrate. This would imply that the transamination pathway must be quite active in enterocytes.

APPENDICES

Table A-1 Plasma arterial, portal venous and A-V differences for amino acids in rats fed 20% casein + 0.3% cystine containing diet for 7 days.

20% Casein + 0.3% cystine diet group (μmol/L)			
Amino acid	**A**	**V**	**A-V**
Phosphoserine	6.1±1.9	8.9±2.4	-2.8±2.2*
Taurine	119.4±21.6	124.8±25.3	-5.3±14.7
Aspartic acid	9.7±4.6	11.8±6.3	-2.1±4.8
Hydroxyproline	28.4±5.5	25.1±4.0	3.3±3.9
Threonine	290.0±49.5	321.1±78.3	-31.1±40.5
Serine	149.1±46.9	200.7±112.1	-51.6±69.1
Asparagine	47.9±14.8	61.1±15.3	-13.2±16.0
Glutamic acid	49.6±17.1	63.2±28.7	-13.5±12.5*
Glutamine	329.7±67.6	305.6±102.5	24.0±46.2
Proline	316.3±78.8	400.8±167.5	-84.5±91.6
Glycine	60.0±18.9	89.2±8.2	-29.1±20.3*
Alanine	382.4±101.7	589.9±238.0	-207.5±144.7*
Citrulline	44.1±8.5	65.6±17.9	-21.4±11.4*
a-amino-n-butyric acid	6.3±2.2	5.7±2.6	0.67±1.9
Valine	194.7±62.5	232.5±106.9	-37.8±46.3
Methionine	46.3±15.6	59.5±32.3	-13.1±17.2
Isoleucine	78.3±29.5	103.0±60.8	-24.6±32.2
leucine	125.2±63.4	165.8±115.6	-40.6±52.9
Tyrosine	98.2±36.8	114.8±56.6	-16.5±23.7
Phenylalanine	43.8±15.5	60.6±36.6	-16.7±21.4
Tryptophan	46.1±25.1	70.5±25.5	-24.4±46.3
Ethanolamine	6.3±3.3	5.5±2.8	0.75±4.3
Ornithine	35.0±9.2	36.3±5.6	-1.3±5.6
Lysine	421.5±116.3	473.3±188.2	-51.7±88.3
Histidine	37.4±7.0	49.1±25.1	-11.6±18.8
Arginine	54.6±24.2	67.7±38.7	-13.1±16.5

Values are means ± SD for n = 6 rats. * Significantly different from zero ($P < 0.05$) by paired t test.

Table A-2 Plasma arterial, portal venous and A-V differences for amino acids in rats fed 20% casein + 0.6% cystine containing diet for 7 days.

20% Casein + 0.6% cystine diet group (μmol/L)			
Amino acid	**A**	**V**	**A-V**
Phosphoserine	4.9±0.6	6.6±1.1	-1.7±1.2*
Taurine	152.5±23.5	166.9±30.6	-14.4±28.2
Aspartic acid	7.2±2.2	10.9±3.2	-3.6±1.8*
Hydroxyproline	27.4±5.2	26.2±5.5	1.1±4.4
Threonine	250.2±63.4	252.5±52.6	-2.3±35.3
Serine	129.3±30.2	145.6±27.9	-16.2±15.2*
Asparagine	39.2±10.0	55.7±11.2	-16.4±7.5*
Glutamic acid	43.8±9.8	52.2±9.8	-8.3±6.2*
Glutamine	280.5±59.9	235.8±43.3	44.7±31.9*
Proline	266.9±81.7	304.3±65.0	-37.3±38.0
Glycine	56.4±13.4	91.6±21.5	-35.1±15.2*
Alanine	331.4±94.1	445.4±88.2	-114.1±54.4*
Citrulline	43.6±8.8	59.5±10.4	-15.8±8.8*
a-amino-n-butyric acid	5.9±2.1	4.9±1.3	0.94±2.0
Valine	173.2±53.3	179.9±46.0	-6.7±21.5
Methionine	40.4±10.4	45.0±9.5	-4.5±4.8
Isoleucine	70.8±25.9	79.5±24.6	-8.6±8.0*
leucine	110.2±42.1	126.1±40.1	-15.9±9.8
Tyrosine	84.9±14.9	90.5±13.2	-5.5±8.0
Phenylalanine	37.7±11.3	45.8±11.5	-8.1±3.6*
Tryptophan	59.4±15.1	60.0±12.6	-0.6±12.4
Ethanolamine	4.7±0.36	5.4±1.5	-0.7±1.4
Ornithine	34.8±14.2	35.3±12.0	-0.56±5.8
Lysine	328.4±78.8	343.5±66.4	-15.1±44.9
Histidine	34.7±8.5	39.0±9.1	-4.2±5.3
Arginine	53.1±15.5	60.3±13.7	-7.1±8.1

Values are means ± SD for n = 6 rats. * Significantly different from zero ($P < 0.05$) by paired t test.

Table A-3 Plasma arterial, portal venous and A-V differences for amino acids in rats fed 60% casein + 0.3% cystine containing diet for 7 days.

60% Casein + 0.3% cystine diet group (μmol/L)			
Amino acid	**A**	**V**	**A-V**
Phosphoserine	5.4±1.1	7.7±1.2	-2.3±2.3
Taurine	80.2±25.8	78.6±23.2	1.5±23.9
Aspartic acid	6.5±1.3	12.8±3.3	-6.3±3.7*
Hydroxyproline	13.1±7.6	12.0±5.3	1.1±7.7
Threonine	133.5±34.8	158.5±49.6	-25.0±43.3
Serine	83.4±22.4	119.7±34.3	-36.2±33.6
Asparagine	32.1±8.5	61.9±19.5	-29.8±17.1*
Glutamic acid	45.0±9.1	66.4±17.1	-21.4±14.7*
Glutamine	238.4±55.0	209.9±46.2	28.5±75.1
Proline	331.5±110.7	445.1±158.6	-113.6±145.9
Glycine	43.4±13.3	100.3±28.0	-56.8±27.6*
Alanine	295.9±89.1	451.4±112.7	-155.6±116.1*
Citrulline	36.6±8.7	47.2±11.2	-10.6±13.0
a-amino-n-butyric acid	11.3±3.8	9.6±3.2	1.6±4.2
Valine	383.3±76.5	379.9±116.9	3.3±83.4
Methionine	35.0±7.6	40.8±8.8	-5.7±11.1
Isoleucine	119.7±31.7	135.6±46.8	-15.8±27.3
leucine	191.6±49.0	211.3±70.1	-19.6±41.6
Tyrosine	57.9±13.0	66.0±16.9	-8.1±16.3
Phenylalanine	35.9±6.9	50.1±13.1	-14.1±13.0*
Tryptophan	50.6±9.1	51.8±11.3	-1.1±15.9
Ethanolamine	3.2±1.4	4.8±1.5	-1.6±1.7
Ornithine	31.3±11.9	29.6±9.2	1.7±10.0
Lysine	361.5±66.1	366.7±90.8	-5.2±90.8
Histidine	30.3±6.2	37.6±9.5	-7.2±9.3
Arginine	59.0±12.2	62.6±16.9	-3.6±17.1

Values are means ± SD for n = 6 rats. * Significantly different from zero ($P < 0.05$) by paired t test.

Table A-4 Plasma arterial, portal venous and A-V differences for amino acids in rats fed 20% casein + 0.3% cystine + 0.5% methionine containing diet for 7 days.

20% Casein + 0.3% cystine + 0.5% Met diet group (μmol/L)			
Amino acid	**A**	**V**	**A-V**
Phosphoserine	4.6±0.4	6.8±1.1	-2.2±1.0*
Taurine	124.5±29.5	150.0±44.4	-25.5±21.8*
Aspartic acid	6.3±1.4	8.7±2.5	-2.3±1.2*
Hydroxyproline	19.2±5.7	23.0±4.3	-3.7±3.5*
Threonine	175.8±38.8	198.3±45.6	-22.4±12.5*
Serine	89.3±13.5	106.7±17.6	-17.2±6.6*
Asparagine	28.1±4.2	41.3±5.6	-13.2±2.9*
Glutamic acid	39.5±11.1	48.6±13.4	-9.1±3.0*
Glutamine	219.8±44.9	195.9±38.8	23.8±20.0*
Proline	186.8±43.3	232.3±55.4	-45.5±16.5*
Glycine	39.1±7.1	69.6±16.7	-30.4±9.8*
Alanine	274.7±57.9	397.5±75.3	-122.8±28.1*
Citrulline	31.9±10.1	51.5±15.5	-19.5±6.7*
a-amino-n-butyric acid	6.0±3.6	8.2±2.3	-2.2±3.7*
Valine	140.0±34.6	157.3±40.6	-17.3±10.6
Methionine	47.8±9.1	54.8±11.5	-6.9±4.3*
Isoleucine	54.4±15.0	66.0±16.6	-11.6±3.0*
leucine	85.3±24.1	102.7±28.7	-17.3±6.6*
Tyrosine	57.4±12.8	65.1±13.3	-7.6±4.6*
Phenylalanine	29.5±8.1	36.8±10.7	-7.2±3.4*
Tryptophan	48.6±15.4	56.8±15.7	-8.1±4.9*
Ethanolamine	2.5±2.2	4.7±2.2	-2.1±0.8*
Ornithine	33.9±12.1	41.3±16.9	-7.4±6.8*
Lysine	280.2±60.3	317.1±67.3	-36.9±19.3*
Histidine	27.0±6.1	33.0±7.8	-6.0±2.5*
Arginine	37.9±13.3	46.0±17.9	-8.0±6.0*

Values are means ± SD for n = 6 rats. * Significantly different from zero ($P < 0.05$) by paired t test.

Table A-5 Plasma arterial, portal venous and A-V differences for amino acids in rats fed 20% casein + 0.3% cystine + 1% methionine containing diet for 7 days.

20% Casein + 0.3% cystine + 1% Met diet group (μmol/L)			
Amino acid	**A**	**V**	**A-V**
Phosphoserine	5.3±1.0	9.7±3.0	-4.4±2.8*
Taurine	154.9±38.9	179.4±41.8	-24.5±17.2*
Aspartic acid	6.2±1.8	8.7±2.3	-2.5±0.7*
Hydroxyproline	24.2±6.4	24.6±5.0	-0.3±3.2
Threonine	221.7±46.9	223.7±43.8	-1.9±23.5
Serine	97.2±18.4	97.7±15.6	-0.4±14.0
Asparagine	25.0±5.1	32.0±7.2	-7.0±4.6*
Glutamic acid	42.9±11.4	48.0±11.0	-5.1±4.4*
Glutamine	212.5±49.7	176.4±27.0	36.0±30.2*
Proline	163.4±31.5	181.5±34.0	-18.1±19.7
Glycine	43.9±8.6	71.6±12.2	-27.6±5.8*
Alanine	265.6±55.9	345.1±57.3	-79.5±31.7*
Citrulline	38.2±12.2	53.3±17.7	-15.1±7.2*
a-amino-n-butyric acid	12.6±3.5	13.4±4.7	-0.7±2.4
Valine	116.6±37.4	116.9±33.0	-0.3±14.0
Methionine	267.1±180.3	275.0±190.1	-7.8±33.4
Isoleucine	49.7±18.6	50.7±16.0	-0.9±6.8
leucine	77.6±29.4	78.8±25.2	-1.2±11.0
Tyrosine	59.1±21.1	59.9±17.0	-0.8±7.9
Phenylalanine	32.5±9.3	34.3±8.2	-1.8±4.3
Tryptophan	44.1±14.7	47.2±15.5	-3.0±3.8
Ethanolamine	4.1±2.3	4.7±1.2	-0.6±3.0
Ornithine	32.9±15.7	40.8±17.0	-7.8±13.0
Lysine	305.7±61.8	312.1±39.3	-6.3±32.6
Histidine	33.4±10.0	35.4±7.8	-1.9±5.1
Arginine	42.7±8.0	44.1±8.6	-1.3±6.2

Values are means ± SD for n = 6 rats. * Significantly different from zero ($P < 0.05$) by paired t test.

Table A-6 Amino acid fluxes across the portal-drained viscera of rats fed different diets for 7 days.

A-V across the Portal Drained Viscera in μmol/min/100g body weight Mean ± SD					
Amino acid	**D_1**	**D_2**	**D_3**	**D_4**	**D_5**
Phosphoserine	-0.011±0.006	-0.007±0.005	-0.010±0.010	-0.009±0.004	-0.020±0.013
Taurine	-0.024±0.070	-0.063±0.113	0.014±0.123	-0.110±0.104	-0.116±0.098
Aspartic acid	-0.008±0.017[a]	-0.016±0.008	-0.023±0.018[a]	-0.009±0.005	-0.011±0.006
Hydroxyproline	0.0148±0.018	0.0038±0.020	0.008± 0.039	-0.016±0.016	-0.003±0.014
Threonine	-0.139±0.168	-0.005±0.143	-0.110±0.222	-0.097±0.061	-0.031±0.098
Serine	-0.207±0.219	-0.070±0.061	-0.167±0.168	-0.073±0.033	-0.015±0.055
Asparagine	-0.072±0.077	-0.073±0.030	-0.138±0.083[b]	-0.055±0.015	-0.034±0.029[b]
Glutamic acid	-0.059±0.041	-0.036±0.024	-0.097±0.069[c]	-0.037±0.014	-0.027±0.030[c]
Glutamine	0.1325±0.187	0.2081±0.142	0.146± 0.394	0.093± 0.08	0.129± 0.08
Proline	-0.354±0.300	-0.162±0.149	-0.503±0.720	-0.194±0.082	-0.098±0.108
Glycine	-0.152±0.108	-0.154±0.054	-0.262±0.127	-0.128±0.046	-0.124±0.063
Alanine	-0.915±0.454	-0.505±0.205	-0.705±0.556	-0.520±0.154	-0.376±0.257
Citrulline	-0.098±0.040	-0.071±0.033	-0.046±0.063	-0.082±0.033	-0.067±0.047
a-amino-n-butyric acid	0.003± 0.009	0.0043±0.010	0.009± 0.022	-0.010±0.018	-0.005±0.009
Valine	-0.156±0.161	-0.028±0.089	0.038± 0.437	-0.074±0.050	-0.015±0.057
Methionine	-0.053±0.054	-0.020±0.018	-0.025±0.056	-0.029±0.020	-0.069±0.163
Isoleucine	-0.099±0.105	-0.038±0.031	-0.068±0.139	-0.049±0.017	-0.011±0.027
leucine	-0.162±0.167	-0.069±0.035	-0.083±0.213	-0.073±0.031	-0.016±0.047
Tyrosine	-0.067±0.084	-0.024±0.031	-0.035±0.083	-0.032±0.022	-0.011±0.030
Phenylalanine	-0.068±0.069	-0.035±0.011	-0.065±0.065	-0.031±0.016	-0.012±0.019
Tryptophan	-0.087±0.149	-0.001±0.047	-0.002±0.082	-0.035±0.024	-0.017±0.019
Ethanolamine	0.0051±0.019	-0.002±0.005	-0.007±0.007	-0.009±0.004	-0.004±0.014
Ornithine	-0.009±0.028	-0.002±0.023	0.0106±0.051	-0.031±0.029	-0.037±0.055
Lysine	-0.213±0.328	-0.062±0.179	-0.004±0.476	-0.159±0.093	-0.058±0.134
Histidine	-0.045±0.067	-0.018±0.022	-0.032±0.046	-0.026±0.012	-0.013±0.023
Arginine	-0.052±0.057	-0.029±0.029	-0.016±0.088	-0.034±0.025	-0.011±0.028

Values are means ± SD for n = 6 rats in each group. Values with similar letter superscripts are significantly different ($P < 0.05$) from each other by one way ANOVA, and Newman-Keuls Multiple Comparison Test.

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