

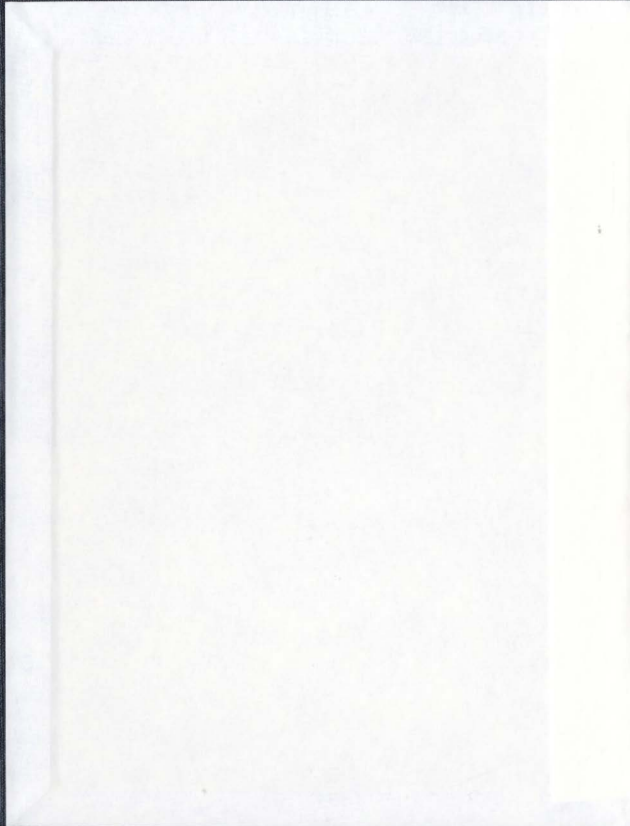
HORMONAL REGULATION OF HOMOCYSTEINE
METABOLISM

CENTRE FOR NEWFOUNDLAND STUDIES

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Hormonal Regulation of Homocysteine Metabolism

by

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A thesis submitted to the
School of Graduate Studies
in partial fulfilment of the
requirements for the degree of
Doctorate of Philosophy

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Abstract

An elevated plasma concentration of homocysteine, a sulfur-containing amino acid derived from methionine, has been recognized as an independent risk factor for the development of vascular disease (Kang *et al.*, 1992). Methionine is adenylated by methionine adenosyltransferase to form S-adenosylmethionine, an important biological methyl donor. Numerous methyltransferases catalyze the transfer of a methyl group from S-adenosylmethionine to a methyl acceptor, producing a methylated product and S-adenosylhomocysteine which is subsequently hydrolysed to form adenosine and homocysteine. Homocysteine has several possible fates: 1) remethylation to form methionine via either the cobalamin-dependent methionine synthase (using N⁵-methyltetrahydrofolate as a methyl donor) or betaine:homocysteine methyltransferase (using betaine as a methyl donor); 2) catabolism by the transsulfuration pathway, ultimately forming cysteine; 3) export to the extracellular space. Two vitamin B₆-dependent enzymes comprise the transsulfuration pathway: cystathionine β -synthase, which condenses homocysteine with serine to form cystathionine, and cystathionine γ -lyase, which cleaves cystathionine to cysteine, NH₄⁺ and α -ketobutyrate.

Altered flux through the remethylation or transsulfuration pathways as a result of genetic mutations or impaired vitamin status has been shown to affect plasma homocysteine levels (Ubbink *et al.*, 1993; Kluijtmans *et al.*, 1999). In recent years it has also become apparent that certain hormones can affect homocysteine metabolism. It has been shown that hypothyroid patients tend to have elevated plasma homocysteine and that these levels are normalized when thyroid levels are restored by thyroxine treatment

(Nedrebo *et al.*, 1998; Hussein *et al.*, 1999). Altered homocysteine metabolism has been observed in diabetes mellitus. Diabetic patients (Types 1 and 2) with signs of kidney dysfunction (i.e. elevated creatinine levels) tend to have increased plasma homocysteine (Hultberg *et al.*, 1991). However in the absence of renal dysfunction, Type 1 diabetic patients exhibit decreased plasma homocysteine relative to normal subjects (Robillon *et al.*, 1994). In this thesis we investigated the hormonal regulation of homocysteine metabolism in rats in hope that we could illuminate a possible mechanism for altered plasma homocysteine levels in human hypothyroidism and Type 1 diabetes mellitus.

In **Chapter 3**, hypothyroidism was induced in one study by addition of propylthiouracil (PTU) to the drinking water for 2 weeks. In a second study, thyroidectomized and sham-operated rats were used with thyroid hormone replacement via mini-osmotic pumps. Unlike the human hypothyroid patients, both groups of hypothyroid rats exhibited decreased total plasma homocysteine (30% in PTU rats, 50% in thyroidectomized rats) versus their respective controls. Thyroid replacement normalized homocysteine levels in the thyroidectomized rat. Increased activities of the hepatic trans-sulfuration enzymes were found in both models of hypothyroidism. These results provide a possible explanation for the decreased plasma homocysteine concentrations. The hypothyroid rat cannot be used as a model to study homocysteine metabolism in hypothyroid patients.

The purpose of our second study (**Chapter 4**) was to investigate homocysteine metabolism in a type 1 diabetic animal model and to examine whether insulin plays a role in its regulation. Diabetes was induced by intravenous administration of streptozotocin

(100 mg/kg) to rats. Depending on the experiment, we observed a 30-70 % reduction in plasma homocysteine in the untreated diabetic rat. Treatment with insulin of the diabetic rat raised plasma homocysteine concentrations. Transsulfuration and remethylation enzymes were measured in both liver and kidney. We observed an increase in the activities of the hepatic transsulfuration enzymes (cystathionine β -synthase and cystathionine γ -lyase) in the untreated diabetic rat. Insulin treatment normalized the activities of these enzymes. The renal activities of these enzymes were unchanged as was the proportion of plasma homocysteine metabolized by the kidney. These results suggest that insulin is involved in the regulation of plasma homocysteine concentrations by affecting the hepatic transsulfuration pathway.

The increased hepatic cystathionine β -synthase activity in the untreated-diabetic rat was associated with elevated mRNA levels. Similar to its activity, cystathionine β -synthase mRNA levels were reduced by insulin administration. To further investigate the regulation of cystathionine β -synthase we incubated rat hepatoma cells (H4IIE) with various hormones. Cystathionine β -synthase mRNA, protein, and activity were induced in triamcinolone stimulated H4IIE cells. This induction was prevented by insulin incubation. CPT-cAMP, an analogue of cAMP, also induces cystathionine β -synthase mRNA levels in cultured cells. Co-incubation of insulin with CPT-cAMP prevents any increase in mRNA levels. These experiments provide evidence of the direct regulation of cystathionine β -synthase by insulin and its counter-regulatory hormones.

Given the broad regulatory effects of glucagon on amino acid metabolism and the fact that plasma glucagon levels are often elevated in Type 1 diabetes we

investigated the effect of glucagon on homocysteine metabolism in the rat (**Chapter 5**). Male Sprague Dawley rats were treated with glucagon (4 mg/kg/day in three divided doses) for 2 days while control rats received vehicle injections. Glucagon treatment resulted in a 30% decrease in total plasma homocysteine and increased hepatic activities of glycine N-methyltransferase, cystathionine β -synthase and cystathionine γ -lyase. Enzyme activities of the remethylation pathway were unaffected. The 90% elevation in activity of cystathionine β -synthase was accompanied by a two-fold increase in its mRNA level. Hepatocytes prepared from glucagon-injected rats exported less homocysteine, when incubated with methionine, than did hepatocytes of saline-treated rats. Flux through cystathionine β -synthase was increased five-fold in hepatocytes isolated from glucagon-treated rats as determined by production of $^{14}\text{CO}_2$ and $1\text{-}^{14}\text{C-}\alpha$ -ketobutyrate from $\text{L-[1-}^{14}\text{C]methionine}$. Methionine transport was elevated two-fold in hepatocytes isolated from glucagon-treated rats resulting in increased hepatic methionine levels. Hepatic concentrations of S-adenosylmethionine and S-adenosylhomocysteine, allosteric activators of cystathionine β -synthase, were also increased following glucagon treatment. These results indicate that glucagon can regulate plasma homocysteine through its effects on the hepatic transsulfuration pathway.

This thesis provides clear evidence that glucagon lowers plasma homocysteine while insulin has the opposite effect. The mechanism for this reciprocal regulation has been outlined in detail. Taken together, it is clear that these metabolic hormones can be very important in controlling plasma homocysteine levels and that the liver is the site of this hormonal regulation.

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List of Abbreviations

ATP: Adenosine triphosphate

BHMT: Betaine:homocysteine methyltransferase

BSA: Bovine serum albumin

cAMP: Cyclic adenosine monophosphate

CBS: Cystathionine β -synthase

CTP: Cytidine triphosphate

DMEM: Dulbecco's Modified Eagle's Medium

DPBS: Dulbecco's phosphate buffered saline

DTT: Dithiothreitol

EDTA: Ethylenediaminetetracetic acid

EGTA: Ethylene glycol-bis(aminoethylether)-N,N,N',N'tetraacetic acid

ESRD: End-stage renal disease

FAD: Flavin adenine dinucleotide

FBS: Fetal Bovine Serum

FMN: Flavin mononucleotide

GFR: Glomerular filtration rate

GNMT: Glycine N-methyltransferase

GSH: Reduced glutathione

GSSG: Oxidized glutathione

HPLC: High pressure liquid chromatography

INS: Insulin

LDH: Lactate Dehydrogenase

MAT: Methionine adenosyltransferase

MTHFR: Methylene tetrahydrofolate reductase

NADH: Nicotinamide adenine dinucleotide

NADPH: Nicotinamide adenine dinucleotide phosphate

PEPCK: Phosphoenolpyruvate carboxykinase

PTU: Propylthiouracil

RPF: Renal plasma flow

SAH: S-Adenosylhomocysteine

SAM: S-Adenosylmethionine

SDS: Sodium dodecyl sulphate

SSA: Sulfosalicylic acid

T₃: Triiodothyronine

T₄: Thyroxine

THF: Tetrahydrofolate

TMC: Triamcinolone

Chapter 1: Introduction

1.1 Historical Perspective

The study of sulfur amino acids began in 1810 when Wollaston isolated "cystic acid" from bladder stones taken from a human patient (Finkelstein, 2000). Two decades later, "cystic acid" was determined to be cysteine. However, a century would pass until du Vigneaud would chemically synthesize homocysteine from methionine.

The events leading to the discovery of homocysteine are fascinating. Du Vigneaud was intrigued by the chemical nature of insulin, which had been isolated by Banting and Best in 1922. His particular interest was of the chemical nature of sulfur in this protein. In his early work he concluded that cysteine could not account for the entire sulfur content of insulin; therefore he started experiments on the newly isolated amino acid, methionine. In 1932, du Vigneaud reported that homocysteine was formed when methionine reacted with concentrated acid (Butz and Du Vigneaud, 1932). Having isolated homocysteine, du Vigneaud and his colleagues began nutritional and metabolic studies using the three sulfur-containing amino acids. This was the beginning of the study of sulfur amino acid metabolism and in the following decades the biochemical intermediates, enzymes, and regulators would be discovered and characterized. Today we are still investigating the factors regulating sulfur amino acid metabolism, as is evident from this thesis. The importance of du Vigneaud's discovery cannot be overstated; however there is a delightful footnote to his work. He misinterpreted his initial findings on insulin: cysteine does account for all the sulfur in insulin!

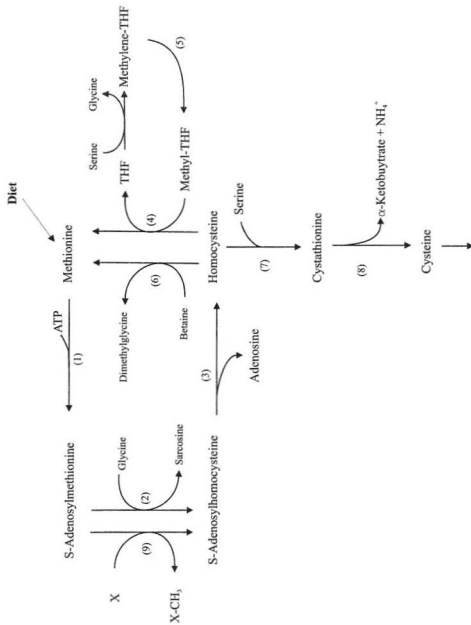
1.2 Overview

Methionine is an indispensable, sulfur-containing amino acid and, as such, in mammals its dietary availability is essential for the normal growth and metabolism of animals. Methionine is involved in protein synthesis, in the synthesis of S-adenosylmethionine, an important biological methyl donor and intermediate in polyamine synthesis, and in the formation of homocysteine. The conversion of homocysteine back to methionine is necessary for the catabolism of choline and betaine, and in the recycling of intracellular folates, while the catabolism of homocysteine yields cysteine (a substrate for protein and glutathione synthesis) which is a precursor of taurine (important in bile synthesis). The role of sulfur amino acids in cellular processes is indeed impressive.

Figure 1-1 summarizes the reactions involved in methionine metabolism. In the first step dietary methionine is activated to the high-energy sulfonium compound, S-adenosylmethionine (SAM), via the enzyme methionine adenosyltransferase. S-adenosylmethionine is the primary biological methyl donor and is a substrate for hundreds of transmethylation reactions. For example, SAM can methylate DNA, phospholipids, and guanidinoacetate, forming S-adenosylhomocysteine and a methylated product. S-adenosylhomocysteine is then hydrolysed to adenosine and homocysteine. Homocysteine has many metabolic fates: 1) it can be remethylated back to methionine via methionine synthase (using methyltetrahydrofolate as a methyl donor) or via betaine:homocysteine methyltransferase (using betaine as a methyl donor); or 2) it can be catabolized to cysteine via the transsulfuration pathway

Figure 1.1: Methionine/Homocysteine metabolism in mammals.

The numbers beside the arrows represent the following enzymes: 1- Methionine adenosyltransferase, 2- Glycine N-methyltransferase, 3- S-adenosylhomocysteine hydrolase, 4- Methionine synthase, 5- Methylene tetrahydrofolate reductase, 6- Betaine:homocysteine methyltransferase, 7- Cystathionine β -synthase, 8- Cystathionine γ -lyase, 9- various transmethylation reactions.



Further Metabolism: Glutathione, Taurine, Protein Synthesis, Catabolism

which contains two vitamin B6-dependent enzymes, cystathionine β -synthase and cystathionine γ -lyase; or 3) it can be exported out of the cell.

As alluded to above, the metabolism of methionine and homocysteine is the combination of two distinct metabolic sequences, the methionine cycle and the transsulfuration pathway. All enzymes involved in methionine metabolism are found within the cytoplasmic compartment; however the tissue profiles of these enzymes are very different. Human and rat livers possess significant activities of all the enzymes shown in Figure 1.1, which is not surprising given the importance of hepatic amino acid metabolism. Besides the liver, only the pancreas and kidney have significant activities of the transsulfuration pathway (Finkelstein, 1990). Unlike the transsulfuration pathway, the methionine cycle is ubiquitous in nature. All cells contain methionine synthase and methylenetetrahydrofolate reductase; however betaine:homocysteine methyltransferase, the other remethylation enzyme, is not as prevalent. In humans, betaine:homocysteine methyltransferase can be found in liver, brain and kidney (Finkelstein, 1974). Unlike humans, rats do not have renal betaine:homocysteine methyltransferase (Delgado-Reyes *et al.*, 2001).

It is clear that methionine metabolism is important in every tissue. Given that most of the experiments have utilized liver tissue and that the liver has a full complement of relevant enzymes, this introduction will focus mainly on hepatic methionine/homocysteine metabolism; however, other tissues will be mentioned when appropriate. The discussion will begin with a description of the methionine cycle and the transsulfuration pathway and the interactions between them. The main

theme of this thesis is the regulation of homocysteine metabolism, so the regulation of individual enzymes and of flux through pathways will be highlighted. The discussion begins at the gateway of methionine metabolism, the synthesis of S-adenosylmethionine.

1.3 Methionine Adenosyltransferase

Methionine adenosyltransferase, or S-adenosylmethionine synthase, (EC 2.5.1.6, Figure 1.1- reaction 1) is a ubiquitous enzyme that catalyzes the formation of S-adenosylmethionine from ATP and methionine. The synthesis of SAM is achieved via two sequential reactions: in the first reaction, the methionine sulfur nucleophilically attacks the C5' carbon of ATP, producing an enzyme-bound SAM and triphosphosphate; secondly, the triphosphosphate is hydrolysed to pyrophosphate and orthophosphate, a step which is rate-limiting for the overall sequence and is required for product release (Markham *et al.*, 1980; Sanchez del Pino *et al.*, 2002).

Two genes, MAT1A and MAT2A, encode for the catalytic subunit of methionine adenosyltransferase (Alvarez *et al.*, 1993). MAT1A is expressed only in adult liver, whereas MAT2A is expressed in all non-hepatic tissues. In addition, MAT2A is found in fetal liver and is replaced by MAT1A during organ maturation. The two MAT genes encode for different isozymes of the catalytic subunit, named α , which have a high degree of homology (approximately 85%). MAT1A gene encodes for the $\alpha 1$ protein while the MAT2A gene encodes for the $\alpha 2$ protein (Kotb *et al.*, 1997).

In the adult liver, MAT1A gives rise to two isoforms of methionine adenosyltransferase: a 210 kDa $\alpha 1$ tetramer (MAT I) and a 110 kDa $\alpha 1$ dimer (MAT III) (Sullivan and Hoffman, 1983). MAT I displays Michaelis-Menten kinetics with a K_m for methionine of 3–40 μM and is allosterically inhibited by the reaction product S-adenosylmethionine. In contrast, MAT III is labelled the “high K_m ” isoform with its K_m for methionine ranging between 0.03–1.4 mM, and is allosterically activated by S-adenosylmethionine (Finkelstein, 1990). Thus the reaction velocity of MAT III increases in response to elevated methionine and S-adenosylmethionine levels, which permits rapid clearance of excess methionine in the liver. The regulation of hepatic MAT will be discussed in later sections.

The extrahepatic MAT II is described as the “low K_m ” enzyme with values for methionine ranging from 4 to 23 μM ; like MAT I, S-adenosylmethionine inhibits the reaction (Finkelstein, 1990). Unlike the liver enzymes, MAT II is a heterodimer of α_2 (catalytic) and β (regulatory) subunits. When the β subunit, the gene product of MAT2B, associates with the α subunit, it alters its kinetic properties thus making MAT II more susceptible to inhibition by S-adenosylmethionine (Halim *et al.*, 1999). Stimulation of T cells results in up regulation of MAT2A gene and down-regulation of MAT2B gene. These conditions favour increased formation of α_2 oligomers. This form of MAT II has a 3-fold higher specific activity and results in a 6-fold increase in intracellular S-adenosylmethionine concentrations (LeGros *et al.*, 1997). Similar results were observed using a transfected human fibroblast cell line (Halim *et al.*, 1999). Indeed, differential expression of the MAT II β subunit may be very important

in regulating MAT activity in extrahepatic cells and may represent a mechanism that modulates S-adenosylmethionine concentrations during different stages of growth and development.

1.4 S-Adenosylmethionine and transmethylation reactions

The liver is the site of 85% of all methylation reactions in the body (Wyss and Walliman, 1994). Given this information it is not surprising that the formation of S-adenosylmethionine (SAM) is vital for normal function. S-adenosylmethionine is the primary methyl donor in virtually all biological methylation reactions with the exception of those involved in the methylation of homocysteine, and participates in a wide range of cellular processes through the methylation of nucleic acids, proteins, phospholipids, and small molecules (Stipanuk, 1986). Alternatively, the propylamine moiety of SAM can be used in the synthesis of polyamines. Despite their important biological function, it is estimated that only 10% of the available S-adenosylmethionine is utilized in this sequence (Mudd and Poole, 1975). Since, S-methylthioadenosine can be recycled back to methionine it is assumed that polyamine synthesis has a minimal effect on cellular methionine metabolism (Backlund and Smith, 1981).

In a series of balance studies Mudd and Poole (1975) attempted to quantify the demands of transmethylation reactions on the availability of S-adenosylmethionine. In these studies, humans were fed cysteine-free diets containing various levels of methionine and choline. As methionine and choline comprised the

dietary methyl group supply, the availability of labile methyl groups could be easily determined. Their initial findings showed that when males were fed a normal diet the excretion of creatinine was greater than the dietary supply of methyl groups. From this key observation it was concluded that *de novo* synthesis of methyl groups must be important in humans (Mudd and Poole, 1975). The *de novo* source of these methyl moieties is methyltetrahydrofolate which can methylate homocysteine to form methionine. Further experiments showed that male subjects, under normal dietary conditions, cycled the homocysteinyI moiety between methionine and homocysteine approximately 1.9 times before converting it to cystathionine. This value was 1.5 for female subjects. In essence, these studies indicated that approximately half of the homocysteine was converted to cystathionine with the remainder being remethylated. However, when humans were fed a diet low in methionine and choline, each homocysteinyI moiety, on average, passed through the methionine cycle at least 4 times in males and 3 in females. These studies suggested that the dietary content of methyl donors influence the *de novo* synthesis of methyl groups (Mudd and Poole, 1975). Specifically, when methyl groups are limiting, the remethylation pathway is activated; however, when methionine is in excess catabolism via the transsulfuration pathway is favoured.

1.4.1 Glycine N-methyltransferase

The results from Mudd and Poole's studies suggest an important role for glycine N-methyltransferase in the metabolism of excess methionine. Glycine N-

methyltransferase (GNMT, EC 2.1.1.20, Figure 1.1- reaction 2) catalyses the methylation of glycine, by S-adenosylmethionine, to form sarcosine and S-adenosylhomocysteine (SAH). Sarcosine, which has no other known physiological role, may be transported into the mitochondrion where it is converted, by sarcosine dehydrogenase, to glycine and methylenetetrahydrofolate. Thus by the coupled action of GNMT and sarcosine dehydrogenase, SAM may be converted to SAH without the net consumption of glycine (Heady and Kerr, 1973). GNMT is found predominantly in liver of mammals (Blumenstein and Williams, 1960), comprising 1-3% of soluble protein; however high levels are also found in kidney cortex and pancreatic exocrine cells (Heady and Kerr, 1973; Kerr, 1972).

GNMT can be distinguished from other methyltransferases by its protein structure and kinetic properties. GNMT is an oligomeric protein consisting of 4 identical 32 kDa subunits, and displays Michaelis-Menten kinetics with respect to glycine with a K_m around 230 μM (Ogawa and Fujioka, 1982; Ogawa *et al.*, 1997). However, S-adenosylmethionine exhibits sigmoidal kinetics with a $[S]_{0.5}$ equalling 60 μM (Ogawa and Fujioka, 1982). It should be noted that the $[S]_{0.5}$ (SAM) is higher for GNMT than for the other methyltransferase reactions which vary between 10-20 μM (Duerre *et al.*, 1977; Hoffman *et al.*, 1979). Furthermore, unlike the other methyltransferases, GNMT is not inhibited by S-adenosylhomocysteine. Given these kinetic and regulatory properties, along with its abundance in liver cells, GNMT may serve as a mechanism to metabolize SAM when methyl supply is in excess. Likewise,

when SAM is limiting, the activity of GNMT is reduced, thus preserving the valuable methyl supply.

1.5 S-adenosylhomocysteine hydrolase

As described previously, the reaction product (S-adenosylhomocysteine) inhibits most biological transmethylation reactions. For this reason, the removal of SAH is essential for normal cellular function. S-adenosylhomocysteine hydrolase (EC 3.3.1.1, Figure 1.1- reaction 3) is responsible for the intracellular removal of SAH by converting it to homocysteine and adenosine. The enzyme is ubiquitous in mammalian tissues and is composed of 4 identical 47 kDa subunits (Coulter-Karis and Hershfield, 1989).

The hydrolysis of SAH is a reversible reaction; in fact, the equilibrium ($K_{eq} = 10^{-6}$ M) greatly favours SAH synthesis (Stipanuk, 1986). However, SAH hydrolysis normally occurs, *in vivo*, due to the removal of homocysteine and adenosine. Intracellular elevation of either product results in accumulation of SAH, which has far-reaching consequences. The ratio of SAM to SAH, which is normally around 5, may be very important in regulating transmethylation reactions (Hoffman *et al.*, 1979). For example, when SAM/SAH ratios approach 2, DNA and histone methyltransferases are inhibited, whereas phosphotidylethanolamine methyltransferase is inhibited when the SAM/SAH ratio approaches 3 (Hoffman *et al.*, 1979). Although methyltransferases behave differently at various SAM/SAH ratios, it is clear that an elevation of SAH will inhibit at least some methyltransferase

reactions and its removal is imperative for normal cellular function. This phenomenon can be observed in patients with adenosine deaminase deficiency, who cannot deaminate adenosine to inosine (Hershfield *et al.*, 1985). The elevation of adenosine inhibits SAH hydrolase activity resulting in a multitude of problems. The ensuing elevation in SAH levels inhibits transmethylation reactions, while the decreased cysteine production, via the transsulfuration pathway, disrupts glutathione synthesis and perpetuates cellular oxidative damage (Atmaca and Fry, 1996).

Two other mechanisms can remove intracellular SAH. First, SAH can bind to specific intracellular proteins, thus becoming kinetically inactive (Svardal and Ueland, 1987). Secondly, elevated SAH may also be exported out of the cell (Duerre *et al.*, 1969; Hoffinan *et al.*, 1980).

1.6 Remethylation of Homocysteine

The importance of homocysteine removal for the normal functioning of methyltransferase reactions was outlined in the previous section. The cell has three major routes to remove intracellular homocysteine: 1) it can export it out of the cell; 2) it can irreversibly catabolise it via the transsulfuration pathway to form cysteine; or 3) it can remethylate it back to methionine. The remethylation of homocysteine can be catalyzed by methionine synthase or by betaine:homocysteine methyltransferase, representing the conservation of the carbon skeleton and sulfur atom from the original methionine molecule.

1.7 Methionine Synthase

Methionine synthase (EC 2.1.13, Figure 1.1- reaction 4), a cytoplasmic enzyme which is widely distributed in mammalian tissues, is responsible for the methylation of homocysteine, by methyltetrahydrofolate, to form methionine and tetrahydrofolate (Xue and Snoswell, 1985). An enzyme-bound cobalamin moiety is vital for methionine synthase activity since it facilitates the methyl transfer (Chen *et al.*, 1995). Catalytic amounts of S-adenosylmethionine are also required to maintain cobalamin in its active form (Burke *et al.*, 1971).

The importance of methionine synthase in homocysteine metabolism was confirmed in human patients with inborn errors of metabolism that reduce methionine synthase activity (Fenton and Rosenberg, 1989). The consequence of such mutations were elevated levels of plasma homocysteine and low plasma methionine (van der Put *et al.*, 1997). Methionine synthase may also be inactivated in animals by exposure to nitrous oxide, resulting in decreased tissue levels of methionine and SAM, and impaired transmethylation reactions (Lumb *et al.*, 1980).

Methionine synthase is also involved in folate metabolism. Folate is essential for the biosynthesis of both purines and pyrimidines; thus, a folate deficiency compromises the cell's ability to synthesise DNA and RNA (Shane and Stokstad, 1985). This can have devastating effects on quickly dividing cells, such as bone marrow (Koury and Horne, 1994), often resulting in macrocytic anemia (Matthews *et al.*, 1990). Cells obtain folate from the blood principally in the form of methyltetrahydrofolate. Only methionine synthase can use this form of folic acid

(Shane, 1989). The conversion of methyl-THF to THF results in the formation of a more useable form of cellular folate, which can be polyglutamated and retained by the cell (Shane and Stokstad, 1985). When methionine synthase activity is impaired, either by vitamin B₁₂ deficiency or mutation, methyltetrahydrofolate accumulates at the expense of other cellular folates (Shane, 1989). This condition is called the “folate trap”, since, under normal cellular conditions, methyltetrahydrofolate cannot be oxidized by methylenetetrahydrofolate reductase (Finkelstein *et al.*, 1978). The decrease in functional cellular folate also explains why clinical symptoms of folate and vitamin B₁₂ deficiency are similar.

1.8 Methylenetetrahydrofolate Reductase

Remethylation of homocysteine by methionine synthase is dependent on the availability of methyltetrahydrofolate, which can be obtained from the circulation or from pre-existing cellular folates. Methylenetetrahydrofolate reductase (MTHFR, E.C.1.7.9.9.5, Figure 1.1- reaction 5) catalyses the NADPH-dependent reduction of 5',10'-methylenetetrahydrofolate to methyltetrahydrofolate, representing the only methyltetrahydrofolate-generating reaction in the cell (Green *et al.*, 1988). This enzyme is a dimer of identical 77 kDa subunits containing a non-covalently bound FAD (Daubner and Matthews, 1982). Tryptic cleavage of the subunit produces a N-terminal 40 kDa catalytic region and a 37 kDa regulatory region (Matthews *et al.*, 1984).

In recent years, there has been an extensive search for mutations in the human methylenetetrahydrofolate reductase gene. Several mutations associated with severe enzyme deficiency in humans have been identified; two such mutations are Arg157Gln and Thr227Met (Matthews *et al.*, 1998). One polymorphism, Ala222Val, has a high incidence in humans (Frosst *et al.*, 1995). Patients homozygous for this polymorphism have reduced enzyme activity in fibroblasts, and the enzyme demonstrates increased susceptibility to heat inactivation (Engbersen *et al.*, 1995). This mutation is of importance since it has been linked to increased risk for neural tube defects (Whitehead *et al.*, 1995), and development of cardiovascular diseases (Engbertsen *et al.*, 1995), although the latter correlation is under dispute (deFranchis *et al.*, 1996). These patients seem to be very sensitive to folate status, with folate deficient diets resulting in massive increases in plasma homocysteine (Jacques *et al.*, 1996). The Ala222Val mutation may result in loss of enzyme-bound FAD (Matthews *et al.*, 1998), a phenomenon that may be prevented by folate supplementation (Yamada *et al.*, 2001). Studies on a mutated bacterial enzyme (Matthews *et al.*, 1998) and human mutants (Yamada *et al.*, 2001) are consistent with this possibility. Thus the decrease in activity in cells from these patients may reflect the presence of inactive apo-enzyme.

1.9 Betaine-Homocysteine Methyltransferase

The second enzyme that methylates homocysteine to give methionine is betaine-homocysteine methyltransferase (BHMT; E.C. 2.1.1.5, Figure 1.1- reaction

6). In this reaction, betaine donates a methyl group to homocysteine forming dimethylglycine and methionine, respectively. This reaction is required for the irreversible oxidation of choline, the metabolic precursor of betaine. The enzyme is a hexamer of 45 kDa subunits with each monomer containing a Zn-binding site (Garrow, 1996). The kinetics of the rat liver enzyme have been investigated. K_m values for homocysteine and betaine were reported as 15-20 and 50-60 μM , respectively (Stipanuk, 1986). In contrast to methionine synthase, BHMT is not widely distributed in mammalian tissues. In humans, BHMT can be found at high activity in liver and at lower activity in brain and kidney (Finkelstein, 1974). Unlike humans, rats do not have renal betaine-homocysteine methyltransferase.

1.10 Transsulfuration Pathway: Cystathionine β -Synthase

The carbon and sulfur groups of methionine/homocysteine are removed from the methionine cycle via the transsulfuration pathway. The first reaction in this sequence is mediated by the vitamin B6-dependent and heme-containing enzyme, cystathionine β -synthase (CBS; E.C. 4.2.1.22, Figure 1.1- reaction 7), which catalyses the condensation reaction of homocysteine and serine to form cystathionine. The presence of heme is essential for activity (Kery *et al.*, 1994). This condensation reaction may be reversed if homocysteine is rapidly removed; however, under physiological conditions the equilibrium favours cystathionine formation (Finkelstein, 1990).

Mammalian cystathionine β -synthase is a tetrameric protein of identical 63 kDa subunits with K_m values for serine of about 1 mM and for homocysteine ranging from 1 to 25 mM (Taoka *et al.*, 1998). Although quite a range, it is clear that these values far exceed those of the remethylation enzymes. In contrast to the methionine cycle enzymes, cystathionine β -synthase is only found in a few tissues. The liver has the highest level of activity, followed by the pancreas, kidney and small intestine.

Abnormal synthase expression has been extensively studied in humans. Cystathionine β -synthase deficiency is the most common disorder of sulfur amino acid metabolism, with an incidence of 1:75000 (Mudd and Levy, 1978). This inborn error was first reported in 1963 during screening tests of mentally retarded patients in Northern Ireland (Carson *et al.*, 1963). Since that first observation, over 100 distinct mutations in the CBS gene have been reported worldwide. In a survey of 630 homocysteinuric patients, Mudd *et al.* (1985) showed that 50% of the patients displayed a large decrease in homocysteine concentration associated with increased CBS activity when they were given pharmacological doses of pyridoxine. This suggests that about half of the mutations result in decreased pyridoxal-5-phosphate binding affinity; thus increased concentration of the co-factor is required for activity. Studies using tissue samples from vitamin B6-nonresponsive patients found that certain mutations cause CBS to fold improperly because it cannot bind heme (Janosik *et al.*, 2001). Furthermore, abnormally folded CBS disfavours tetramer formation and makes the mutant prone to subunit aggregation, thus inactivating the enzyme.

1.11 Cystathionine γ -Lyase

The last enzyme involved in the catabolism of methionine to cysteine, cystathionine γ -lyase (C γ L; EC 4.4.1.1, Figure 1.1-reaction 8), catalyzes the conversion of cystathionine into cysteine, α -ketobutyrate and ammonia. Rat cystathionine γ -lyase is composed of four 43 kDa subunits and its activity is pyridoxal-5-phosphate dependent (Braunstein and Goryachenkova, 1984). The K_m for cystathionine has been reported to be in the range of 0.3 to 3.5 mM. The liver has the highest activity of cystathionine γ -lyase, whereas the kidney and pancreas exhibit approximately half the hepatic level (Nishi *et al.*, 1994).

It has been shown that cystathionine γ -lyase protein and activity are absent in fetal liver (Gaulle *et al.*, 1972; Sturman *et al.*, 1970). The rate of glutathione synthesis from methionine is considerably lower in fetal compared with adult hepatocytes, and thus can be attributed to the low enzyme activity (Heinonen, 1973). Although cystathionine γ -lyase activity is absent in fetal liver, its mRNA can be detected as early as the nineteenth gestational week (Levonen *et al.*, 2000). The reason for the discrepancy between mRNA and enzyme activity is currently unknown. However, it has been suggested that posttranscriptional regulation, rather than the presence of an inhibitor or improper protein folding, is the most plausible explanation (Levonen *et al.*, 2000).

1.12 Regulation of Homocysteine metabolism

Early studies on both animals and human patients with inborn errors of metabolism illustrated that interconversion of methionine and homocysteine was a cycle, with irreversible removal of homocysteine from the cycle achieved by cystathionine β -synthase. The balance between the two pathways ensures that sufficient methionine is available for protein synthesis and SAM-dependent methylation reactions while preventing methionine toxicity and supplying adequate levels of cysteine for glutathione synthesis and other functions. In his early experiments, Finkelstein (1978) provided evidence for the regulation of these pathways. In a delicate experiment he measured the radioisotope disappearance of [^3H -methyl, ^{35}S]methionine in liver slices taken from rats fed either a 3.5% or 55% casein diet. From this experiment, Finkelstein could measure the half-life of methionine and the proportion of homocysteine catabolised by the transsulfuration pathway. The half-life of ^3H -methyl groups was reduced from 9.3 to 4.8 minutes when animals were fed a high protein diet. This change in half-life corresponded to an increased conversion of homocysteine to cystathionine, from 10 % in the control diet to 70% in the high-protein. These data obtained *in vitro* validated the human methylation studies (discussed in section 1.4) completed just a few years earlier by Mudd and Poole (1975). The next section discusses how competing pathways are controlled in tandem to achieve metabolic balance between methionine conservation and catabolism. The two major mechanisms that regulate homocysteine metabolism

are the level of enzyme in the tissue and the kinetic properties of the relevant enzymes.

1.12.1 Tissue level of enzymes

The liver has been recognized as an important site for amino acid metabolism. With this in mind, it should not be surprising that alterations in dietary methionine, either by methionine supplementation or increased protein intake, can affect the tissue content of the relevant enzymes. This can be clearly seen in rats fed a low (3.5%) versus high (55%) casein diet. The activities of all hepatic enzymes responsible for converting methionine to cysteine were altered by methionine excess. The activities of methionine adenosyltransferase, glycine N-methyltransferase, and S-adenosylhomocysteine hydrolase were elevated 1-2 fold, while cystathionine β -synthase and cystathionine γ -lyase were 3-4 times higher in livers taken from rats fed a high-protein diet (Finkelstein and Mudd, 1967; Ogawa and Fujioka, 1982; Finkelstein and Harris, 1973). These findings are consistent with the decreased methyl group half-life and increased transsulfuration flux observed in liver slices isolated from high-protein fed rats (Finkelstein, 1978). The behaviour of the remethylation enzymes was quite different from those that catabolize methionine. Methionine synthase was drastically decreased during methionine excess, which is consistent with the enzyme's role as a conserver of methyl groups (Finkelstein *et al.*, 1971). Methylene tetrahydrofolate reductase activity was unaltered by fluctuations in dietary methionine (Finkelstein, 1978). However, betaine:homocysteine

methyltransferase activity was elevated in rats fed either a methionine deficient or supplemented diet. It has been hypothesized that betaine:homocysteine methyltransferase functions to maintain tissue methionine concentration when intake is limited and to remove homocysteine when methionine is in excess (Finkelstein *et al.*, 1983). Recently the interactions between dietary methionine and methyl donor intake (choline and betaine) were investigated with respect to betaine:homocysteine methyltransferase. It was found that methionine-deficient diets could elevate levels of betaine:homocysteine methyltransferase mRNA, protein and activity (Park and Garrow, 1999). However, maximum betaine:homocysteine methyltransferase induction was only observed when high levels of betaine or choline were also present in the diet.

The dietary content of cysteine also affects the hepatic content of enzymes. Womack and Rose (1941) were the first to demonstrate that cysteine could replace approximately 70% of the dietary requirement for methionine. The enzymatic basis for the methionine-sparing effect of cysteine was first studied by Finkelstein and Mudd (1967). They observed that replacement of dietary methionine with cysteine resulted in marked decrease in the hepatic activity of cystathionine β -synthase, but had little effect on the level of remethylation enzymes. The decreased activity of cystathionine β -synthase was later shown to occur at the gene level since cystathionine β -synthase mRNA levels were reduced in rats fed a low methionine/high cysteine diet (Yamanoto *et al.*, 1995).

1.12.2 Enzyme kinetics and allosteric regulators

In the long term, altering the flux through a pathway may be achieved by changing the tissue content of individual enzymes; however the kinetic properties of the enzymes, their relative affinities for substrates and their response to effectors, can provide a rapid mechanism for responding to intracellular perturbations.

The enzymes that metabolize methionine and its derivatives can be classified according to their K_m values (Table 1.1). The methionine “conserving” enzymes have low K_m values and tend to be inhibited by their products. The enzymes in this category include MAT I, SAH hydrolase, betaine:homocysteine methyltransferase, methionine synthase, and methylenetetrahydrofolate reductase. In contrast, the methionine “catabolizing” enzymes have relatively high K_m values, and are not inhibited by their reaction product. MAT III, cystathionine β -synthase and cystathionine γ -lyase are included in this group. Furthermore, Glycine N-methyltransferase can also be considered a member of this group. There are two properties that make GNMT ideal for its role in methionine catabolism: it is not inhibited by S-adenosylhomocysteine; and it produces sarcosine, a non-toxic recyclable product (Cook *et al.*, 1989). The “low” K_m values of the methionine “conserving” enzymes and the “high K_m ” values of the “catabolizing” enzymes ensure that when methionine is required, homocysteine is conserved; however, when methyl groups are in excess, flux through the transsulfuration pathway is favoured.

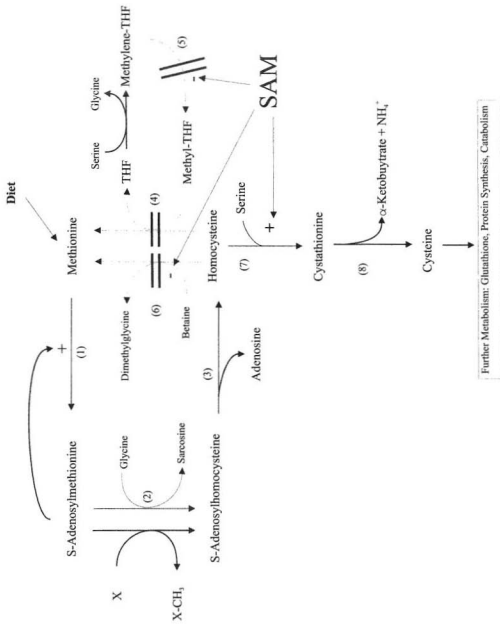
Table 1.1: Classification of methionine metabolizing enzymes

Methionine Conserving Enzymes	K_m (μ M)	Methionine Catabolizing Enzymes	K_m (mM)
MAT I	Met: 3-40	MAT III	Met: 0.03-1.4
SAH hydrolase	SAH: 8-60	Cystathionine β- synthase	Ser: 1.0 HCY: 1-25
BHMT	HCY: 15-20 Betaine: 50-60	Cystathionine γ- lyase	CYST: 0.3-3.5
Methionine Synthase	HCY: 60	Glycine N- methyltransferase	Gly: 0.23 SAM: 0.06
MTHFR	Not available		

S-adenosylmethionine and S-adenosylhomocysteine also play a significant role in regulating the proportion of homocysteine that is remethylated versus the proportion which is catabolized. The cellular level of S-adenosylmethionine is dependent on the supply of methionine, as would be expected. As summarized in Figure 1.2, elevated levels of SAM inhibit betaine-homocysteine methyltransferase and methylenetetrahydrofolate reductase, which limits the availability of methyltetrahydrofolate, while activating methionine adenosyltransferase III and cystathionine β -synthase (Finkelstein and Martin, 1984; Kutzbach and Stokstad, 1971; Finkelstein, 1990). Shan *et al.* (2001), using site-directed mutagenesis, has shown that S-adenosylmethionine binds to the C-terminal domain of cystathionine β -synthase. The binding of SAM removes the inhibition provided by the regulatory domain resulting in a decreased K_m value for homocysteine and an increase in specific activity. Taken together, the regulatory effects of elevated SAM promote transsulfuration flux, while limiting homocysteine remethylation.

Like SAM, S-adenosylhomocysteine levels regulate enzymes involved in methionine metabolism. The major effect of elevated levels of SAH is inhibition of transmethylation reactions (Cantoni, 1985). However, SAH may also activate cystathionine β -synthase by binding to the same site as SAM (Finkelstein and Martin, 1984). SAH's effect on the remethylation pathway is less clear. SAH activates methylenetetrahydrofolate reductase, when SAM levels are normal, providing a

Figure 1.2: Allosteric regulation of methionine metabolism by S-adenosylmethionine. The positive and negative allosteric regulation sites are represented by + and – symbols, respectively.



mechanism for increased substrate production for methionine synthase (Kutzbach and Stokstad, 1971). However, elevated levels of SAH inhibit methionine synthase. Furthermore, SAH only regulates betaine:homocysteine methyltransferase when SAM is normal (Finkelstein *et al.*, 1974). Clearly, the regulatory effects of SAH need additional investigation.

The level of cellular methyltetrahydrofolate may also act as a metabolic regulator. Hepatic levels of methyltetrahydrofolate are inversely proportional to the availability of methionine. Typically when methyltetrahydrofolate is elevated, methionine levels are low and thus need to be conserved (Cook *et al.*, 1984). In such a situation methyltetrahydrofolate inhibits glycine N-methyltransferase, thereby preventing the removal of the vital S-adenosylmethionine supply (Yeo and Wagner, 1994; Wagner *et al.*, 1985). In contrast, when methyltetrahydrofolate concentrations are low, a sign of excess methionine, the inhibition of glycine N-methyltransferase is removed, permitting the removal of excess SAM (Balaghi *et al.*, 1993).

1.12.3 Coordination of Regulatory Mechanisms

In 1984, Finkelstein and Martin attempted to investigate the role of each regulatory mechanism in an assay that would emulate the *in vivo* situation. To do so they developed an *in vitro* system which could simultaneously measure the reaction rates of the homocysteine metabolizing enzymes (i.e CBS, BHMT, and methionine synthase). These measurements provided an accurate picture of metabolic flux since the system was designed to mimic *in vivo* conditions. Furthermore, the model allowed

for independent variation of enzymes, substrates and related metabolites. The only discrepancy from the *in vivo* circumstance is the loss of any specific spatial relationship or compartmentalization that could occur in the cell. Obviously, their model was homogenous in nature.

In their first set of experiments, Finkelstein and Martin (1984) evaluated the effects of dietary methionine on the distribution of homocysteine between competing enzymes. In hepatic extracts prepared from control rats, methionine synthase, betaine-homocysteine methyltransferase and cystathionine β -synthase accounted for 27, 27, and 46% of the homocysteine consumed, respectively. Under low-protein conditions the percentage of homocysteine converted to cystathionine was decreased to 30%, while a corresponding increase was observed in flux through betaine-homocysteine methyltransferase. Conversely, under high-protein conditions 90% of available homocysteine was catabolized by cystathionine β -synthase.

To further investigate the pathways' adaptation to methionine excess, Finkelstein and Martin (1986) fed rats diets with varying levels of methionine. The transsulfuration flux was 12-times higher in the livers taken from rats fed a high-methionine diet. However, the activity of cystathionine β -synthase was only increased 2-fold. The elevated transsulfuration flux was inhibited when the concentration of substrates and regulators, such as methionine, S-adenosylmethionine, S-adenosylhomocysteine, and serine were reduced in the assay. In the low-substrate conditions there was an increase in remethylation reactions. Finkelstein and Martin concluded that the large increase in transsulfuration flux, in response to methionine

excess, was primarily due to increased substrate concentrations rather than changes in enzyme levels.

1.13 Redox Regulation

Studies with purified mammalian methionine synthase and cystathionine β -synthase have revealed that both enzymes are oxidatively sensitive, leading to the hypothesis that redox regulation of this pathway may have physiological significance. Methionine synthase was activated under reducing conditions since more cobalamin was kept in its kinetically active state (Chen *et al.*, 1995). When cystathionine β -synthase was incubated with a reducing agent, such as titanium citrate, enzyme activity decreased by 50% (Taoka *et al.*, 1998). This activity was normalized when the enzyme's heme was reoxidized. Since the product of transsulfuration, cysteine, is a glutathione precursor redox regulation of its supply is an attractive mechanism in response to changes in cellular redox potential.

Mosharov *et al.* (2000) examined metabolite production in HepG2 cells, to test whether redox changes serve any regulatory role under physiological conditions. Addition of H_2O_2 or t-butyl hydroperoxide increased cystathionine production by 2-fold. This increase in transsulfuration flux showed a dose-dependent response to both oxidants. It is important to note that increased flux was not due to alterations in methionine synthase or cystathionine β -synthase protein levels. Furthermore, the concentration of cellular glutathione was increased following t-butyl hydroperoxide incubation with about half of the glutathione's cysteine being derived from

homocysteine. This is consistent with early work performed by Krebs, who showed that methionine supply was vital in maintaining hepatic cellular glutathione (Nina *et al.*, 1978). Thus activation of the transsulfuration pathway may provide an adaptive mechanism to deal with oxidative stress.

1.14 Importance of Homocysteine in Health and Disease

Over 30 years ago, McCully described a child who was homocysteinuric, secondary to a defect in vitamin B12 metabolism. The child had arterial lesions that were similar to those observed in patients with cystathionine β -synthase deficiency (McCully, 1969). He proposed that elevated plasma homocysteine found in homocysteinuric patients was responsible for the development of vascular disease. In 1976, Wilcken and Wilcken presented work supporting the homocysteine theory of arteriosclerosis. They found evidence of abnormal methionine metabolism in patients with unexplained and premature heart disease. These findings have sparked over 2 decades of intense research. In the past decade alone, over 110 studies have investigated the relationship between vascular disease and total homocysteine, defined as the sum of free, oxidized and protein-bound forms. Normal total plasma concentrations of homocysteine are generally in the range of 5 to 15 μM . While a consensus on precise cut off values has yet to be established, mild hyperhomocysteinemia may be defined as concentrations ranging from 15-25 μM , moderate between 25-50 μM and severe > 50 μM (Green and Jacobsen, 1995)

In 1995 a meta-analysis of 27 studies showed that homocysteine was an independent, graded risk factor for occlusive vascular disease, including coronary, cerebral, and peripheral vessels. A 5 μM increment in total plasma homocysteine was associated with an increased risk of coronary heart disease of 60 and 80% for men and women, respectively. It was concluded that the same 5 μM increment was associated with the same risk as a 0.5 mM increase in cholesterol (Boushey *et al.*, 1995). In a more recent study, plasma homocysteine was measured in patients with confirmed coronary arterial disease with a mean follow-up time of 4.6 years (Nygard *et al.*, 1997). After this time, only 4% of those patients with plasma homocysteine <9.0 μM died, whereas patients with homocysteine >15 μM had a mortality rate of 25%. In the elderly Framingham study population, those in the highest quartile of plasma homocysteine demonstrated a two-fold increase in cardiovascular disease mortality compared to those in the lowest (Bostom *et al.*, 1999).

In recent years, the majority of the sulfur amino acid research has been directed to the precise mechanism whereby homocysteine may cause vascular lesions. The proposed deleterious effects of homocysteine have included increased platelet adhesion (Dardik *et al.*, 2000), oxidation of low-density lipoproteins (Voutilainen *et al.*, 1999), inhibition of nitric oxide synthase (Upchurch *et al.*, 1997), interference with endothelium-dependent vasodilation (Tawakol *et al.*, 1997), and even homocysteinylation of proteins (Jakubowski, 1999). To date, consensus on this subject has not been reached. It is clear that additional studies are required to pinpoint the mechanism(s) by which homocysteine induces atherosclerosis. It is also

abundantly clear that homocysteine may interact with a variety of systems, resulting in different outcomes. Of course, the possibility still exists that homocysteine is a marker for the actual atherogenic agent.

1.15 Renal Homocysteine metabolism

Increased plasma homocysteine is a common finding in human patients with end-stage renal disease (ESRD) (Bostom and Lathrop, 1997). Furthermore, these patients have a severely reduced life expectancy, mainly because of increased risk of atherothrombotic vascular disease. The level of plasma homocysteine is inversely related to the glomerular filtration rate (GFR) (Arnadottir *et al.*, 1996). This dependency is observed in normal subjects, in ESRD patients, and even extends to hyperfiltering diabetic patients (Wollesen *et al.*, 1999). Of course, a change in GFR is only one determinant of plasma homocysteine in renal disease. As one might expect, folate and vitamin B6 status are major concerns for ESRD patients, due to increased loss following dialysis (Descombes *et al.*, 1993). However, superphysiological doses of B-vitamins, while normalizing vitamin status, only partially reduce plasma homocysteine and many patients still have levels greater than normal subjects (Bostom *et al.*, 1996). Indeed, the renal handling of homocysteine is an important topic and much work has been carried out on how the human and rat kidneys metabolize plasma homocysteine.

As mentioned earlier, various forms of homocysteine are present in plasma. In humans, about 80% of homocysteine is protein bound with the remaining 20%

consisting of mixed disulfides, homocystine and reduced homocysteine (Green and Jacobsen, 1995; van Guldener and Robinson, 2000). In contrast, rat plasma consists only of 20-30% protein-bound homocysteine (House *et al.*, 1998). The fraction of free homocysteine is important since protein-bound homocysteine is not available for filtration by the glomerulus. Homocysteine filtered by the glomerulus is almost completely reabsorbed by tubular cells with only 1% of the filtered load being excreted (House *et al.*, 1998). Studies performed *in vivo* in our laboratory showed that rat kidneys have substantial capacity for reabsorbing homocysteine. In fact, when plasma homocysteine was elevated 6-fold, either by homocysteine infusion or exposure to nitrous oxide, there was a proportional increase in filtered homocysteine; however the percentage of total homocysteine excreted in the urine was unchanged (House *et al.*, 1998). Taken together with human data, impairment of homocysteine excretion cannot account for hyperhomocysteinemia in end-stage renal disease.

Our laboratory has shown, in rats, that approximately 20% of the arterial homocysteine was removed on passage through the kidney (House *et al.*, 1998). Since the excretion of homocysteine is negligible in healthy rats, the kidney appears to be a major site for removal and metabolism of plasma homocysteine. Furthermore, when isolated renal cortical tubules were incubated with homocysteine, only 2% was converted to methionine (House *et al.*, 1997). The production of cystathionine, and subsequently cysteine, accounted for the greater part of the homocysteine metabolized. In addition, the flux through the transsulfuration pathway, and thus the disappearance of homocysteine, was enhanced by serine.

In contrast to rats, normal fasted humans have no significant arteriovenous difference in homocysteine concentration across the kidney (van Guldener *et al.*, 1998). The very large free fraction of homocysteine in rat plasma, as compared to humans, may explain why rats have increased homocysteine uptake and subsequent metabolism. The renal complement of enzymes is another striking difference between humans and rats. While rat kidney has methionine synthase, most of the homocysteine removal occurs via the transsulfuration pathway (House *et al.*, 1997). Humans, however, have significant levels of betaine:homocysteine methyltransferase (McKeever *et al.*, 1991). Even more important is the low renal activity of cystathionine β -synthase, although the human kidney does express CBS mRNA (Sturman *et al.*, 1970; Quere *et al.*, 1999). It is apparent that, while rat kidneys metabolize homocysteine via the transsulfuration pathway, humans remove homocysteine, if it happens at all, via remethylation. It is curious that human kidneys do contain cystathionine γ -lyase suggesting that this enzyme has a metabolic role independent of the synthase, such as in cysteine metabolism (Sturman *et al.*, 1970). Of course cystathionine γ -lyase may metabolize cystathionine imported from the plasma. Although it is clear that hyperhomocysteinemia occurs in end-stage renal disease, indisputable evidence for the removal of homocysteine by human kidneys has yet to be documented.

1.16 Thyroid hormone in humans

Hypothyroid patients have an increased risk for cardiovascular diseases (Vanhaelst *et al.*, 1967). Autopsy studies have shown that the atherosclerotic process is twice as common in hypothyroid patients compared with normal subjects, and is decreased in hyperthyroidism (Steinberg, 1968; Grytting and Salvesen, 1957). The increase in cardiovascular disease in hypothyroidism has been linked to lipid abnormalities and hypertension, such as increased concentration of plasma low-density lipoprotein cholesterol and elevated diastolic blood pressure (Williams and Braunwald, 1984; Streeten *et al.*, 1988). However, these factors do not explain atherogenesis in all hypothyroid patients, suggesting that another mechanism may be involved.

There are now consistent reports that thyroid status can influence plasma homocysteine levels in humans. Nedrebo *et al.* (1998) observed that total plasma homocysteine concentrations were elevated in hypothyroid patients versus controls, while hyperthyroid patients tend to have lower plasma homocysteine levels. Furthermore, Hussein *et al.* (1999) determined that restoration of euthyroidism, by L-thyroxine treatment, normalized plasma homocysteine; however the mechanism by which thyroxine elicits its effects is currently under debate. Altered vitamin status in hypothyroid patients is one possibility since retention of liver folate has been observed in the hypothyroid rat (Stokstad *et al.*, 1980). Patients with hyperthyroidism tend to have higher plasma folate levels than do hypothyroid patients (Nedrebo *et al.*, 1998, Diekman *et al.*, 2001). Functional riboflavin deficiency has also been reported

in the hypothyroid rat, with decreased levels of FAD and FMN observed in the liver (Rivlin, 1970). This deficiency has been associated with decreased activities of many riboflavin-dependent enzymes. It is not known, however, if these defects are related to homocysteine metabolism. In a longitudinal study, Nedrebo *et al.* (2001) reported that folate and cobalamin status could not explain the change in plasma homocysteine following thyroxine treatment. Furthermore, they observed parallel changes in serum creatinine concentrations, suggesting that changes in renal function rather than vitamin status may account for the altered plasma homocysteine levels. Both animal and human studies have demonstrated that hypothyroid patients tend to have low glomerular filtration rates, while hyperthyroidism is associated with an elevated GFR (Capasso *et al.*, 1999; Kreisman *et al.*, 1999). Although evidence suggests that altered renal homocysteine handling may be responsible for the changes in plasma homocysteine observed in hypothyroidism, it is still plausible that altered metabolism in other tissues may contribute to this change.

1.17 Diabetes Mellitus

Vascular disease is the scourge of any diabetic patient. Diabetics are two, two to ten, and five to ten times more likely than healthy individuals to have strokes, myocardial infarctions, and peripheral vascular problems, respectively (Ellenberg and Rifkin, 1990). The survival rate following a stroke or myocardial infarction is also much lower in diabetics compared to non-diabetics (Smith *et al.*, 1984; Oppenheimer *et al.*, 1985). Many factors contribute to accelerated atherosclerosis in diabetics

including genetic pre-disposition and diet. In addition, the amplifying role of other risk factors, such as high blood pressure, smoking, and high plasma cholesterol has been documented (Ellenberg and Rifkin, 1990). As homocysteine is a recognized risk factor for vascular disease, much attention has been given to the possible relationship between diabetes mellitus and plasma homocysteine.

In 1991, Hultberg *et al.* were the first to report altered homocysteine metabolism in diabetic patients. They observed that Type 1 diabetics, who showed signs of renal damage, had elevated plasma homocysteine compared to diabetic patients without renal complications. In their follow-up study, Hultberg *et al.* (1997) reported that Type 1 adult patients, who had the longest duration of disease and poorest metabolic control, were the most prone to increased levels of plasma homocysteine. Similar relationships were not observed in adolescent diabetic patients. However, the duration of disease in those patients was far shorter than in the adult study. Hyperhomocysteinemia is not normally observed in diabetic patients until the onset of renal disease or microalbuminuria (Pavia *et al.*, 2001; Chiarelli *et al.*, 2000). Furthermore, Robillon *et al.* (1994) observed that the Type 1 diabetic patients, without cardiovascular disease or renal impairment, had decreased plasma homocysteine compared to healthy individuals. This result was confirmed in a larger study by Cronin *et al.* (1998). These studies could not provide an explanation for the subnormal levels of plasma homocysteine; however the possible regulation of homocysteine metabolism by insulin could afford an explanation.

1.18 Objectives of Investigation

Clinical studies on diabetics and hypothyroid patients have suggested that insulin and thyroid hormone may have a role in regulating homocysteine metabolism. To date, no studies have been undertaken to investigate possible mechanism(s), if any exist, by which insulin and thyroid hormone regulate homocysteine metabolism. We therefore completed a series of studies with the following questions in mind:

1. Does the streptozotocin-diabetic rat mimic human type 1 diabetes mellitus with respect to plasma homocysteine?
2. What role does insulin play in regulating homocysteine metabolism? What is/are the mechanism(s) of action?
3. Does hyperglucagonemia, a common characteristic of diabetes mellitus, have a role in regulating homocysteine metabolism?
4. Does the hypothyroid rat mimic human hypothyroidism with respect to plasma homocysteine? What role, if any, does thyroid hormone play in regulating homocysteine metabolism.

Chapter 2: Methods and Materials

2010 2011 2012 2013 2014

2.1 Chemicals

All chemicals were of the highest quality available and were purchased from Sigma (Oakville, ON, Canada), except where noted in the text.

2.2 Animal treatment

Male Sprague-Dawley rats (supplied by the University's breeding colony) weighing between 200-300 grams were fed *ad libitum* either an AIN 93 growth diet or laboratory chow, and had free access to water. The rats were exposed to a 12-hour light: 12-hour dark cycle beginning with lights on at 8 am. All studies were conducted immediately following termination of the dark cycle. All procedures were approved by Memorial University's Institutional Animal Care Committee and are in accordance with the principles and guidelines of the Canadian Council on Animal Care.

2.3 Enzyme assays

Kidneys and liver were rapidly removed from an anaesthetized rat (65 mg/kg sodium pentobarbital, i.p.) and placed in ice-cold 50 mM potassium phosphate buffer, pH 6.9. Tissues were diluted (5:1 vol/wt) with the phosphate buffer, then homogenized with a Polytron homogenizer (Brinkman Instruments, Toronto, Canada) for 20 seconds at 50% output. The homogenates were centrifuged at 18,000g at 4°C for 30 minutes and the supernatant retained. All enzyme assays were carried out on this 18,000g (postmitochondrial) supernatant. Assays were shown to be linear with

respect to time and protein before use in any experiment. The conditions used for each enzyme assay are given in Table 2-1.

2.3.1 Methionine adenosyltransferase

Methionine adenosyltransferase activity was determined using the assay developed by Mudd *et al.* (1965). The incubation mixture consisted of 133 mM Tris-HCl (pH 7.6), 177 mM KCl, 266 mM MgCl₂, 16 mM ATP, 3.5 mM glutathione, 0.26 mM S-adenosylmethionine, 0.131 mM ¹⁴CH₃-methionine (150 x 10³ dpm), and cytosol. The reaction was started with the addition of cytosol and incubated at 37°C for 30 minutes in a final volume of 0.45 ml. The reaction was stopped by the addition of ice-cold water to a final volume of 11.5 ml. A 5 ml-aliquot of the resulting solution was added to a column (0.9 x 2.0 cm) of Dowex 50-x4 (NH₄⁺). The column was washed with 10 ml of water and the radioactive S-adenosylmethionine was eluted with 10 ml 3 N NaOH. A sample was taken and radioactivity measured. The value obtained from incubation with heat-inactivated enzyme was used as a blank. Linearity with time and protein was demonstrated previously in our laboratory (Stead, unpublished data).

2.3.2 Glycine N-methyltransferase

This assay is a modified version of Wagner's method (Cook and Wagner, 1984). The reaction mixture (1 ml) consisted of 0.2 mM [³H-CH₃]-S-adenosylmethionine (1.0 mCi/mmol), 10 mM Tris-HCl buffer (pH 9.0), 0.5 mM

Table 2-1: Summary of enzyme assay conditions of time and protein for both liver and kidney samples.

	Liver		Kidney	
	Time (min)	Protein (mg)	Time (min)	Protein (mg)
Methionine Adenosyltransferase	30	0.75	nd	nd
Glycine N-methyltransferase	20	0.15	nd	nd
Cystathionine β -synthase	40	0.3	30	0.4
Cystathionine γ -lyase	10	1.5	10	3
Methionine Synthase	30	1.0	30	0.75
Betaine:homocysteine methyltransferase	30	0.3	nd	nd
Methylenetetrahydrofolate reductase	15	0.15	15	0.1

Assay conditions are given in the text. All assays were carried out at 37°C, except that for glycine N-methyltransferase which was at 25 °C. nd- activity not determined.

dithiothreitol (DTT), 0.2 mM glycine and cytosol. Following the addition of glycine, to start the reaction, samples were incubated at 25°C for 15 mins at which point the reaction was stopped by the addition of 50 µl of ice-cold 10% w/v TCA. 250 µl of an acid-washed charcoal suspension (38 mg/ml, in 0.1 M acetic acid) was added to bind all unreacted S-adenosylmethionine. The samples were incubated on ice for 15 mins and then spun for 10 mins at 3200g in a Brinkman benchtop centrifuge. 200 µl of the supernatant was added to 10 ml of scintillation cocktail and the radioactivity determined. This assay was shown to be linear between 50-250 µg of cytosolic protein and up to 30 mins of incubation time (Williams, 1998).

2.3.3 Cystathionine β-Synthase

Cystathionine β-synthase was measured by the method of Miller *et al.* (1994). Incubation mixtures (120 µl) consisted of 16.6 mM serine, 0.83 mM EDTA, 0.42 mM pyridoxal 5-phosphate, 33 mM d,l-homocysteine and 0.42 mM propargylglycine in 1 M Tris buffer, pH 8.4. The reaction was started with the addition of cytosol, incubated at 37°C, and stopped with the addition of 100 µl of 10% SSA. Each sample was then centrifuged at 14,000g for 5 mins. Cystathionine concentrations in the supernatants, following neutralization, were determined by HPLC. Linearity with respect to time and protein are shown for the liver in Figure 2.1 and the kidney in Figure 2.2.

Figure 2.1: Dependence of cystathionine production by hepatic cystathionine β -synthase on time and protein concentration. Linearity with respect to protein was determined using a 30 minute time period. The time curve was obtained using 0.3 mg of protein. Typical graph is shown.

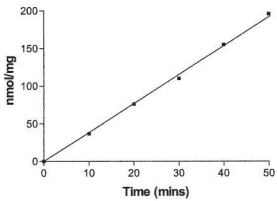
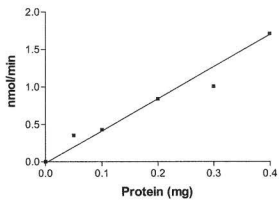
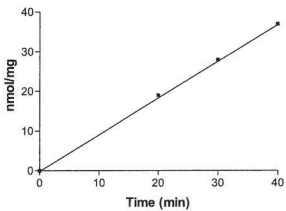
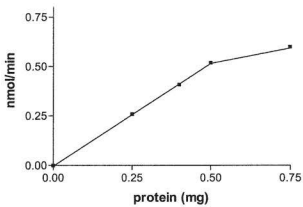


Figure 2.2: Dependence of cystathionine production by renal cystathionine β -synthase on time and protein concentration. Protein linearity was evaluated using a 30 minute time period. The time curve was obtained using 0.3 mg of protein. Typical graphs are shown.



2.3.3.1 Determination of cystathionine and methionine by HPLC

Concentrations of methionine and cystathionine were determined using a Waters automated HPLC system which includes the following equipment: a Model 600E System Controller, a Model 712 WISP sample injector, a Model 420 Fluorescence Detector, and a 743 Data Module. Following derivatization with O-phthalaldehyde (OPA), the samples were injected into a Hypersil ODS-C18 column (4.6 x 150 mm). The chromatographic conditions were described previously by Jones and Gillingham (1983). Buffer A contained 0.05 M sodium acetate and 0.05 sodium phosphate (pH 7.5), which was mixed with methanol and tetrahydrofuran in a ratio of 96:2:2, respectively. Buffer B contained methanol and water in a ratio of 65:35. The amino acids were resolved using a 47-minute linear gradient from 100% buffer A to 100% buffer B at a flow rate of 1.5 ml/min. The fluorescence intensities were measured with excitation at 334 nm and emission at 425 nm. The level of methionine and cystathionine was calculated by comparing the area under the peak with that of AEC (aminoethyl-cysteine), an internal standard.

2.3.4 Cystathionine γ -Lyase

Cystathionine γ -lyase activity was measured using the method of Stipanuk (1979). Cytosolic protein was added to a 2 ml cocktail consisting of 100 mM potassium phosphate (pH 7.5), 4.0 mM cystathionine, 0.125 mM pyridoxal 5-phosphate, 0.32 mM NADH, and 1.5 units of lactate dehydrogenase (LDH). In this

assay, α -ketobutyrate serves as a substrate for LDH. Therefore, NADH oxidation is measure of α -ketobutyrate production by cystathionine γ -lyase. The decrease in optical density at 340 nm was monitored spectrophotometrically at 37 °C. All measurements were taken from the linear portion of the graph. Blank reactions were performed using a cystathionine-free cocktail. Linearity with respect to protein is shown for the liver in Figure 2.3 and the kidney in Figure 2.4.

2.3.5 Methylene tetrahydrofolate reductase

Methylene tetrahydrofolate reductase activity was determined radiochemically by the method of Engbersen *et al.* (1995). The reaction cocktail (600 μ l) consisted of 0.18 M potassium phosphate buffer (pH 6.8), 1.15 mM EDTA, 11.5 mM ascorbic acid, 54 μ M FAD, 20 μ M [14 C-CH $_3$]methyltetrahydrofolate (0.20 μ Ci), 3.5 mM menadione, and cytosolic protein (100 μ l). A zero-time point was used as a blank for this assay. The reactions were started with the addition of menadione and incubated in the dark at 37°C, and stopped by the addition of 10 μ l of 1.0 M formaldehyde, 50 μ mol dimedone in 200 μ l ethanol:water (1:1), and 100 μ l 3.0 M potassium acetate, pH 4.5. The test tubes were heated at 95 °C for 15 mins then cooled in an ice-bath for 10 mins. 3 mls of toluene were added to the tubes, mixed vigorously, centrifuged at low speed for 5 mins, and then 2.0 ml of the toluene phase was taken for measurement of radioactivity. Linearity with time and protein is presented for the liver in Figure 2.5 and in the kidney in Figure 2.6.

Figure 2.3: Dependence of hepatic cystathionine γ -lyase on protein concentration. The activity was evaluated over a 10 minute period. A typical graph is shown.

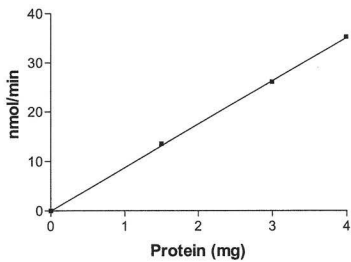


Figure 2.4: Dependence of renal cystathionine γ -lyase on protein concentration.

The activity was evaluated over a 10 minute period. A typical graph is shown.

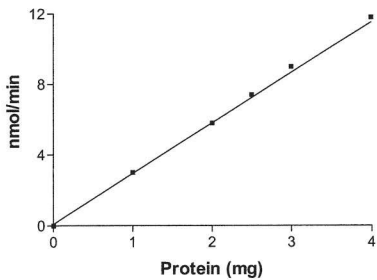


Figure 2.5: Linearity with protein concentration and time for hepatic methylenetetrahydrofolate reductase activity. The protein curve was obtained using a 15 minute time period. The linearity with respect to time was determined using 0.15 mg of protein. Typical graphs are shown.

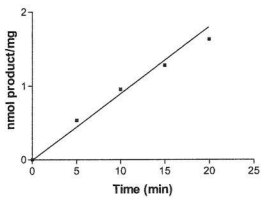
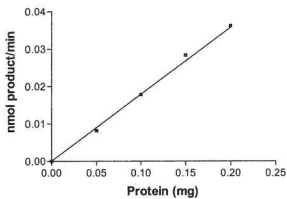
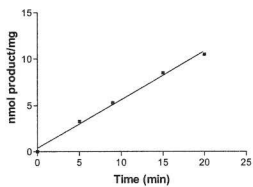
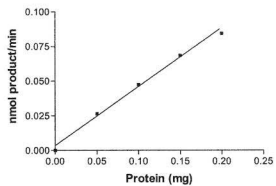


Figure 2.6: Linearity with protein concentration and time for renal methylenetetrahydrofolate reductase activity. The protein curve was obtained using a 15 minute time period. The linearity with respect to time was determined using 0.10 mg of protein. Typical graphs are shown.



2.3.6 Methionine synthase

Methionine synthase was measured using the method described by Koblin *et al.* (1981). The reaction mixture consisted of 87.5 mM potassium phosphate buffer (pH 7.5), 7.5 mM homocysteine, 29 mM dithiothreitol, 0.25 mM S-adenosylmethionine, 10 μ M cyanocobalamin, 7 mM β -mercaptoethanol, 0.5 mM [5- 14 C]methyltetrahydrofolate (0.25 μ Ci), and cytosolic protein. The reaction was started by addition of cytosol and was then gassed with nitrogen, capped, and incubated in the dark at 37°C. The reactions were stopped with 0.5 ml of ice-cold water and immediately added to the top of a Biorad AG1-X8 (200-400 mesh) column (Cl⁻). The column was washed 3 times with 0.5 ml water. The resulting effluent was added to 10 ml Scintiverse and the radioactivity measured. Linearity with respect to time and protein are shown for the liver (Figure 2.7) and kidney (Figure 2.8).

2.3.7 Betaine:homocysteine methyltransferase

Betaine:homocysteine methyltransferase activity was measured using a method developed by Wang *et al.* (1991) with modifications. The reaction mixture (200 μ l) contained 75 mM sodium phosphate (pH 7.4), 3.25 mM betaine, 10 mM d,l-homocysteine, and cytosol. Samples were incubated at 37°C and stopped by the addition of 100 μ l of 10% SSA. The samples were kept on ice for 10 mins, then centrifuged for 5 mins at 14,000 rpm. The supernatant was retained and methionine measured by HPLC, as described in section 2.3.3.1. Linearity with respect to time and protein are shown in Figure 2.9.

Figure 2.7: Dependence of ^{14}C -methionine production by hepatic methionine synthase on time and protein concentration. The protein curve was obtained using a 30 minute incubation period. The time curve was determined using 1.0 mg of protein. Typical graphs are shown.

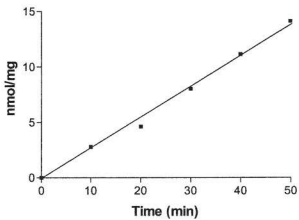
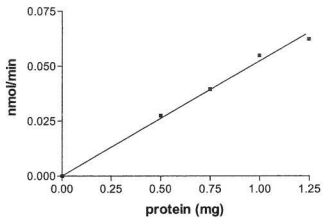


Figure 2.8: Dependence of ^{14}C -methionine production by renal methionine synthase on time and protein concentration. The protein curve was determined using a 30 minute incubation period. The time curve was obtained using 0.75 mg of protein. Typical graphs are shown.

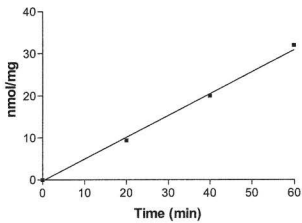
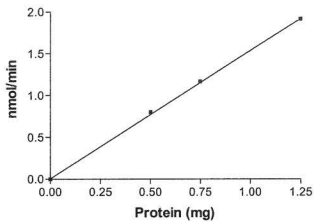
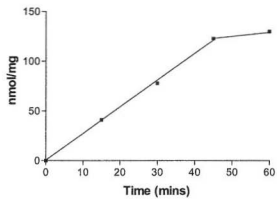
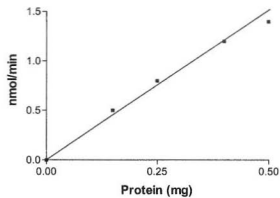


Figure 2.9: Dependence of methionine production by betaine:homocysteine methyltransferase on protein and time. The protein curve was obtained using a 30 minute incubation period. Linearity with respect to time was demonstrated using 0.3 mg of protein. Typical curves are shown.



2.4 Plasma hormones, glucose and creatinine

Blood samples were collected from the abdominal aorta of anaesthetized rats (65 mg/kg sodium pentobarbital, i.p.). Heparinized tubes containing the blood samples were placed on ice until plasma was separated by centrifugation in a clinical centrifuge (15 mins, 3700 x g). The plasma was then frozen (-20°C) for later use. Plasma levels of total T₃ and free T₄ were measured by the St. John's General Hospital clinical laboratory (autoDELFA kit, Pharmacia, Baie d'Urfe, Que.). Insulin and glucagon levels were measured by Linco Research Inc. (St. Charles, MO) using rat insulin and glucagon, respectively, as standards. Glucose (Bergmeyer *et al.*, 1981) and creatinine (Sigma kit) concentrations were determined using standard methods.

2.5 Total homocysteine measurements

Total homocysteine concentrations were determined by the method of Vester and Rasmussen (1991). 20 µl of 10% tri-n-butyl-phosphine in dimethylformamide was added to 150 µl of sample (plasma, liver, or hepatocytes). Following a 30 minute (4°C) reduction period, the samples were deproteinized by the addition of 125 µl of 0.6 M perchloric acid. 50 µl of 0.2 mM 8-aminonaphthalene-1,3,6-trisulfonic acid was added and the tubes centrifuged at 14,000g (Brinkman Instruments, Rexdale, ON, Canada) for 5 min (4°C). Then, 100 µl of the supernatant was added to an Eppendorf tube containing 200 µl of 2 M potassium borate, pH 10.5, containing 5 mM EDTA and 100 µl of ammonium 7-fluorobenzo-2-oxo-1,3-diazole-4-sulphonate solution (1.0 g/l in 2 M potassium borate, pH 9.5). Following an incubation period (60°C, 60 min),

the samples were cooled in an ice bath (15 mins), at which point 20 μ l of the derivatized sample was analysed by HPLC.

2.5.1 HPLC apparatus and conditions

A Man-Tech Shimadzu system (Pump GT-104, SCL-10A Controller, SIL-10A Auto Injector, LC-10AD Liquid Chromatograph, RF-535 Fluorescence Monitor) equipped with a Phase Separations Hypersil C18-ODS analytical column (4.6 x 150 mm) was used to measure homocysteine. The fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm. The detection signal was recorded and peaks quantified using a Man-Tech Shimadzu CR501 Chromatopac integrator.

The chromatographic conditions were modified from those used by Vestor and Rasmussen (Vestor and Rasmussen, 1991). The buffers consisted of 0.1 M acetate (pH 4.0) mixed with 20 or 200 ml/L methanol for solution A and B, respectively. These buffers were filtered through a 0.45 μ m Millipore filter before use. A linear gradient was run from buffer A to buffer B over 12.5 minutes, then 2.5 minutes buffer B, a three minute linear gradient back to buffer A, and finally 2 minutes with buffer A, at a flow rate of 1.00 ml/min.

2.6 Amino acid analysis

Plasma and liver amino acids were determined by the Department of Biochemistry's amino acid facility. Plasma or freeze-clamped liver were first deproteinized with 10% sulfosalicylic acid. Following centrifugation, the resulting

supernatant was adjusted to pH 2.2 with lithium citrate buffer. The samples were then analyzed on a Beckman 121 MB Amino Acid Analyser using Benson D-X, 0.25 Cation Xchange Resin and a single column, three buffer lithium method as per Beckman 121MB-TB-017 application notes. Results were quantitated using a Hewlett Packard Computing Integrator Model 3395A.

2.6.1 Calculation of intracellular hepatic amino acid concentrations

Intracellular hepatic amino acid concentrations were calculated and are reported in Chapter 5 using published data from our laboratory on hepatic cellular spaces (Qian and Brosnan, 1996). The measured hepatic concentration for an amino acid is the sum of the amino acid in the intracellular fluid (ICF) and the amino acid in the extracellular fluid (ECF). The following equation was used to determine the hepatic intracellular concentration of an amino acid.

$$\text{Total liver AA (nmol/g)} = [\text{Plasma AA (nmol/ml)} \times \text{ECF (ml/g liver)}] + \text{Intracellular AA (nmol/ml)} \times \text{ICF (ml/g liver)}$$

Intracellular water was previously found to be 0.45 ml/g liver while the extracellular water was 0.25 ml/g liver. These values were obtained in control rats. It should be noted that glucagon, *in vitro*, has been shown to decrease hepatocyte volume by 5-10%, we did not take this into account (vom Dahl *et al.*, 1991).

2.7 S-Adenosylmethionine and S-adenosylhomocysteine determination

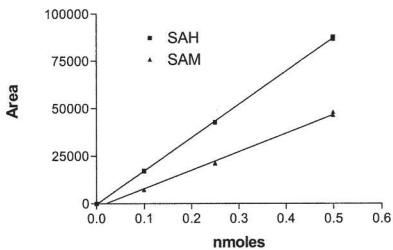
S-Adenosylmethionine and S-adenosylhomocysteine were measured in the liver using the method of Wagner with minor modifications (personal

communication). Freeze-clamped liver was quickly added to 5 volumes of 8% ice-cold trichloroacetic acid. Samples were homogenized with a Polytron for 10 seconds at 20% output and were placed on ice for 10 minutes. The samples were then centrifuged for 10 minutes at 13,000g. The supernatant was retained and analysed by HPLC using a VYDAC C18 column (#2187P54) that was equilibrated with 96% 50 mM NaH_2PO_4 , 10 mM heptane sulfonic acid (adjusted to pH 3.2 with concentrated sulfuric acid) and 4% acetonitrile. A 15 minute gradient from 4% to 20% acetonitrile was used to separate SAM and SAH. Peaks were monitored by UV detection at 258 nm and quantified using a 3390A Hewlett Packard Integrator. The concentration of SAM and SAH was calculated using a standard curve shown in Figure 2.10.

2.8 Isolation of Primary Hepatocytes

Primary rat hepatocytes were isolated via the method of Berry *et al.* (1991). An anaesthetised rat was injected with 100 μl heparin (1000 units/ml) through the femoral vein. A cannula was then inserted into the portal vein and perfusion commenced immediately with 500 ml calcium-free Krebs-Henseleit medium (144 mM Na^+ , 6 mM K^+ , 1.2 mM Mg^{2+} , 126 mM Cl^- , 1.2 mM H_2PO_4^- , 1.2 mM SO_4^{2-} , 25 mM HCO_3^-) containing 2 mM EGTA, 20 mM glucose, 2.1 mM lactate, 0.3 mM pyruvate (pH 7.4). The flow rate was 40 ml/minute and the medium was gassed with 19:1 O_2/CO_2 for 20 minutes before use. Following a 12 minute flow through period, the medium was switched to 500 ml Krebs-Henseleit medium containing 1.3 mM Ca^{2+} , 20 mM glucose, 2.1 mM lactate, and 0.3 mM pyruvate (also gassed for 20 min

Figure 2.10: Standard curve of S-adenosylmethionine and S-adenosylhomocysteine. The graph illustrates that both S-adenosylmethionine ($y = 97199X - 1925$) and S-adenosylhomocysteine ($y = 174900X - 426$) were linear between 0 and 0.5 nanomoles. A typical graph is shown.



before use). A cannula was inserted through the right atrium and into the inferior vena cava. Krebs-Henseleit medium supplemented with 0.25 % BSA and collagenase (50 mg/100 mL) was then recirculated through the liver. To start the flow through the liver the inferior vena cava was cut below the kidney. The recirculation of perfusate continued until the liver became soft (15-20 minutes), at which point the liver was transferred to a petri dish containing collagenase medium and massaged gently to liberate cells. The resulting suspension was incubated at 37°C for 10 minutes in a shaking water bath under constant gassing (19:1 O₂/CO₂). Following filtration through a cheesecloth, the cells were centrifuged at 600 rpm for 2 minutes and the cells resuspended in Krebs-Henseleit medium containing calcium, and the process repeated. A final wash with Krebs-Henseleit medium containing 2.5% BSA was completed, and the cell pellet was resuspended in this medium. Hepatocytes were quantified by drying 3.0 ml of resuspended cells and 3.0 ml of 2.5% BSA Krebs-Henseleit medium in tared vessels at 50°C for 24 hours. The difference in the weights represented the dry weight of the cells. Cell viability was determined by 0.1% trypan blue exclusion.

2.8.1 Homocysteine export from isolated hepatocytes

Hepatocytes were preincubated for 20 minutes at 4-6 mg dry weight of cells/ml (1ml final volume) in Krebs-Henseleit medium containing 1.25 % (w/v) BSA. Following preincubation, 1 mM methionine with or without 1 mM serine was added and cells were incubated for another 30 minutes. Cells were gassed with 19:1 O₂/CO₂

at the beginning of preincubation and at the addition of substrates. At the end of the incubation, the contents of the flasks were immediately centrifuged at 14,000g for 30 seconds to sediment the cells. The supernatant, containing any exported homocysteine, was then frozen at -20°C until analysis (Stead *et al.*, 2000).

2.8.2 $^{14}\text{CO}_2$ production by isolated hepatocytes

The second step of the transsulfuration pathway involves the production of α -ketobutyrate. Incubation with $[1-^{14}\text{C}]$ methionine will give rise to $[1-^{14}\text{C}]\alpha$ -ketobutyrate. $[1-^{14}\text{C}]\alpha$ -Ketobutyrate is a substrate for pyruvate dehydrogenase, which will release $^{14}\text{CO}_2$. Label in unmetabolised $[1-^{14}\text{C}]\alpha$ -ketobutyrate can be released with hydrogen peroxide. The sum of these two numbers can be used as a measure of flux through the transsulfuration pathway.

Hepatocytes were incubated with 1 mM L- $[1-^{14}\text{C}]$ methionine (American Radiolabeled Chemicals, Inc., St. Louis, MO) in the presence and absence of propargylglycine (an inhibitor of cystathionine γ -lyase) and α -cyanocinnamate (an inhibitor of the mitochondrial α -ketoacid transporter). Following the addition of methionine, flasks were incubated for 30 minutes, after which they were equipped with rubber septa with suspended plastic centre-wells containing NCS tissue solubilizer. Incubations were terminated with 0.3 ml 30% (wt/vol) perchloric acid, added through the septa with a syringe, and $^{14}\text{CO}_2$ was collected for 1 hour. The centre-wells were added to scintillation vials containing Omnifluor scintillation fluid. A new centre-well was then added to each flask and 0.3 ml 30% (wt/vol) hydrogen

peroxide was added through the septum with a syringe. $^{14}\text{CO}_2$ was collected for 1 hour and measured as described above.

2.8.3 Methionine transport into isolated hepatocytes

Methionine transport into isolated hepatocytes was measured as described by Salter *et al* (1986). Cells were pre-incubated for 20 minutes and then 1- ^{14}C -methionine was added at a final concentration of 0.5 mM (400 dpm/nmole). At 5, 35, 65, 95, and 125 seconds following the addition of methionine, 1 ml aliquots were transferred to 1.5 ml microcentrifuge tubes containing 0.25 ml of silicone oil mixture (2:1 (v/v) Dow Corning 550 silicone oil and dinonyl phthalate) layered on top of 0.1 ml 6% (v/v) perchloric acid. The tubes were centrifuged at 14,000g for 15 seconds to sequester cells through the silicone oil and into the acid layer, leaving the extracellular component on top of the oil. Following centrifugation, the tubes were frozen in liquid nitrogen and then were cut through at the bottom of the silicone oil layer. The bottom layer, containing the intracellular ^{14}C -methionine, was placed in a scintillation vial containing 10 ml Omnifluor scintillation cocktail and radioactivity determined in a scintillation counter. The volume of extracellular space that was sequestered through the silicone oil was determined by measuring the bottom layer following parallel cell incubations with carboxyl- ^{14}C -inulin. This value was used to correct rates of methionine transport. Methionine transport rates were determined by subtracting 5 second rates, representing primarily amino acids binding to membranes, from the values obtained at subsequent time points.

2.9 Isolation of total RNA and Northern blot analysis

Total RNA was isolated from rat livers by the acid guanidinium thiocyanate-phenol-chloroform method as described by Chomczynski and Sacchi (1987). 15 µg of total RNA were separated on a 1% agarose gel containing formaldehyde and transferred to a Hybond-Nylon membrane (Amersham Life Sciences). cDNAs for cystathionine β-synthase, cystathionine γ-lyase, and β-actin (Clontech) were labeled with [α -³²P]dCTP using a random-labeling kit (Ambion). The probes (specific activity ~10⁹ dpm/ug) were allowed to hybridize to the membrane for 16 hours at 42°C in Northern Max™ ultrahybridization buffer (Ambion). Autoradiography was by exposure (8 hours to 3 days) of the blot to Kodak XAR film. The results were analyzed by densitometry of the bands on autoradiograms. The levels of cystathionine β-synthase and cystathionine γ-lyase mRNA were normalised to the amount of RNA applied, using the housekeeping gene β-actin, by setting the ratio of cystathionine β-synthase mRNA to that of β-actin in the control animals to 1.

2.10 Cell Culture

H4IIE rat hepatoma cells (American Tissue Culture Collection, Rockville, MD) were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% v/v Fetal Bovine Serum (FBS), 100 units/ml penicillin and 100 µg/ml of streptomycin (Canadian Life Technologies, Burlington, ON) under 5% CO₂/95% O₂ in a humidified incubator at 37°C until they reached near confluency (70%). Cells were

made quiescent by an overnight incubation with medium containing 0.5% FBS. Prior to the addition of hormones (triamcinolone, insulin, glucagon), cells were washed once with DPBS, then the fresh medium containing 0.5% FBS was replaced. The plates were incubated at 37°C for various time periods ranging from 0 to 24 hours. Cell viability was monitored by trypan blue exclusion.

2.10.1 Reverse transcriptase/Polymerase Chain Reaction (RT-PCR)

2 µg of total RNA were reverse transcribed using a one step reverse transcription kit (Qiagen) and amplified for 30 cycles. The upstream primer corresponding to 41 to 53 base pairs (5'-GCCTTCAGGACATCCAGTGT-3') and a down stream primer (5'-TCTTCCGGGTCTGCTCACGGGC-3') corresponding to 1661-1684 base pairs of the type 3 rat CBS cDNA (gift from Dr. J. Kraus) were used to amplify 1644 base pair PCR fragment. A 768 bp fragment of the rat β-actin was co-amplified using primers obtained from Clontech Laboratories (Palo Alto, CA). PCR products were separated on a 0.8% agarose gel and ethidium bromide-stained bands were visualized by UV illumination. Before transfer to a membrane (S and S nytran SuPer charge® nylon) the gel was immersed in 0.25 N HCl for 30 min, incubated in denaturing solution (1.5M NaCl, 0.5M NaOH) for 30 mins, rinsed with distilled water, soaked in neutralization buffer (0.5 M Tris-HCl, pH 7.0, 1.5 M NaCl), and washed in 10xSSC. The transferred DNA was immobilized by UV crosslinking (Hoefer UVC 500 UV crosslinker). The blots were simultaneously probed with ³²P labelled CBS cDNA, and radiolabelled β-actin cDNA probe (Clontech Laboratories,

Palo Alto, CA). Autoradiography was by exposure of the blot to Kodak XAR or Biomax film.

2.10.2 Western blot

At the end of the incubation with hormones, the medium was aspirated and cells were washed once with PBS. Cells were then lysed in sodium dodecyl sulphate (SDS) sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50mM dithiothreitol and 0.1% bromophenol blue), scraped off the plate and sonicated for 10-15 seconds to shear DNA and reduce viscosity. Protein concentration of the cell lysates was determined by BCA method (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard. Following a 5 minute (95-100°C) incubation period, protein bands were separated on a 12% SDS-PAGE and transferred to 0.2µ pore size nitrocellulose membranes (BioRad, Hercules, CA). Membranes were washed with blocking buffer (5% non fat dry milk in TBS containing 0.1% Tween-20), incubated with rabbit anti-CBS polyclonal antibody (1:5000 dilution in blocking buffer), rinsed, and incubated with anti-rabbit IgG conjugated to horse-radish peroxidase (1:2000 dilution in blocking buffer). The amount of CBS protein was determined by a chemiluminescent detection system containing hydrogen peroxide and LumiGLO reagent, followed by exposure of membranes to Kodak Biomax ML film.

2.11 Renal Homocysteine Flux

Studies were carried out to measure homocysteine removal by the kidneys. Previous work in our lab has shown that the kidneys are very important tissues in the removal of plasma homocysteine (House *et al.*, 1998). Two measurements are required to quantify renal homocysteine flux: an arterial-venous (A-V) difference for homocysteine and renal plasma flow. The A-V difference across the kidney for homocysteine gives only a qualitative picture and therefore requires quantification. When the A-V difference is multiplied by the plasma flow one has a quantitative measure of homocysteine uptake.

The methodology used was similar to that previously employed in Lowry *et al.* (1987) and more recently by House *et al.* (1998). Briefly, an anaesthetised rat was placed on a heating pad and the trachea was cannulated with a small piece (2.5 cm long x 2.5 mm ID) of polyethylene tubing. The right jugular vein was then catheterised with PE-50 tubing for the infusion of inulin. A priming dose of 1.75 μ Ci of [carboxyl- 14 C]inulin (New England Nuclear, Lachine, Quebec) in 0.8 ml of 10% mannitol-0.45% NaCl was given through the saphenous vein and followed by the continuous infusion of the same solution at a rate of 0.037 ml/min using a Harvard Apparatus model 975 compact infusion pump. The left ureter was then catheterised with a short piece of PE-10 tubing fitted inside a length of PE-50 tubing. Urine was collected between 20 and 40 minutes. At the end of the urine collection period, 1-ml blood samples were taken from the left renal vein and the abdominal aorta. The blood samples were quickly spun in an Eppendorf centrifuge and the plasma retained. An

aliquot of plasma (25 μ l) was used for the determination of inulin radioactivity. Samples of urine (10 μ l) were treated in a similar manner.

2.11.1 Calculations

Glomerular filtration rate (GFR) was calculated from urinary inulin excretion in the 20-40 min clearance period. Renal plasma flow was calculated using the expression derived by Wolf (1941). The calculations that are involved in the measurement of renal plasma flow and GFR are as follows:

1. U/P (inulin) = inulin counts in urine (dpm/ml) / inulin counts in arterial plasma (dpm/ml).
2. GFR (ml/min) = U/P (inulin) \times urine flow rate (ml/min); the rate of excretion of inulin is directly proportional to, and a linear function of, the plasma concentration of inulin.
3. RPF = [(urine (dpm/ml)) / (arterial plasma (dpm/ml) - renal venous plasma (dpm/ml))] \times urine flow rate

The values of GFR, and renal plasma flow, are multiplied by 2 (for 2 kidneys) and are expressed as ml/min/100 g body weight.

2.12 Statistical analysis

All results are expressed as means \pm standard deviation. Student's unpaired t-test was used to determine significant differences in studies with only two groups (Glucagon

and PTU studies). In studies involving three or more experimental groups (Diabetic and Thyroidectomy studies) one-way ANOVA, followed by Neuman-Keul multiple means comparison test, was used to compare data. A value $P < 0.05$ was taken to indicate a significant difference.

Chapter 3: Homocysteine Metabolism in the Hypothyroid Rat

3.1 Background

A recent study by Nedrebo *et al.* (1998) investigated homocysteine status in hypothyroid patients. They observed that total plasma homocysteine was elevated in hypothyroid patients compared with healthy controls. Hussein *et al.* (1999) observed that hypothyroid patients who received L-thyroxine treatment have normal plasma homocysteine. These observations are of particular importance since hypothyroid patients have an increased risk for cardiovascular diseases (Steinberg, 1968), which is often attributed to elevated low-density lipoprotein cholesterol. However, altered lipid levels do not fully explain the accelerated pathogenesis (Masaki *et al.*, 1992; Ishikawa *et al.*, 1989, Mamiya *et al.*, 1989).

The present study was undertaken to investigate the role of thyroid hormones on homocysteine metabolism in the rat. To this end, we utilised two hypothyroid models.

3.2 The Hypothyroid Rat

Our first hypothyroid model was the propylthiouracil-treated rat. Propylthiouracil, a thiocarbamide drug, is a potent inhibitor of the thyroid peroxidase, an important enzyme involved in thyroid hormone synthesis. In this study, rats ($n=5$) received water containing 0.05% propylthiouracil (PTU) for 2 weeks, while a control group of ten rats received untreated water (Helton and Magner, 1994).

In our second study, thyroidectomized and sham-operated rats were purchased from Charles River, Montreal. Since the rats' parathyroid glands may have been

damaged during the operation they received 0.1% calcium gluconate in the water supply for the duration of the study. After 28 days, mini-osmotic pumps (MCL2, Alzet, Newark, Del.) were placed subscapularly. The osmotic pumps placed in the sham-operated rats and half of the thyroidectomized rats contained saline. The remainder of the thyroidectomized rats received T₄ (0.9 µg/100 g b.w./day) and T₃ (0.15µg/100 g b.w./day). This regimen of thyroid hormone supplementation has been shown to ensure euthyroidism in the thyroidectomized rat (Escobar-Morreale *et al.*, 1996). Homocysteine metabolism was examined 14 days after pump implantation. There were 6 rats in each group.

3.2 Results

3.2.1 Propylthiouracil Study

Table 3.1 gives information on body weight, plasma thyroid hormone and plasma creatinine levels for the propylthiouracil experiment. The total plasma T₃ and free plasma T₄ concentrations were both decreased substantially in the PTU-treated rats, thus establishing that they were hypothyroid. Plasma creatinine was unaltered by PTU treatment suggesting that kidney function was unaffected by hypothyroidism. This is germane since plasma creatinine, an indirect index of glomerular filtration rate, is positively correlated with homocysteine levels (Bostom *et al.* 1995).

Table 3.1. Body weight, plasma total T₃, and free T₄ concentrations in propylthiouracil-treated and control rats.

Variable	Units	Control (n=10)	PTU-treated (n=5)
Initial weight	g	312 ± 13	317 ± 12
Final weight	g	455 ± 11	370 ± 38*
Total T ₃	nM	0.80 ± 0.13	0.21 ± 0.15*
Free T ₄	pM	14.5 ± 1.9	0.9 ± 0.8*
Creatinine	μM	63.7 ± 7.8	59.9 ± 9.8

Note: Data are expressed as mean ± SD. * Represents significant difference compared with controls. P< 0.05 (Student's t-test).

Plasma total homocysteine levels were significantly decreased in the PTU-treated rats compared to the controls: 6.86 ± 1.35 vs 11.54 ± 1.85 μM (Fig 3.1). This suggests that thyroid hormones may be involved in regulating plasma homocysteine levels. Our next goal was to explain the observed decrease in homocysteine levels. A decrease in plasma homocysteine concentrations could be explained by an increased flux through enzymes of the transsulfuration and/or remethylation pathways. The liver is the focal organ of amino acid metabolism and contains all of the relevant enzymes (Finkelstein, 1990). The kidney appears to be the major organ involved in homocysteine removal from plasma in the rat. In one pass, 20% of plasma homocysteine is removed by the kidney (Brosnan *et al.* 1995). Given the involvement of these tissues in homocysteine metabolism, enzymes were assayed in both liver and kidney. In the PTU-treated rat, there was a 70% increase in hepatic cystathionine β -synthase activity; hepatic cystathionine γ -lyase activity was elevated approximately two fold (Table 3.2). No difference was observed in liver methionine synthase and betaine:homocysteine methyltransferase activity. Hepatic methylenetetrahydrofolate reductase activity was decreased in the PTU-treated rats. These results suggest that the reduction of plasma homocysteine concentration was not the result of increased activity of the remethylation pathway but they are consistent with increased flux through the transsulfuration enzymes.

Unlike the liver, the kidney showed no increase in the activity of the transsulfuration enzymes (Table 3.3). In fact, cystathionine γ -lyase was somewhat decreased in the hypothyroid rat. There was also a small increase in renal methionine

Figure 3.1: Comparison of plasma total homocysteine concentrations in control and PTU-treated rats. The asterisk represents significant difference ($P < 0.05$, Student's t-test).

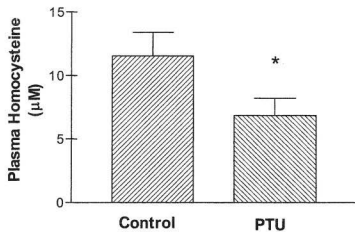


Table 3.2: Hepatic enzyme activities of homocysteine metabolism in PTU-treated and control rats.

Liver	Control (n=10)	PTU-treated (n=5)
Cystathionine β-synthase	2.89 \pm 0.58	4.87 \pm 0.50*
Cystathionine γ-lyase	14.5 \pm 4.1	27.4 \pm 3.5*
Methionine synthase	0.22 \pm 0.04	0.20 \pm 0.05
Methylenetetrahydrofolate reductase	0.14 \pm 0.03	0.11 \pm 0.01*
Betaine:homocysteine methyltransferase	4.71 \pm 1.36	4.33 \pm 0.65

Note: All assays were performed on post-mitochondrial supernatant. Enzyme activity is expressed as nmol/mg/min. Data are expressed as means \pm SD. The asterisk signifies difference from control, $P < 0.05$.

Table 3.3: Renal enzyme activities of homocysteine metabolism in PTU-treated and control rats

Kidney	Control (n=10)	PTU-treated (n=5)
Cystathionine β -synthase	1.71 \pm 0.58	1.81 \pm 0.78
Cystathionine γ -lyase	5.30 \pm 0.79	4.04 \pm 0.51*
Methionine synthase	0.65 \pm 0.04	0.78 \pm 0.10*
Methylenetetrahydrofolate reductase	0.45 \pm 0.07	0.49 \pm 0.03

Note: All assays were performed on post-mitochondrial supernatant. Enzyme activity is expressed as nmol/mg/min. Data are expressed as means \pm SD. The asterisk signifies difference from control, $P < 0.05$.

synthase activity in the PTU-treated rat. Methylene-tetrahydrofolate reductase activity was unchanged. Betaine-homocysteine methyltransferase is not present in the rat kidney (Finkelstein, 1990).

3.2.2 Thyroidectomized Rat

Because these results were completely different from the human situation (where hypothyroidism is associated with elevated plasma homocysteine) we considered whether the PTU model might produce an artifactual result. We, therefore, employed a different model of hypothyroidism- the thyroidectomized rat. Table 3.4 contains data concerning body weight, food intake, and plasma thyroid hormone levels. Before insertion of the osmotic pumps, the thyroidectomized rats consumed less food and were much smaller. Thyroid hormone replacement increased food intake of the thyroidectomized rats, which grew at a rate similar to the control rats (5 g/day). Total T₃ concentrations were normalised following thyroid hormone replacement; however, free plasma T₄ levels did not return to normal levels.

Total plasma homocysteine (Fig 3.2.) was also decreased in this hypothyroid model (9.4 ± 0.6 in thyroidectomized rats compared with 14.5 ± 2.2 μ M in controls). Thyroid treatment restored homocysteine to control levels (13.2 ± 1.6 μ M). This strongly implicates thyroid hormone in the regulation of homocysteine metabolism.

As in the PTU-treated rats, the activities of liver cystathionine β -synthase and cystathionine γ -lyase were elevated in the hypothyroid rat (Table 3.5). Methylene-tetrahydrofolate reductase activity was decreased in the thyroidectomized

Table 3.4: Body weight, total plasma T₃, and free T₄ in thyroidectomized (\pm thyroid treatment) and sham-operated control rats

Parameter	Units	Control	Thyroidectomized	Thyroid treatment
Weight on day 28	g	406 \pm 46 ^b	254 \pm 41 ^a	252 \pm 23 ^a
Final weight	g	487 \pm 57 ^c	270 \pm 15 ^a	335 \pm 31 ^b
Food intake	g/day	31.5 \pm 2.4 ^a	18.3 \pm 3.0 ^b	25.5 \pm 2.5 ^a
Total plasma T ₃	nM	0.79 \pm 0.09 ^b	0.49 \pm 0.04 ^a	0.78 \pm 0.05 ^b
Free plasma T ₄	pM	26.0 \pm 4.4 ^b	5.84 \pm 0.45 ^a	9.06 \pm 2.66 ^a

Note: Initial weights of rats were equal for all groups (~ 215 g). Data are expressed as mean \pm SD. Different superscripts within a horizontal row represent a statistically significant difference among means ($P < 0.05$). There are 6 rats in each test group.

Figure 3.2: Comparison of total plasma homocysteine levels in thyroidectomized (\pm thyroid treatment) and control rats. Columns with different letters (a,b) show significant difference (one-way Anova, $P < 0.05$).

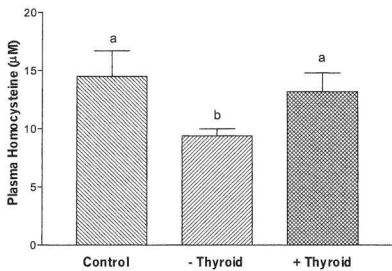


Table 3.5: Hepatic enzyme activities of homocysteine metabolism in thyroidectomized (\pm thyroid treatment) and control rats.

Liver	Control	Thyroidectomized	Thyroid treatment
Cystathionine β -synthase	2.70 ± 0.64^b	4.86 ± 0.49^a	2.83 ± 0.66^b
Cystathionine γ -lyase	14.9 ± 3.1^a	29.6 ± 7.7^b	13.8 ± 4.9^a
Glycine N-methyltransferase	3.05 ± 1.09	2.34 ± 0.70	2.2 ± 0.55
Methionine synthase	0.29 ± 0.09	0.38 ± 0.09	0.38 ± 0.11
Methylenetetrahydrofolate reductase	0.24 ± 0.08^a	0.14 ± 0.06^b	0.25 ± 0.07^a
Betaine:Homocysteine methyltransferase	3.43 ± 0.56	3.78 ± 1.01	3.94 ± 0.95

Note: All assays were performed on post-mitochondrial supernatant. Enzyme activity is expressed as nmol/mg/min. Data are expressed as means \pm SD. Different superscripts within a horizontal row represent a statistically significant difference among means ($P < 0.05$). There were 6 rats in each test group.

rat. Thyroid treatment normalised the activity of these enzymes. No change in activity was observed in any of the other liver enzymes measured.

The renal activity of methylenetetrahydrofolate reductase was reduced in the thyroidectomized rat compared to the other groups. No change in renal activity of cystathionine β -synthase or methionine synthase was observed. Cystathionine γ -lyase was unaltered in the thyroidectomized rat compared with controls. However, enzyme activity was slightly increased in thyroid-treated rats (Table 3.6).

3.3 Discussion

The purpose of this study was to investigate homocysteine metabolism in the hypothyroid rat in an effort to provide a metabolic explanation for increases in plasma homocysteine in the human hypothyroid patient. However, the hypothyroid rat exhibits decreased plasma homocysteine, unlike its human counterpart. In order to eliminate the possibility that the observed decreases in homocysteine were an artefact of a specific hypothyroid rat model, we examined both chemical and surgical models of hypothyroidism. Similar decreases in plasma total homocysteine levels were observed in both models. However, increased activities of liver cystathionine β -synthase and cystathionine γ -lyase were observed in both hypothyroid rat models. This result provides a possible explanation for the decrease in plasma homocysteine concentration. Thyroid hormone administration to thyroidectomized rats normalised the activities of both transsulfuration enzymes and plasma homocysteine levels,

Table 3.6: Renal enzyme activities of homocysteine metabolism in thyroidectomized (\pm thyroid treatment) and control rats.

Kidney	Control	Thyroidectomized	Thyroid treatment
Cystathionine β-synthase	1.09 ± 0.35	1.42 ± 0.62	1.21 ± 0.43
Cystathionine γ-lyase	7.9 ± 2.0^a	6.2 ± 1.1^a	9.9 ± 1.6^b
Methionine synthase	0.91 ± 0.06	0.90 ± 0.16	0.97 ± 0.14
Methylenetetrahydrofolate reductase	0.67 ± 0.07^b	0.47 ± 0.5^a	0.65 ± 0.17^b

Note: All assays were performed on post-mitochondrial supernatant. Enzyme activity is expressed as nmol/mg/min. Data are expressed as means \pm SD. Different superscripts within a horizontal row represent a statistically significant difference among means ($P < 0.05$). There were 6 rats in each test group.

providing further evidence for the role of thyroid hormone in regulating homocysteine metabolism.

The reason (or reasons) for the discrepancy between the hypothyroid human and rat is not readily apparent, although differences in kidney function may provide a plausible explanation. Nedrebo *et al.* (2001) observed parallel changes in serum creatinine and total homocysteine, suggesting that decreased renal filtration may account for the elevation in plasma homocysteine in the human hypothyroid patient. Some studies have reported decreased GFR in the hypothyroid rat; however, in our study, plasma creatinine levels were unaltered by PTU-treatment. This may be explained by the relatively short (2 weeks) hypothyroid period. One could hypothesize that a longer treatment period might result in decreased GFR and hyperhomocysteinemia in the PTU-treated rat. However, this issue was not addressed.

It is also conceivable that the hormonal control of gene expression of the transsulfuration enzymes differ between the two species. In this connection Ingenbleek *et al.* (1986) reported a decrease in plasma cystathionine levels in hypothyroid patients and suggested that this may be related to a deficiency in cystathionine β -synthase. However, this observation could also be explained by increased cystathionine γ -lyase as was seen in this study. This issue may be resolved by comparative studies on the effect of thyroid hormone on the expression of these enzymes in human and rat hepatoma cells.

Although we have strong evidence for the involvement of the hepatic transsulfuration pathway in reducing plasma homocysteine in the hypothyroid rat, we

cannot exclude the possibility of elevated remethylation of homocysteine to methionine. One argument against this possibility is the consistent decrease in methylenetetrahydrofolate reductase activity in the hypothyroid rat, a result also observed in other studies (Nair *et al.*, 1994; Keating *et al.*, 1988). The activity of methionine adenosyltransferase has been shown by Keating *et al.* (1988) to be elevated in hypothyroidism, resulting in enhanced levels of S-adenosylmethionine (SAM). S-adenosylmethionine is a known allosteric inhibitor of methylenetetrahydrofolate reductase and an activator of CBS (Finkelstein, 1990). Thus, an increase in SAM would favour transsulfuration over remethylation. These findings argue against elevated remethylation in the hypothyroid rat.

The actions of thyroid hormone are primarily the result of the interaction of T₃ with nuclear receptors that bind to regulatory regions of genes and modify their expression (reviewed in Brent, 1994). It is therefore possible that the expression of cystathionine β -synthase and cystathionine γ -lyase genes is induced during hypothyroidism. However, the changes occurring in hypothyroid rats may be secondary to changes in other factors. Cell culture studies are necessary to resolve this issue.

To our knowledge this is the first study that examines homocysteine metabolism and its enzymes in an experimental model of hypothyroidism. We have shown that homocysteine concentrations are consistently decreased in the hypothyroid rat, and this decrease is associated with increased enzyme activities of the hepatic transsulfuration pathway. Normalization of these parameters after thyroid

replacement emphasises the importance of thyroid status in homocysteine metabolism. As it is apparent that hypothyroidism in the rat is not a good model for human hypothyroidism we did not pursue this model any further.

Chapter 4: The streptozotocin-diabetic rat

4.1 Background

Cardiovascular diseases are the major cause of death in diabetic patients (Fein and Scheuer, 1990), and there has recently been much interest in the possible role of homocysteine in the development of cardiovascular diseases in these patients (Schneede *et al.*, 2000). Homocysteine levels in human diabetic patients appear to depend on the presence or absence of nephropathy. Diabetic patients with elevated creatinine levels (an indicator of kidney dysfunction) tend to exhibit an increase in plasma homocysteine (Hultberg *et al.*, 1991). This is consistent with studies that show that the kidney is an important organ in the removal of plasma homocysteine (Bostom *et al.*, 1996). On the other hand, type 1 patients with normal creatinine levels have decreased plasma homocysteine (Robillon *et al.*, 1994). The explanation for decreased homocysteine concentrations is unknown in these cases, but the regulation of homocysteine metabolism by insulin could afford an explanation.

The purpose of this study, therefore, was to examine homocysteine metabolism in a diabetic rat model and to determine whether insulin plays a role in its regulation. For several decades, streptozotocin, an analogue of N-acetylglucosamine, has been used to cause pancreatic β -cell death and the subsequent development of Type 1 diabetes (Konrad *et al.*, 2001). Thus the streptozotocin-diabetic rat provides us with a useful tool in studying homocysteine metabolism in Type 1 diabetes mellitus.

4.2 The streptozotocin-diabetic rat

Diabetes was induced by a single intravenous (tail vein) injection of 100 mg/kg streptozotocin (dissolved in 10 mM citrate buffer, pH 4.5 immediately before use) under light ether anaesthesia, while control rats were administered an equal volume of citrate buffer. Half of the diabetic rats (diabetic untreated) were treated with Novolin®ge ultralente human insulin (Eli Lilly, Indianapolis) subcutaneously for 5 days, whereupon insulin was withdrawn and they received saline injections for 5 days (n=8). The remaining diabetic rats (diabetic treated) received insulin injections for the entire 10 days of the experiment (n=8). The control group received saline injections (n=8). Insulin was administered at 9:00 and 19:00h; the dose was adjusted so as to maintain blood glucose close to control values as measured with an Ames glucometer II, using a drop of blood obtained by tail prick.

Tissue sampling and experimental procedures are as outlined in **Chapter 2**. Variations in experimental designs are indicated, when necessary, throughout the Results section.

4.3 RESULTS

4.3.1 The streptozotocin-diabetic rat model

Table 4.1 gives information on body weight, food intake, plasma glucose and creatinine. Although the final body weights differed, there were no differences in food intake among groups. Given that the groups consumed similar quantities of

TABLE 4.1: Body weight, food intake and plasma glucose and creatinine concentrations in control, diabetic, and insulin-treated diabetic rats.

	Control	Untreated Diabetic	Insulin-treated Diabetic
Initial Weight (g)	316 ± 17 ^a	318 ± 18 ^a	313 ± 23 ^a
Final Weight (g)	365 ± 22 ^c	275 ± 15 ^a	313 ± 27 ^b
Food Intake (g/day)	27 ± 4.1 ^a	30 ± 9.4 ^a	28 ± 4.0 ^a
Plasma Glucose (mM)	7.6 ± 1.2 ^a	34.8 ± 3.5 ^b	7.4 ± 1.7 ^a
Plasma Creatinine (μM)	81 ± 5.9 ^b	61 ± 4.8 ^a	79 ± 7.2 ^b

Food intake was determined daily. Glucose and creatinine measurements were made on day 10, i.e. at the end of the experimental period. Data are expressed as means ± SD. Different superscripts within a horizontal row represent a statistically significant difference among means ($P < 0.05$).

food, we cannot attribute alterations in homocysteine metabolism to differences in vitamin or amino acid intakes. The plasma glucose in the untreated diabetic rat demonstrates frank diabetes which was well controlled by insulin treatment in the diabetic treated group. Plasma creatinine levels in the untreated-diabetic rats were not elevated; in fact, they were slightly lower than the other groups, indicating that renal function was not impaired in the diabetic rats. The decrease in creatinine concentration may be attributed to hyperfiltration.

4.3.2 Effect of streptozotocin and insulin treatment on plasma homocysteine

The question posed is whether streptozotocin-induced diabetes results in decreased plasma homocysteine levels as observed in the human diabetic patient. Connected to this question is whether daily insulin administration could prevent any observed decrease and whether we could provide a satisfactory explanation for altered homocysteine metabolism. Plasma homocysteine levels in the streptozotocin-induced diabetic rats were approximately 40% lower than in the controls (diabetic untreated vs control; 6.97 ± 1.17 vs 10.91 ± 1.14 μM , $P < 0.05$) [Fig. 4.1]. Insulin treatment prevented the decrease in the plasma homocysteine concentrations in diabetic rats (10.33 ± 0.56 μM). This is the first evidence to suggest that insulin may play a role in regulating plasma homocysteine levels.

We measured urinary homocysteine to determine whether the decreased plasma homocysteine in diabetes could be due to increased urinary loss. Figure 4.2 shows no significant difference in the quantity of homocysteine excreted per day

Figure 4.1: Comparison of total plasma homocysteine in control, untreated diabetic and insulin-treated diabetic rats. Data are expressed as means \pm SD. The difference in letters among columns signifies differences ($P < 0.05$) between groups.

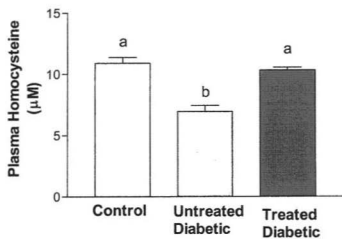
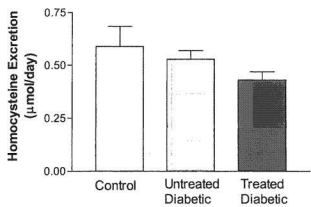


Figure 4.2: Comparison of daily urinary excretion of homocysteine in control, untreated diabetic, and insulin-treated diabetic rats. Data are expressed as means \pm SD.



among the three groups. Bostom *et al.* (1995) showed that only 2% of the total homocysteine removed by the rat kidney appears in the urine. Thus homocysteine excretion does not represent a major route of homocysteine loss from the body and it is not increased by untreated diabetes.

4.3.3 Effect of streptozotocin-induced diabetes and insulin-treatment on hepatic and renal enzyme activities

The liver is a major organ in methionine metabolism, as evidenced by the distribution and specific activities of the enzymes of methionine metabolism as well as its role in creatine synthesis, the major methyl requiring reaction in the body (Stipanuk, 1986). Although unproven, the liver is likely the major producer of plasma homocysteine. Svoldal *et al.* (1986) have shown that hepatocytes release homocysteine into their culture medium. We, therefore, measured the activities of the enzymes in the transulfuration and remethylation pathways in the liver.

Increases in hepatic activities of enzymes involved in the catabolism of methionine to cysteine are seen in the untreated-diabetic rat. Methionine adenosyltransferase, glycine N-methyltransferase, and cystathionine β -synthase activity increased 30% while cystathionine γ -lyase activity was elevated two-fold in liver (Table 4.2). Insulin treatment prevented the increases in the activities of these enzymes. No difference was observed in the activities of methionine synthase, methylenetetrahydrofolate reductase and betaine:homocysteine methyltransferase,

TABLE 4.2: Activities of hepatic enzymes involved in methionine and homocysteine metabolism

	Control	Untreated Diabetic	Insulin-Treated Diabetic
Methionine adenosyltransferase	1.00 ± 0.06 ^a	1.30 ± 0.10 ^b	0.99 ± 0.08 ^a
Glycine N-methyltransferase	1.0 ± 0.05 ^a	1.25 ± 0.10 ^b	0.95 ± 0.15 ^a
Cystathionine β-synthase	0.70 ± 0.10 ^a	0.98 ± 0.31 ^b	0.68 ± 0.14 ^a
Cystathionine γ-lyase	10.4 ± 1.5 ^a	22.5 ± 0.9 ^b	10.0 ± 1.3 ^a
Methionine synthase	0.08 ± 0.01 ^a	0.07 ± 0.01 ^a	0.08 ± 0.02 ^a
Betaine:homocysteine methyltransferase	3.2 ± 0.3 ^a	4.1 ± 1.4 ^a	2.9 ± 1.0 ^a
Methylenetetrahydrofolate reductase	0.33 ± 0.06 ^a	0.26 ± 0.07 ^a	0.25 ± 0.05 ^a

All assays were performed on post-mitochondrial supernatant. Enzyme activity is expressed as nmol/mg protein/min. Data are expressed as means ± SD. Different superscripts within a horizontal row represent a statistically significant difference among means ($P < 0.05$).

suggesting that the reduction of plasma homocysteine was not the result of an activation of one of the remethylation pathways. These data suggest an important role for the hepatic transsulfuration pathway in diabetes and also suggest a role for insulin in regulating this pathway.

Patients with end-stage renal disease have increased plasma homocysteine concentrations (Bostom and Lathrop, 1997). Rat renal tubular cells possess enzymes for both remethylation and transsulfuration of homocysteine (House *et al.*, 1999). Furthermore, our lab reported a significant, positive arteriovenous difference for homocysteine across the rat kidney, representing 20% of the arterial plasma concentration (Brosnan *et al.*, 1995). Given the importance of kidney metabolism in regulating plasma homocysteine and the fact that alterations in kidney function are a common complication in diabetes we performed two separate experiments. First, we measured the activities of renal enzymes involved in homocysteine metabolism and secondly we measured renal homocysteine uptake in the streptozotocin-diabetic rat.

Unlike the liver, the activity of the renal transsulfuration enzymes in the kidney did not change (Table 4.3). A decrease in renal methionine synthase activity was observed in the diabetic rats, which was normalized by insulin treatment. The renal activity of methylenetetrahydrofolate reductase was decreased in the diabetic rats and this was not corrected by insulin treatment. Betaine:homocysteine methyltransferase is not found in the rat kidney.

TABLE 4.3: Renal Activities of enzymes of homocysteine metabolism.

	Control	Untreated Diabetic	Insulin-Treated Diabetic
Cystathionine β-synthase	0.36 ± 0.05^a	0.35 ± 0.08^a	0.35 ± 0.09^a
Cystathionine γ-lyase	6.2 ± 1.1^a	6.4 ± 1.9^a	5.8 ± 1.4^a
Methionine synthase	0.34 ± 0.03^b	0.25 ± 0.01^a	0.36 ± 0.04^b
Methylenetetrahydrofolate reductase	0.79 ± 0.07^b	0.24 ± 0.08^a	0.33 ± 0.06^a

All assays were performed on post-mitochondrial supernatant. Enzyme activity is expressed as nmol/mg protein/min. Data are means \pm SD. Different superscripts within a horizontal row represent a statistically significant difference among means ($P < 0.05$).

4.3.4 Effect of streptozotocin on renal homocysteine flux

Even though the activities of the renal transsulfuration enzymes were normal in the streptozotocin-diabetic rat, the kidney still could be responsible for the decrease in plasma homocysteine in the diabetic rat. Many studies in humans have shown that plasma homocysteine correlates inversely with GFR (Arnadottir *et al.*, 1996). In our study, plasma creatinine was decreased in the diabetic rat suggesting an increase in GFR. If this were the case, increased renal metabolism may be responsible for the decreased plasma homocysteine in the diabetic rat. To address this possibility, we measured the renal homocysteine flux in both diabetic and control rats. If the kidneys were responsible for the decreased plasma homocysteine we would expect to observe an increase in renal homocysteine flux in the diabetic rat.

Renal hemodynamic parameters are presented in Table 4.4. No significant effect of diabetes on glomerular filtration rate or renal plasma flow occurred, which is inconsistent with our plasma creatinine data. Not surprisingly, the urine flow was increased 5-fold in the untreated-diabetic rat versus controls. As a result of increased urination, the U:P ratio for the diabetic rat was reduced by 75%.

Table 4.5 shows the results of our studies of renal homocysteine metabolism in diabetes *in vivo*. We observed a constant, positive arteriovenous difference for homocysteine concentration, equivalent to approximately 15% of the total arterial plasma homocysteine concentration. In no case did we find a zero or negative arteriovenous difference. Consistent with previous measurements from our laboratory, rates of homocysteine excretion were low (<2% of total loads),

Table 4.4: Renal hemodynamic parameters of diabetic and control rats

Parameter	Units	Control	Untreated Diabetic
Body weight	grams	370 ± 40	300 ± 8.0*
Urine flow	μL/min/100g	4.5 ± 1.1	26.6 ± 2.8*
U:P ratio for inulin		195 ± 80	41 ± 10*
Glomerular filtration rate	mL/min/100g	0.83 ± 0.21	1.1 ± 0.1
Renal plasma flow	mL/min/100g	3.5 ± 0.4	4.1 ± 0.9

Values are expressed as means ± SD (n=4). Asterisks represents significant difference from controls (Student's t-test, P<0.05).

Table 4.5: *In vivo* renal homocysteine metabolism in diabetic and control rats.

Parameter	Units	Control	Untreated Diabetic
Arterial Homocysteine	μM	6.7 ± 1.0	$2.9 \pm 1.0^*$
A-V Difference	μM	1.04 ± 0.30	$0.47 \pm 0.18^*$
Net Renal Homocysteine Flux	$\eta\text{moles/min/100g}$	3.7 ± 1.2	$1.8 \pm 0.4^*$
Homocysteine Excretion	$\eta\text{moles/min/100g}$	0.07 ± 0.02	0.06 ± 0.02

Values are expressed as means \pm SD (n=4). Asterisks indicates significant difference from controls (Student's t-test, $P < 0.05$). Net renal homocysteine flux was calculated as the product of the A-V difference and the renal plasma flow.

which illustrates that the bulk of the homocysteine taken up by the kidney is metabolized. The renal homocysteine flux was decreased by 50% in the diabetic rat versus control. This value reflects the 50% decrease in A-V difference for homocysteine concentration in the diabetic rat as the renal plasma flow was unchanged compared to controls values. These data provide direct evidence that the kidneys are not responsible for the decreased plasma homocysteine levels in the diabetic rat.

4.3.5 Effects of fasting on homocysteine metabolism in the diabetic rat

The results of these experiments suggest that the decreased plasma homocysteine, observed in the untreated-diabetic rat, could be due to increased catabolism via the hepatic transsulfuration pathway. However, these experiments were performed on fed animals, which favour transsulfuration flux over the remethylation pathway. We therefore, performed the same experiment on 24 hour fasted rats. In this study insulin was not administered to the diabetic rats due to the risk of hypoglycaemia even though plasma glucose was elevated 40% in the diabetic rat (Figure 4.3).

As in the first experiment, plasma homocysteine was decreased in the diabetic rat versus controls (Figure 4.3). The hepatic enzyme activities involved in homocysteine metabolism were again measured (Table 4.6). Again, we observed increased enzyme activities of the hepatic transsulfuration pathway, while no change was found in the remethylation enzyme activities. This provides further evidence for

Figure 4.3: Plasma glucose and total plasma homocysteine concentrations following a 24-hour fast in control and untreated diabetic rats. Data are expressed as means \pm SD. An asterisk indicates a significant difference between groups, $P < 0.05$.

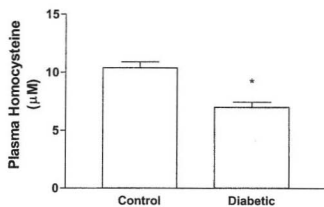
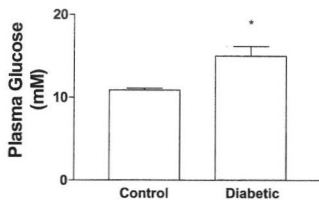


Figure 4.4: Hepatic enzyme activities in control and diabetic rats following a 24 hour fast.

	Control	Untreated Diabetic
Cystathionine β-Synthase	2.99 \pm 0.51	4.44 \pm 0.96*
Cystathionine γ-Lyase	13.3 \pm 2.4	20.6 \pm 0.98*
Methionine Synthase	0.11 \pm 0.03	0.14 \pm 0.04
Betaine:Homocysteine Methyltransferase	2.71 \pm 0.45	2.76 \pm 0.62
Methylenetetrahydrofolate Reductase	0.20 \pm 0.03	0.22 \pm 0.02

All assays were performed on post-mitochondrial supernatant. Enzyme activity is expressed as nmol/mg protein/min. Data are means \pm SD (n=5). Asterisk indicates a significant difference between groups, $P < 0.05$.

the importance of the hepatic transsulfuration pathway in regulating plasma homocysteine in the diabetic rat.

4.3.6 Regulation of plasma homocysteine and hepatic cystathionine β -synthase by insulin

We next examined the time-course of insulin's effect on homocysteine metabolism in diabetic rats. Diabetic rats were without insulin for 5 days, at which point insulin was administered for 1, 3, or 5 days. Rats ate a commercially obtained rodent diet *ad libitum* and had free access to water. Diabetes was induced as outlined previously in this chapter.

Figure 4.4 shows the time-course of the insulin effect on plasma homocysteine in the diabetic rat. Plasma homocysteine was decreased by 60% in the untreated diabetic rat versus controls. Insulin treatment increased plasma homocysteine in the diabetic rat within 1 day. However, plasma homocysteine did not return to control values. This is noteworthy since insulin did normalize plasma homocysteine in our previous experiments. Plasma glucose was normalized following insulin treatment thus dismissing the possibility that the rats were not properly insulinized (Figure 4.4). Since diabetes is characterized by decreased circulating insulin and increased circulating glucagon levels, we also measured plasma glucagon concentrations in control, diabetic and insulin-treated (5-days) diabetic rats (Figure 4.5). Plasma glucagon was elevated by 40% in the untreated diabetic rat versus controls; however, insulin treatment failed to return glucagon levels to normal. The

Figure 4.4: Effect of insulin treatment on plasma glucose and total plasma homocysteine levels in the streptozotocin-diabetic rat. Diabetic rats were treated with insulin for 0, 1, 3 or 5 days. Data are expressed as means \pm SD. Differences in letters (a,b,c) between columns signify differences between group means. (n= 4-6)

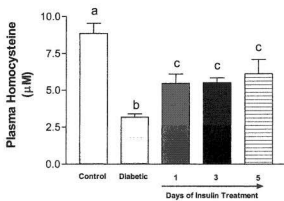
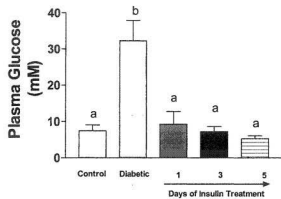
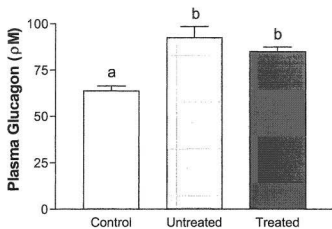


Figure 4.5: Comparison of plasma glucagon levels in control, untreated diabetic, and insulin-treated diabetic rats. Data are expressed as means \pm SD. Differences in letters (a,b) between columns signify difference between group means.



increased glucagon levels may also offer an explanation why homocysteine levels did not completely normalize following insulin treatment.

Hepatic cystathionine β -synthase activity was elevated in the untreated diabetic rat (Figure 4.6) and treatment of these rats with insulin for 1 day decreased the activity of cystathionine β -synthase to control levels. To determine whether these observed changes in cystathionine β -synthase enzyme activity were regulated at the level of transcription, we measured cystathionine β -synthase mRNA levels in rat liver. When normalized for β -actin mRNA, hepatic cystathionine β -synthase mRNA from the untreated diabetic rats was elevated by 2-fold versus controls (Figure 4.7). Insulin treatment decreased the level of cystathionine β -synthase mRNA to nearly 40% of that in the untreated diabetic rat within a day. This level fluctuated little in the 3- and 5-day insulin-treated groups.

4.3.7 Hormonal regulation of cystathionine β -synthase in H4IIE cells

To determine whether the effects of insulin *in vivo* on CBS expression would be evident *in vitro* and whether counter-regulatory hormones might play a role, we examined the effects of hormones on CBS in rat hepatoma (H4IIE) cells. CBS activity increased by 50% in cell extracts obtained from H4IIE cells following an 8 hour treatment with triamcinolone (a glucocorticoid analogue) (Figure 4.8). This increase in enzyme activity was blocked when cells were co-incubated in the presence of insulin.

Figure 4.6: Effect of insulin treatment on hepatic cystathionine beta-synthase activity in the diabetic rat. Diabetic rats, which were insulin-deficient for 5 days were treated with insulin for 0, 1, 3 or 5 days. Data are expressed as means \pm SD. Differences in letters (a,b,c) between columns signify difference between groups.

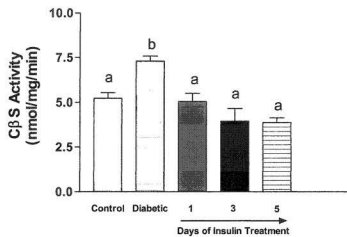
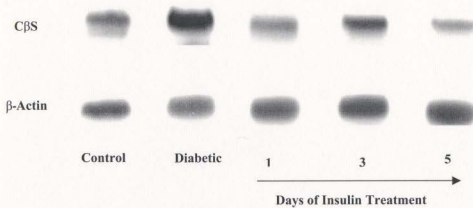
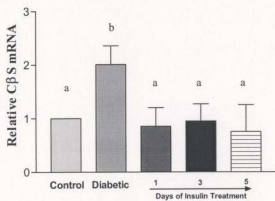


Figure 4.7: Northern Blot analyses of hepatic cystathionine beta-synthase mRNA levels in control, diabetic, and insulin-treated (1,3, and 5 days) diabetic rats. Total RNA, isolated from freeze-clamped livers, was separated on a 1% agarose/formaldehyde gel and transferred to a Nylon membrane. Cystathionine β -synthase and β -actin mRNA levels were determined by Northern Blot analysis.

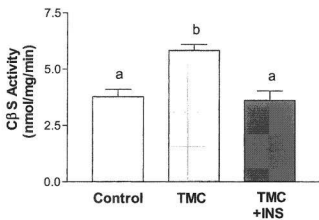
A



B



4.8 Effect of triamcinolone (TMC) and insulin (INS) incubation on cystathionine beta-synthase activity in H4IIE cells. Data are expressed as means \pm SD. Differences in letters (a,b) between columns signify difference between groups ($P < 0.05$).

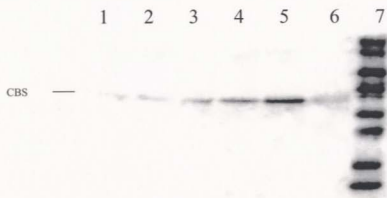


To verify that the triamcinolone-induced increase in cystathionine β -synthase enzyme activity was a reflection of an increased amount of CBS protein, cellular lysates were separated by SDS-PAGE with the amount of cystathionine β -synthase protein determined by Western Blot analysis (performed in collaboration with Dr. Shobhitha Ratnam). The level of cystathionine β -synthase protein increased with increasing triamcinolone with maximal levels reached at a concentration of 1 μ M (Figure 4.9a). H4IIE cells were then stimulated by a fixed concentration of triamcinolone (100 nM) in the presence of various doses of insulin (10 nM to 1 μ M). The glucocorticoid-stimulated increase in CBS protein was partially inhibited by 10 nM insulin and was completely inhibited by 50 nM (Figure 4.9b). Incubation with insulin alone showed no significant changes in the basal expression of cystathionine β -synthase protein in these cells.

To investigate whether the hormone-dependent regulation of cystathionine β -synthase protein and activity levels was associated with relative changes in CBS mRNA, total RNA was isolated from H4IIE cells. Following RT-PCR, the DNA products (CBS and β -actin) were separated on an agarose gel and analyzed by Southern blotting (performed in collaboration with Dr. Shobhitha Ratnam). Control cells expressed low levels of CBS mRNA which was increased by 3-4 fold by triamcinolone (Figure 4.10, lane 2). This glucocorticoid-dependent induction was completely inhibited by insulin (Figure 4.10, lane 3). When cells were incubated with a cyclic-AMP analogue (CPT-cAMP) there was a 1.5-2 fold increase in CBS mRNA which was also suppressed by insulin (Figure 4.10, lane 4). Co-incubation of cAMP

Figure 4.9: Cystathionine beta-synthase protein levels in H4IIE cells. CBS protein was measured by Western Blot. **A.** Effect of increasing concentration of triamcinolone. Lane 1, Control, Lanes 2-5: triamcinolone, 10,50,100 and 1000 nM; lane 6, DMSO control; Lane 7, Biotinylated molecular weight standards. **B.** Suppression of CBS protein by insulin incubation. Cells were stimulated with 100 nM triamcinolone and increasing concentrations of insulin. Lane 1, triamcinolone (100 nM); lanes 2-6, triamcinolone (100 nM) and insulin 1, 10, 50, 100, 1000 nM, respectively; Lane 7, biotinylated molecular weight standard.

A



B

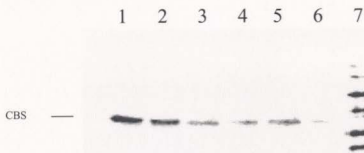
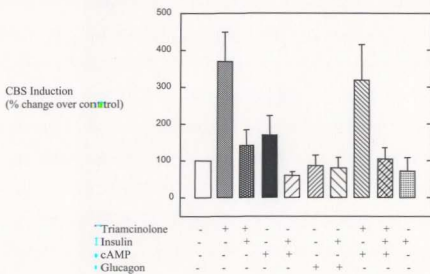


Figure 4.10 Effect of hormone treatment on cystathionine beta-synthase mRNA levels in H4IIE cells. RNA (2 μ g) was reverse transcribed and amplified. Southern blot of the PCR products probed with 32 P labeled CBS cDNA and β -actin cDNA. **A.** Lane 1, control; lane 2, triamcinolone (1 μ M); lane 3, triamcinolone (1 μ M) + insulin (1 μ M); lane 4, CPT cAMP (200 μ M), lane 5, CPT cAMP + Insulin (1 μ M); lane 6, glucagon (1 μ M); lane 7, glucagon (1 μ M) + Insulin (1 μ M); lane 8, triamcinolone (1 μ M) + CPT cAMP (200 μ M); lane 9, triamcinolone (1 μ M) + CPT cAMP (200 μ M) + Insulin (1 μ M); lane, 10, insulin (1 μ M). **B.** Graph represents densitometric quantitation of CBS mRNA when normalized for β -actin. Data represented are mean \pm standard deviation (3-5 experiments).



B



and triamcinolone did not have an additive effect (Figure 10, lane 8). Furthermore, the addition of glucagon to the medium was without effect (Figure 4.10, lane 6); this was possibly due to the common loss of glucagon receptors on hepatoma cells (Fehlmann *et al.*, 1983). Finally, when cells were incubated with insulin alone, constitutive expression of CBS mRNA was unaltered (Figure 4.10, lane 10).

4.4 Discussion

Destruction of β -cells causes Type 1 diabetes mellitus, due to cessation of insulin synthesis and secretion. The effects of insulin deficiency on fuel metabolism have been well documented (Ellenberg and Rifkin, 1990). Hyperglycemia, a characteristic finding, arises from glucose underutilization in peripheral tissues (muscle and adipose) together with hepatic overproduction. Elevated lipolysis from adipocytes increases plasma fatty acids and hepatic ketogenesis. Similarly, muscle proteolysis results in increased release of amino acids, mainly alanine and glutamine, into the plasma. Furthermore, hepatic uptake of gluconeogenic amino acids is increased and urea synthesis sharply elevated in the diabetic rat. Increased catabolism of the most quantitatively important amino acid, glutamine, has been observed in our laboratory. Hepatic glutaminase was induced and activated in the diabetic liver (Brosnan *et al.*, 1995). In addition, insulin treatment returned glutamine catabolism to control values, as measured by flux through glutaminase. That study illustrated the importance of insulin in regulating glutamine metabolism and a similar regulatory effect seems to be important in homocysteine metabolism.

Plasma homocysteine is decreased in the streptozotocin-diabetic rat. This finding is consistent with human Type 1 patients with normal kidney function (Robillon *et al.*, 1994). Given the importance of renal homocysteine metabolism, it was vital to ascertain the role of the kidney in the regulation of plasma homocysteine levels. The activities of the renal transsulfuration enzymes, the major mechanism for homocysteine removal by the kidney (House *et al.*, 1998), were unaltered in the diabetic rat. Furthermore, renal homocysteine flux (an accurate measurement of the amount of homocysteine metabolized by the kidneys) was decreased in the diabetic rat. Together, these data provide convincing evidence that altered renal homocysteine handling was not the cause of the decreased plasma homocysteine in the diabetic rat.

Investigating the hepatic enzyme activities did shed some light on the possible mechanism responsible for the altered plasma homocysteine. The decrease in plasma homocysteine was associated with increased activities of the enzymes that convert methionine to cysteine. Insulin treatment normalized the activity of these hepatic enzymes and elevated the level of plasma homocysteine, emphasizing the importance of insulin in homocysteine metabolism. The increased activity of methionine adenosyltransferase (the first step in methionine metabolism) and cystathionine β -synthase (the first committed step in homocysteine catabolism) may be vital for the increased conversion of methionine to cysteine in the diabetic rat. The hormonal regulation of these enzymes has now been characterized and they respond similarly to insulin and its counter-regulatory hormones.

In this study, we have investigated the hormonal regulation of cystathionine β -synthase in detail. CBS mRNA and activity are elevated in the untreated-diabetic rat. Insulin administration represses CBS mRNA levels *in vivo*. The role of counter-regulatory hormones was observed in cultured hepatoma cells where triamcinolone increased the level of CBS mRNA, protein and activity. This is consistent with an early study by Goss (1986) who showed that CBS activity in hepatoma cells is increased by the combination of dexamethasone and agents that raise intracellular cAMP levels. Glucocorticoids exert their metabolic effects primarily by changing the transcription rate of genes (Beato, 1989; Lee and Tsai, 1994); however they also increase mRNA stability and/or transport to the cytoplasm (Nielsen and Shapiro, 1990). Our laboratory is currently investigating how glucocorticoids stimulate CBS. Although we did not observe any additive effect of TMC and CPT-cAMP it is possible that the actions of glucagon (along with glucocorticoids) play an important role in the induction of this enzyme. Indeed, **Chapter 5** describes a plethora of *in vivo* effects elicited by glucagon on hepatic CBS.

We have shown that insulin inhibits CBS *in vivo* and *in vitro*. The mechanism by which insulin represses glucocorticoid and cAMP-mediated gene expression is complicated and poorly understood. The hormonal regulation of phosphoenolpyruvate carboxykinase (the rate limiting step in hepatic gluconeogenesis) gene expression has been well characterized (Sasaki *et al.*, 1984; Schubart, 1986). The rate of transcription of hepatic PEPCK is stimulated by cAMP and glucocorticoids and is inhibited by insulin (Forest *et al.*, 1990). Insulin primarily

inhibits the initiation of PEPCK gene expression but also reduces the rate of transcript elongation (Scott *et al.*, 1998; Stafford *et al.*, 2001). Furthermore, the action of insulin was shown to be dominant over cAMP and glucocorticoids (a characteristic shared with cystathionine β -synthase) (Sasaki *et al.*, 1984). Recent studies have suggested that insulin may inhibit glucocorticoid stimulated PEPCK gene transcription by interfering with the binding of transcription factors to the steroid response element (O'Brien and Granner, 1996). Similar regulation has been observed for tyrosine aminotransferase (Ganss *et al.*, 1994). Insulin may also activate enzymes that degrade the glucocorticoid receptor, thus linking insulin and glucocorticoid signalling (Kupfer *et al.*, 1994). Whether the regulation of cystathionine β -synthase gene expression is similar to that of PEPCK is unclear. However, it should be noted that the insulin response sequence (T(G/A)TTT(T/G)(G/T) present on the promoters of PEPCK and tyrosine aminotransferase (O'Brien and Granner, 1996) is present on the human CBS gene promoter (-4430 downstream with respect to the start codon). Upon analysis, this sequence was also found on the 5' untranslated region of the rat CBS gene.

The methionine adenosyltransferase response to insulin and glucocorticoid levels is similar to that of CBS. The hormonal regulation of MAT was first observed in adrenalectomized animals in the late 1960s (Pan and Tarver, 1967). However the mechanism for such regulation would remain obscure until the late 1990s when Gil *et al.* (1997) presented convincing evidence that glucocorticoids modulate this enzyme both *in vivo* and in hepatoma cells. Following adrenalectomy in the rat, enzyme activity, protein and mRNA levels were all decreased. When these animals were

treated with triamcinolone all 3 parameters were normalized. Triamcinolone also increased MAT mRNA content in culture cells. This increase in mRNA levels was dependent on induction of the MAT gene since actinomycin D abolished the effects of triamcinolone. Gil *et al.* (1997) also showed that insulin could block the inducing effects of glucocorticoids on MAT mRNA. The coordinated regulation of MAT and CBS may have importance during periods of stress and trauma. In such situations elevated glucocorticoids may stimulate flux through the transsulfuration pathway. The increased supply of cysteine may help maintain the glutathione pool thus preventing oxidative damage. Methionine transsulfuration is increased in the septic rat. Glucocorticoids are often elevated in sepsis which may provide a mechanism for the increased conversion of methionine to cysteine.

Our *in vivo* data suggest that flux through the hepatic transsulfuration pathway is increased in the diabetic rat. Our earlier work showed that the hepatic level of methionine is markedly reduced in diabetic rat liver while the hepatic level of cysteine is increased 3-fold, which is consistent with increased flux through the transsulfuration pathway in the diabetic liver (Brosnan *et al.*, 1983). We have described an induction of CBS in the untreated-diabetic rat, which would increase the capacity of the transsulfuration pathway and may explain any increase flux through this pathway. It is, of course, also possible that increased transsulfuration flux in the diabetic rat would be due to the activation of the cystathionine β -synthase in addition to induction. The actions of many hormones, including glucagon and epinephrine, occur through phosphorylation of cellular proteins. Such an effect has been described

in *in vitro* studies of MAT which have shown that this enzyme can be phosphorylated (Pajares *et al.*, 1994). Indeed, there are at least 12 different putative phosphorylation motifs in the rat-liver MAT sequence (Pajares *et al.*, 1992). Cystathionine β -synthase also contains a phosphorylation (Protein Kinase A) consensus sequence; however, phosphorylation of this enzyme has yet to be shown. Allosteric regulation may play an important role in increasing the flux through cystathionine β -synthase in the diabetic rat. Cabrero *et al.*, (1986) showed a 35-fold increase in the total hepatic S-adenosylmethionine/S-adenosylhomocysteine concentrations in the alloxan-treated rat. These nucleotides activate cystathionine β -synthase while inhibiting remethylation which would favour flux through the transsulfuration pathway (Finkelstein and Martin, 1986). Additional work is necessary to determine the importance of the other mechanisms in our diabetic rat model.

In the diabetic state, the absence of insulin and the increased levels of glucagon signal the liver to supply the body with glucose through glycogenolysis and gluconeogenesis from amino acids released from muscle (Ellenberg and Rifkin, 1990). Pyruvate is the carbon product of cysteine metabolism and is a good gluconeogenic precursor. In view of insulin's well-established role in inhibiting gluconeogenesis and given its inhibitory effects on the hepatic transsulfuration pathway, we suggest that glucose is the likely end product of sulfur amino acid metabolism in the diabetic rat liver.

This study provides convincing evidence that plasma homocysteine concentration is decreased in the Type 1 diabetic rat. Collectively, these findings offer

the first demonstration that insulin has a possible role in its metabolic regulation, and, through its effects on the hepatic transsulfuration pathway, in the metabolic disposal of this highly atherogenic amino acid. While the mode of insulin's action has yet to be completely defined, insulin (along with counter-regulatory hormones) modulates cystathionine β -synthase gene transcription both *in vitro* and *in vivo*.

Chapter 5: The Glucagon-Treated Rat

5.1 Background

The regulatory effects of glucagon on amino acid metabolism are well known. For example, in liver it can activate the glycine cleavage system, stimulate the y^+ transporter, and induce the five urea-cycle enzymes (Mabrouk *et al.*, 1998; Handlogten *et al.*, 1984; Snodgrass *et al.*, 1978). Patients with a glucagonoma have diminished plasma amino acid levels which are related to increased clearance by the liver (Barazzoni *et al.*, 1999). In light of the broad effects of glucagon on amino acid metabolism, it appears likely that homocysteine metabolism would be similarly regulated, given that it is a product of the metabolism of dietary essential methionine, and a precursor to cysteine. In addition, plasma glucagon is frequently elevated in Type 1 diabetes (Unger, 1976; and **Chapter 4**). We therefore examined the role of glucagon in regulating homocysteine metabolism in the rat.

5.2 The glucagon-treated rat

Glucagon treatment followed the procedure of Snodgrass *et al* (1978). Glucagon (2 mg/kg/day, s.c.) was administered in 3 equal daily doses (at 0800, 1600, 2400) for 2 days while control rats received the vehicle (Eli Lilly Canada Inc). Animals were fed a standard laboratory chow. Two hours following the last injection rats were anaesthetized for plasma and tissue sampling (described in **Chapter 2**). There were 3-8 rats in each experimental group. This study was performed in collaboration with Lori Stead; thus some data also will be common to this thesis and hers.

5.3 Results

5.3.1 Alterations in plasma and liver amino acids following glucagon treatment

Table 5.1 gives information on body weight, food intake, plasma glucose, plasma insulin and plasma glucagon. Two day glucagon treatment did not affect weight gain in male rats. Likewise, there was no change in food intake following glucagon treatment. Therefore, none of the metabolic changes observed can be attributed to alterations in vitamin or amino acid intakes. Plasma glucose was increased by 80% (as expected) following glucagon administration. Plasma glucagon was increased 35-fold versus control rats and there was no change in plasma insulin.

Total plasma homocysteine levels were decreased by 30% by glucagon treatment (Table 5.2). This is the first report, to our knowledge, of the homocysteine lowering effects of glucagon. Table 5.2 also shows the effects of the glucagon treatment on plasma and liver concentrations of the other amino acids involved in sulfur amino acid metabolism. All plasma amino acids shown were significantly decreased by glucagon-treatment. Hepatic amino acid levels are also reported. Liver homocysteine and glycine levels were significantly decreased by glucagon treatment, while the concentration of taurine was increased. No change was observed in the hepatic levels of methionine, cysteine and serine. However, calculation of intracellular amino acid concentrations revealed increased methionine and taurine in the glucagon-treated rat, while intracellular homocysteine was decreased. Table 5.3 shows the effects of glucagon treatment on all of the plasma amino acids. All amino

Table 5.1: Body weight, food intake, plasma glucose, insulin and glucagon concentrations in control and glucagon-treated rats.

	Units	Control	Glucagon
Initial Weight	g	240 ± 15	245 ± 7
Final Weight	g	272 ± 10	271 ± 7
Food Intake	g/day	26.3 ± 4.5	27.5 ± 3.7
Plasma Glucagon	pg/ml	79.5 ± 12.1	2630 ± 1040*
Plasma Insulin	ng/ml	8.9 ± 1.7	7.1 ± 2.6
Plasma Glucose	mM	8.0 ± 0.8	14.1 ± 1.3*

Rats were administered glucagon (4 mg/kg/day) for 2 days while control rats received the vehicle. Food intake and body weight were measured daily. Blood samples were taken from the abdominal aorta and centrifuged for plasma separation. Means ± SD are shown for 3-6 rats. An asterisk signifies significant difference versus control rats, $P < 0.05$.

Table 5.2: Plasma and hepatic amino acids involved in homocysteine metabolism.

	Control				Glucagon			
	Plasma (μM)	Liver (nmol/mg)	Hepatic Intracellular concentration (μM)	L:P Ratio	Plasma (μM)	Liver (nmol/mg)	Hepatic Intracellular concentration (μM)	L:P Ratio
Methionine	56.9 \pm 2.9	56.0 \pm 6.0	94.1 \pm 10.1	1.7	14.8 \pm 2.8*	60.6 \pm 6.0	125 \pm 13*	8.5
Homocysteine	9.7 \pm 1.1	9.0 \pm 1.0	14.8 \pm 1.7	1.5	6.4 \pm 0.7*	6.9 \pm 1.0*	11.9 \pm 1.7*	1.9
Cysteine	318 \pm 25	443 \pm 110	808 \pm 200	2.5	223 \pm 28*	358 \pm 158	672 \pm 297	3.0
Taurine	177 \pm 19	2850 \pm 435	6235 \pm 950	35	101 \pm 18*	3600 \pm 557*	7943 \pm 1228*	78
Serine	180 \pm 17	311 \pm 51	591 \pm 97	2.9	85 \pm 31*	330 \pm 48	686 \pm 100	8.0
Glycine	239 \pm 38	920 \pm 141	1912 \pm 293	8.0	96 \pm 34*	830 \pm 77*	1804 \pm 166	19.0

Plasma and liver amino acids were determined using a Beckman amino acid analyzer, except for total homocysteine and total cysteine which were determined by HPLC. Intracellular amino acid concentrations were calculated as described in **Chapter 2**. Liver:Plasma (L:P) ratio is calculated by dividing the hepatic intracellular amino acid concentration (μM) by the plasma amino acid concentration (μM). Means \pm standard deviation for 5 rats are shown. Asterisk indicates significant difference versus control, $P < 0.05$.

Table 5.3: Plasma amino acid levels in control and glucagon-treated rats.

Amino Acid	Control	Glucagon
Tryptophan	89 ± 8.0	98 ± 16
Lysine	430 ± 52	385 ± 34
Glutamic Acid	140 ± 23	56 ± 14*
Glutamine	670 ± 71	147 ± 11*
Phenylalanine	51 ± 5.0	37 ± 10*
Tyrosine	86 ± 19	46 ± 13*
Histidine	59 ± 9.0	36 ± 6*
Arginine	210 ± 25	101 ± 18*
Aspartic Acid	18 ± 3.0	12 ± 6.0
Asparagine	53 ± 6.0	13 ± 2.0*
Threonine	303 ± 30	57 ± 17*
Proline	196 ± 6.0	35 ± 4.0*
Alanine	480 ± 16	113 ± 14*
Valine	182 ± 32	154 ± 19
Isoleucine	87 ± 18	79 ± 12
Leucine	130 ± 20	60 ± 15*
Citrulline	88 ± 13	38 ± 12*
Ornithine	63 ± 5.0	38 ± 10*

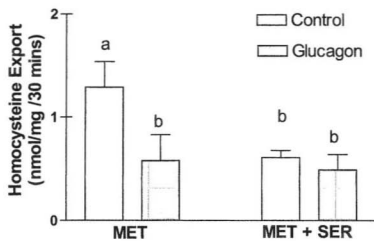
Amino acid concentrations are expressed as $\mu\text{mol/L}$ and are presented as mean \pm SD (n=5). An asterisk indicates significant difference from control (Student t-test, $P < 0.05$).

acids, except for tryptophan, aspartic acid, lysine, valine, and isoleucine, were decreased in the hyperglucagonemic rat. This general decrease in plasma amino acids is consistent with observations made in the human glucagonoma patients (Barazzoni et al., 1999).

5.3.2 Hepatocytes isolated from glucagon-treated rats export less homocysteine

The liver is the central organ in sulfur amino acid metabolism. It contains a full complement of enzymes involved in the methionine cycle and the transsulfuration pathway and is the site of 85% of all methylation reactions in the body (Wyss and Wallimann, 1994). In light of this, it is reasonable to assume that alterations in hepatic homocysteine metabolism would have a profound effect on circulating levels of this atherogenic amino acid. We therefore measured homocysteine output by isolated hepatocytes. Previously, our lab has showed that the half-maximal rate of homocysteine export occurs at a methionine concentration of 0.44 mM and is linear for at least 60 minutes (Stead *et al.*, 2000). A methionine concentration of 1 mM was chosen for all experiments. Following incubation with methionine, hepatocytes isolated from the glucagon-treated rats exported less than half as much homocysteine as the control hepatocytes (Figure 5.1). Our earlier studies have shown that addition of serine (a substrate for cystathionine β -synthase), together with methionine, reduced homocysteine export (Stead *et al.*, 2000). We therefore undertook experiments with both serine and methionine in the incubation medium. Serine incubation decreased

Figure 5.1: Homocysteine export from hepatocytes isolated from glucagon-treated and control rats. Hepatocytes were preincubated for 20 mins at 4-6 mg dry weight of cells/ml in a final volume of 1 ml in Krebs-Henseleit medium containing 1.25 % BSA. Following preincubation, 1 mM methionine \pm 1 mM serine were added and incubation was allowed to continue for an additional 30 minutes. Cells were gassed with 95% O₂/ 5% CO₂ at the beginning of the incubation and on the addition of substrates. At the end of the incubation, the contents of the flasks were centrifuged at 14000g for 2 minutes to sediment the cells. The supernatant was removed for homocysteine analysis. Mean \pm SD for 4 experiments is shown. Data were analysed using ANOVA followed by Newman-Keuls multiple comparison post test, with a P< 0.05 taken to indicate a significant difference. Results with differing superscripts were significantly different from each other.



homocysteine export from the control hepatocytes by 50%. However serine did not reduce, any further, the homocysteine export by hepatocytes from glucagon-treated animals. There was no change in the rate of cysteine export (1.5 nmol/mg/30 mins) in any of the experimental groups.

5.3.3 Glucagon treatment increases hepatic enzyme activities involved in methionine catabolism

Such a decrease in homocysteine export by hepatocytes coupled with a decreased intracellular homocysteine concentration suggests an appreciably altered metabolism. We, therefore, assayed the major enzymes involved in producing (transmethylation) and removing (transsulfuration and remethylation) homocysteine in the liver. Glucagon-treated rats exhibited increased hepatic activities of enzymes involved in the catabolism of methionine to cysteine (Table 5.4). The activities of glycine N-methyltransferase and cystathionine γ -lyase were elevated by 25% while cystathionine β -synthase activity was increased by 90%. These changes are still evident when activities are expressed either as per gram liver or per 100 grams body weight (data not shown). Methionine adenosyltransferase activity was unaffected by glucagon treatment. These data suggest the importance of the hepatic transsulfuration pathway in glucagon's regulation of homocysteine metabolism. No changes were observed in methionine synthase, betaine:homocysteine methyltransferase or methylenetetrahydrofolate reductase activity.

TABLE 5.4: Hepatic enzymes of methionine and homocysteine metabolism in glucagon-treated rats

	Control	Glucagon
S-Adenosylmethionine synthase	1.20 ± 0.05	1.25 ± 0.10
Cystathionine β-synthase	5.3 ± 0.9	9.3 ± 1.2*
Cystathionine γ-lyase	15.2 ± 2.0	19.6 ± 3.2*
Glycine N-methyltransferase	1.02 ± 0.04	1.25 ± 0.10*
Methionine synthase	0.11 ± 0.01	0.11 ± 0.02
Betaine:homocysteine methyltransferase	2.6 ± 0.7	2.2 ± 0.1
Methylenetetrahydrofolate reductase	0.16 ± 0.05	0.14 ± 0.02

Liver samples were homogenized in potassium phosphate buffer and centrifuged at 17000g for 30 minutes. All assays were performed on the resulting post-mitochondrial supernatant. Enzyme activity is expressed as nmol/mg protein/min. Means ± SD for 6 rats are shown. The asterisk indicates a significant difference versus control rats, $P < 0.05$.

5.3.4 mRNA levels in the glucagon-treated rats

We measured the mRNA levels for cystathionine β -synthase and cystathionine γ -lyase following glucagon treatment by comparing their abundance to that of β -actin. Glucagon treatment increased cystathionine β -synthase mRNA level by 90% (Figure 5.2). This increase is similar to the increase in enzyme activity. No change was observed in cystathionine γ -lyase mRNA levels.

5.3.5 Allosteric regulators of cystathionine β -synthase

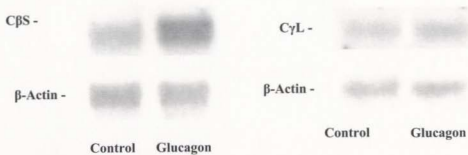
Cystathionine β -synthase can also be regulated allosterically by S-adenosylmethionine and S-adenosylhomocysteine (Finkelstein *et al.*, 1975). We therefore measured the hepatic levels of these modulators. Hepatic concentrations of both S-adenosylmethionine and S-adenosylhomocysteine were elevated following glucagon administration (Figure 5.3). However, the ratio of SAM:SAH, often described as the “methylation ratio”, was unchanged.

5.3.6 Increased flux through transsulfuration following glucagon treatment

Since increased enzyme activity alone cannot be used as a measure of total flux through the transsulfuration pathway, we devised a new method to measure the effect of glucagon treatment on the flux through the transsulfuration pathway in hepatocytes. The second step of the transsulfuration pathway involves the cleavage of cystathionine to produce cysteine, NH_4^+ and α -ketobutyrate (Refer to pathway in

Figure 5.2: Northern Blot analysis of hepatic cystathionine β -synthase and cystathionine γ -lyase following glucagon treatment. Total RNA, isolated from freeze-clamped livers, was separated on a 1% agarose/formaldehyde gel and transferred to a Nylon membrane. Cystathionine β -synthase (C β S), cystathionine γ -lyase (C γ L) and β -actin mRNA levels were determined by Northern Blot analysis (shown in A). mRNA intensities were quantified by densitometry and normalized to β -actin mRNA (shown in B). The data are expressed as mean \pm SD for 4 rats. The asterisk signifies a difference between groups, $P < 0.05$.

(A)



(B)

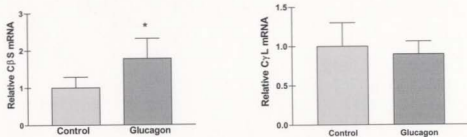


Figure 5.3: S-adenosylmethionine, S-adenosylhomocysteine, and SAM/SAH ratios in control and glucagon-treated rats. Freeze-clamped liver samples were homogenized in 8% trichloroacetic acid. The samples were then centrifuged for 10 minutes at 13000g. S-adenosylmethionine and S-adenosylhomocysteine were determined by HPLC using UV detection. Values are expressed as nmol/g liver. Means \pm SD for 6 samples are shown. An asterisk indicates a significant difference versus control, $P < 0.05$.

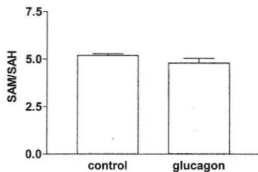
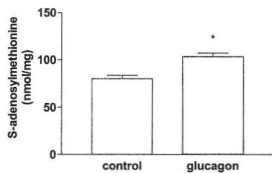
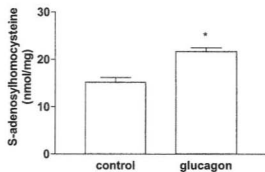


Figure 5.5). Incubating cells with L-[1- ^{14}C]methionine will give rise to α -[1- ^{14}C]-ketobutyrate which may be metabolized via pyruvate dehydrogenase to produce $^{14}\text{CO}_2$, which can be released with acid and readily collected and counted. Label in unmetabolized α -[1- ^{14}C]-ketobutyrate can be released with H_2O_2 . Such $^{14}\text{CO}_2$, released from α -ketobutyrate, must also be included in measures of flux through the transsulfuration pathway (Wyss and Wallimann, 1994). Following incubation with L-[1- ^{14}C]-methionine there was a five-fold increase in $^{14}\text{CO}_2$ production from hepatocytes isolated from the glucagon-treated rats as compared to control cells, indicating a profound activation of flux through the transsulfuration pathway (Table 5.5). To ensure that $^{14}\text{CO}_2$ produced was actually a product of the transsulfuration pathway, propargylglycine, an irreversible inhibitor of cystathionine γ -lyase, was included in the incubations. Blocking cystathionine γ -lyase resulted in an 80% reduction in $^{14}\text{CO}_2$ production from hepatocytes isolated from control and glucagon-treated rats. When hepatocytes were incubated with cyanocinnamate, an inhibitor of the mitochondrial α -ketoacid transporter, there was a reduction in $^{14}\text{CO}_2$ released after the addition of perchloric acid with a corresponding elevation of $^{14}\text{CO}_2$ release after addition of peroxide. These results further confirm $^{14}\text{CO}_2$ production from 1- ^{14}C -methionine as a measure of flux through the transsulfuration pathway and emphasize the need to determine ^{14}C in α -ketobutyrate to fully quantify flux through this pathway.

5.3.7 Glucagon activates methionine transport in isolated hepatocytes

Our calculations show that the hepatic intracellular concentration of methionine was elevated in the glucagon-treated rats (Table 5.2). Of particular interest is that the ratio of liver cellular methionine to plasma methionine was five-fold greater in the glucagon-treated animal. This could be explained by an effect of glucagon on methionine transport. Methionine transport rates were, therefore, measured from hepatocytes isolated from control and glucagon-treated rats. Glucagon treatment resulted in a doubling of methionine uptake into hepatocytes (Figure 5.4).

5.4 DISCUSSION

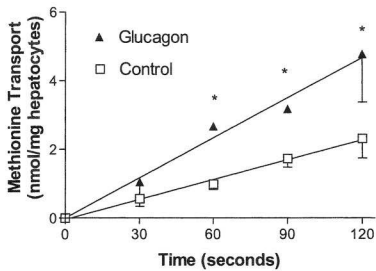
The regulatory effects of glucagon on amino acid metabolism have been well documented. It increases the catabolism of a variety of amino acids (e.g. glycine, glutamine, arginine, and phenylalanine), it increases gluconeogenesis from amino acids, and it increases the rate of ureagenesis (Mabrouk *et al.*, 1998; Squires *et al.*, 1997; Carr and Pogson, 1981; O'Sullivan *et al.*, 2000; Wasserman *et al.*, 1989; Meijer *et al.*, 1990). With this in mind, an investigation of the specific effects of glucagon on homocysteine metabolism is warranted. The dose of glucagon used in this study was indeed pharmacological. We observed a 35-fold increase in plasma glucagon using this dosage, well above circulating levels in control animals. Although these levels are not observed in a healthy organism, patients with glucagon-producing tumours have been reported to have 60-fold increase in plasma glucagon (Barazzoni *et al.*, 1999). Therefore our model is a good reflection of human glucagonoma. This view is

Table 5.5: $^{14}\text{CO}_2$ and 1- ^{14}C - α -ketobutyrate production by isolated hepatocytes incubated with 1-[^{14}C]-methionine.

	Control			Glucagon		
	$^{14}\text{CO}_2$	1- ^{14}C - α -ketobutyrate	Total	$^{14}\text{CO}_2$	1- ^{14}C - α -ketobutyrate	Total
Methionine	3.31 \pm 0.26	2.10 \pm 0.11	5.41 \pm 0.54	8.90 \pm 3.65 [*]	13.7 \pm 4.01 [*]	22.9 \pm 7.61 [*]
Methionine + Propargylglycine	0.62 \pm 0.12 ^a	1.26 \pm 0.11 ^a	1.89 \pm 0.20 ^a	0.49 \pm 0.09 ^{b*}	3.42 \pm 0.09 ^{b*}	3.90 \pm 0.20 ^{b*}
Methionine + Cyanocinnamate	1.1 \pm 0.18 ^a	4.17 \pm 0.58 ^a	5.27 \pm 0.74	5.23 \pm 1.93 ^{b*}	24.2 \pm 3.1 ^{b*}	29.9 \pm 4.99 ^{b*}

Hepatocytes were preincubated for 20 minutes in BSA-supplemented Krebs-Henseleit medium and in the presence and absence of propargylglycine (an inhibitor of CBS) and cyanocinnamate (an inhibitor of the mitochondrial ketoacid transporter). Following the addition of 1 mM 1-[^{14}C]-methionine the flasks were incubated for 30 minutes. The flasks were then equipped with rubber septae in which plastic centre wells containing NCS tissue solublizer were suspended. Incubations were terminated by injection of PCA and $^{14}\text{CO}_2$ was collected for 1 hour. Then a new centre well was added to the flask and H_2O_2 was injected. $^{14}\text{CO}_2$ was collected for another hour and radioactivity measured. The total $^{14}\text{CO}_2$ produced was calculated by adding the $^{14}\text{CO}_2$ collected after the addition of PCA with the $^{14}\text{CO}_2$ collected after addition of H_2O_2 . Data are expressed as nmol $^{14}\text{CO}_2$ produced/mg dry hepatocytes/ 30 minutes. Means \pm SD for 3 measurements are shown. * $P < 0.05$ versus same measurement in control group (Student's t-test), ^a $P < 0.05$ versus control cells incubated with methionine alone (Student's t-test), ^b $P < 0.05$ versus glucagon cells incubated with methionine alone (Student's t-test)

Figure 5.4: Methionine transport in hepatocytes isolated from glucagon-treated and control rats. Hepatocytes were preincubated for 20 mins at 4-6 mg dry weight of cells/ml in a final volume of 2 ml in Krebs-Henseleit medium containing 1.25 % BSA. Following preincubation, 1-¹⁴C-methionine was added to a final concentration of 0.5 mM. At designated time points 1 ml aliquots were transferred to 1.5 ml microcentrifuge tubes containing 0.25 ml silicone oil mixture layered on top of 0.1 ml 6% (v/v) perchloric acid. The tubes were centrifuged at 14000g for 15 seconds to sediment cells through the silicone oil and into the acid layer. The tubes were frozen in liquid nitrogen and then the tubes were cut at the end of the frozen silicone oil layer. The radioactivity in the bottom layer was measured. Means \pm SD are shown for 4 experiments. The transport of methionine was linear in hepatocytes isolated from both the control ($y = 0.019x - 0.042$, $r^2 = 0.78$) and glucagon-treated ($y = 0.039x - 0.002$, $r^2 = 0.89$) rats. An asterisk indicates a value significantly different ($P < 0.05$) from the corresponding control time point value.



supported by the similar decreases in total and specific plasma amino acids in both the glucagon-treated rat and the human glucagonoma patient (Barazzoni et al., 1999). The decrease in amino acids in glucagonoma patients has been linked to increased clearance from the plasma, likely by the liver, rather than to decreased export from the muscles (Barazzoni et al., 1999).

Regulation of the methionine cycle and of the transsulfuration pathway are thought to be exerted primarily through alterations in substrate availability and in the levels of the effector molecules, SAM and SAH. This work demonstrates, for the first time, the effects of the catabolic hormone, glucagon, on these regulatory parameters and on the expression of the rate-limiting transsulfuration enzyme, cystathionine β -synthase. Our *in vitro* and *in vivo* experiments show a dramatic effect of glucagon on the metabolism of the atherogenic amino acid, homocysteine. It is clear, from our data, that glucagon administration intervenes at a number of sites (reviewed in Figure 5.5) in methionine and homocysteine metabolism and, thus, the observed changes in homocysteine are a reflection of these different actions. Firstly, glucagon affects methionine transport. This is evident from the fact that the hepatic intracellular concentration of methionine was increased by 25% and, more impressively, that the ratio of intracellular to plasma methionine was elevated 5-fold. This suggests a marked stimulation of methionine transport into liver cells and, indeed, we found that this was increased two-fold in the glucagon-treated rats. This is consistent with the observation that glucagon up-regulates the System A amino acid transporter since this

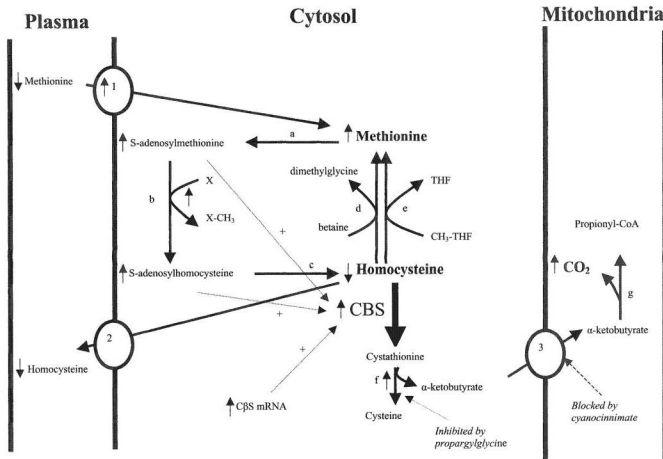
Figure 5.5: Effects of glucagon on hepatic sulphur amino acid metabolism.

↑ Indicates elevation following glucagon treatment. ↓ Indicates reduction following glucagon treatment. + Represents positive regulator of cystathionine β -synthase.

Key:

Transport Processes: (1) glucagon-sensitive methionine transporter; (2) reduced-homocysteine carrier; (3) monocarboxylate transporter

Enzymes: (a) methionine adenosyltransferase; (b) methyltransferases; (c) S-adenosylhomocysteine hydrolase; (d) betaine-homocysteine methyltransferase; (e) methionine synthase; (f) cystathionine γ -lyase; (g) pyruvate dehydrogenase.



is the major hepatic transporter of methionine (McGivan and Pastor-Anglada, 1994). It is likely that the increased hepatic methionine level has important metabolic consequences. In an elegant theoretical analysis of the control of hepatic methionine metabolism, Martinov *et al.* (2000) showed that this system is extraordinarily sensitive to small changes in methionine concentration, largely due to the kinetic properties of the liver-specific S-adenosylmethionine synthetase. Therefore, even in the absence of a detectable increase in activity of this enzyme, it is expected that the increased intracellular methionine concentration will stimulate flux through S-adenosylmethionine synthase which can account for the increased hepatic SAM levels.

The markedly decreased plasma methionine concentration, at a constant dietary intake, implies increased methionine clearance, which is consistent with the general plasma amino acid picture in our rat model, as well as in human glucagonoma patients. This would involve increased flux through glycine N-methyltransferase, whose activity is modestly, though significantly, increased. S-adenosylhomocysteine, produced in the glycine N-methyltransferase reaction, is also increased in the livers of these glucagon-treated rats. This may be partially as a result of the increased flux through glycine N-methyltransferase but must also, in part, reflect its removal through S-adenosylhomocysteine hydrolase. This reaction is thought to operate close to thermodynamic equilibrium *in vivo* (Kloor *et al.*, 1998). Since SAH levels are increased and homocysteine levels are decreased after glucagon treatment, the maintenance of an equilibrium would require the concentration of adenosine, the

other product of S-adenosylhomocysteine hydrolase, to be increased. However, we did not examine this issue.

The increased hepatic SAM and SAH levels, alone, would tend to stimulate flux through the transsulfuration pathway, since both of these nucleotides are allosteric activators of cystathionine β -synthase. They both decrease cystathionine β -synthase's K_m for homocysteine (Finkelstein *et al.*, 1975). However, the increased maximal activities of the two enzymes of the transsulfuration pathway, especially that of the rate-limiting cystathionine β -synthase, also play a major role. Glucagon treatment increased both cystathionine β -synthase activity and mRNA levels. This is likely to be a result of a direct effect on the induction of cystathionine β -synthase. Goss *et al.* (1986) showed that increased cAMP, glucagon's intracellular messenger, leads to an increased cystathionine β -synthase activity in cultured hepatoma cells. As mentioned in chapter 4, we have shown that cAMP increases cystathionine β -synthase mRNA in H4IIE cells. Insulin levels were unchanged in our glucagon-treated rats so that changes in this hormone cannot account for the increased activity of cystathionine β -synthase, although we have shown that insulin treatment of diabetic rats can reduce cystathionine β -synthase activity to normal levels (Chapter 4). It is currently unknown whether glucagon's effects on cystathionine β -synthase are mediated by an increase in gene transcription, enhancement of mRNA stability, or both. It has been reported that liver cystathionine β -synthase mRNA is increased in rats fed a high-protein diet (Yamamoto *et al.*, 1996). Since such a diet is known to elevate plasma glucagon (Peret *et al.*, 1981) our results might provide an explanation

for this dietary regulation of cystathionine β -synthase. Furthermore, hepatic transsulfuration flux and thus cysteine production is stimulated by a high protein meal. This activation of the transsulfuration pathway may be mediated, in part, by the physiological effects of glucagon.

That flux through the transsulfuration pathway is stimulated by glucagon treatment is directly shown in isolated hepatocytes (Table 5.5). These data also provide evidence that the α -ketobutyrate produced by cystathionine γ -lyase must enter mitochondria on the mitochondrial ketoacid transporter for decarboxylation. A key finding is that glucagon treatment caused a four-fold increase in flux through the transsulfuration pathway even though the increased maximal activities of cystathionine β -synthase and cystathionine γ -lyase were less than two-fold. Clearly, additional factors must play a role. In this context, the doubling of the rate of methionine transport into the hepatocyte is key. It is possible that glucagon exerts effects at other sites, since it activates cellular protein kinases. Cystathionine β -synthase does contain a protein kinase A consensus sequence but phosphorylation of this enzyme has yet to be shown. We can eliminate the idea that flux through the transsulfuration pathway is mediated by changes in serine, the co-substrate for cystathionine β -synthase. Indeed, hepatocytes isolated from the glucagon-treated rats exported less homocysteine, when incubated with methionine, than did control hepatocytes. When serine was included in the incubation, homocysteine export was reduced in control hepatocytes but not in the hepatocytes isolated from glucagon-treated rats. However, the possibility that such a mechanism is important *in vivo* is

eliminated by the finding that the hepatic concentration of serine was unaffected by glucagon treatment.

The decreased plasma homocysteine, upon glucagon treatment, follows from the decreased hepatic homocysteine concentration. Very little is known about the transport of homocysteine across hepatocyte membranes. In rat cortical tubules homocystine has been shown to share the γ^+ system transporter, with cystine and the dibasic amino acids (Foreman *et al.*, 1982). The uptake of homocystine is plausible since most of the free homocysteine in plasma exists in an oxidized disulfide form. However, uptake of homocystine by liver cells has not been reported. Furthermore, since hepatocytes have such low activity of the γ^+ transporter another mechanism may be required for homocyst(e)ine uptake (White, 1985). Given the reduced nature of the intracellular milieu (The liver cytoplasmic NADPH/NADP⁺ ratio is about 100 and that of GSH/GSSG is about 150 (Veech *et al.*, 1969; Lakritz *et al.*, 1997)) most of the intracellular homocysteine is believed to be in the reduced form. This necessitates a transporter that recognizes reduced homocysteine to effect homocysteine export. At present this carrier has not been identified. However, glucagon treatment caused no change in the liver/plasma ratio for total homocysteine. Thus it appears that as hepatic homocysteine fell, that in the plasma followed. It is not necessary to ascribe any role to changes in the homocysteine transporter.

Activated flux through the transsulfuration pathway also requires increased disposal of cysteine as this substance does not accumulate (Table 5.2). Pyruvate is the carbon product of cysteine catabolism and is a good gluconeogenic precursor. In view

of glucagon's well-established role in stimulating gluconeogenesis and that gluconeogenesis is the likely fate of much of our dietary amino acids (O'Sullivan *et al.*, 2000; Jungas *et al.*, 1992) we suggest that glucose production is the carbon end-product of cysteine oxidation in these glucagon-treated rats.

This chapter illustrates the effects of glucagon on sulfur amino acid metabolism and provides a plausible explanation of how this catabolic hormone affects circulating homocysteine concentrations. Our previous work (Chapter 4) emphasized the role of insulin in regulating plasma homocysteine. Injection of this anabolic hormone increases plasma homocysteine in the Type 1 diabetic rat. Taken together it is clear that the opposing effects of these metabolic or "fuel" hormones can be very important in controlling plasma homocysteine metabolism and that the liver is the site of this hormonal regulation.

Chapter 6: General Discussion and Conclusions

6.1 General Discussion

Elevated plasma homocysteine is a well-established risk factor for the development of vascular disease (Boushey *et al.*, 1995). Both hypothyroid (Vanhaelst *et al.*, 1967) and diabetic (Ellenberg and Rifkin, 1990) patients have high incidence of vascular disease, therefore much research has focused on the relationship between these diseases and total plasma homocysteine. Several studies have described increased plasma homocysteine concentrations in the hypothyroid patient (Nedrebo *et al.*, 1998; Hussein *et al.*, 1999). Furthermore, homocysteine levels are normalized when a euthyroid state is achieved (Hussein *et al.*, 1999). Similarly, type 1 diabetes mellitus patients have compromised homocysteine metabolism. Diabetic patients with kidney dysfunction tend to exhibit increased plasma homocysteine (Hultberg *et al.*, 1991; Hultberg *et al.*, 1997). On the other hand, type 1 diabetic patients with normal kidney function have decreased plasma homocysteine (Robillon *et al.*, 1994; Cronin *et al.*, 1998). The mechanism for such perturbations was unknown; however, we hypothesized that hormonal regulation of homocysteine metabolism played an important role in the regulation of this atherogenic amino acid. Therefore the objective of this thesis was to investigate the effects of these hormones on homocysteine metabolism using various rat models.

We have shown that the plasma levels of insulin, glucagon, and thyroid hormone influence total plasma homocysteine concentrations in the rat. Our initial purpose was to see if the diabetic rat mimics human type 1 diabetes with respect to homocysteine. Indeed, the streptozotocin-diabetic rat exhibited decreased plasma

homocysteine, as does the human patient (Robillon *et al.*, 1994). Furthermore, insulin-treatment raised plasma homocysteine in the diabetic rat. We also investigated homocysteine metabolism in two hypothyroid rat models. Both hypothyroid rats exhibited decreased total plasma homocysteine. This result contrasts with the human situation where hypothyroidism is associated with increased homocysteine levels (Nedrebo *et al.*, 1998). The reason for this discrepancy was not clear. It is possible that the genes related to homocysteine metabolism respond differently to thyroid hormone in rats as compared to humans. However, it is more likely attributed to kidney dysfunction (Hussein *et al.*, 1999), a common complication in hypothyroid patients, that was not present in our rat models.

Although not immediately germane to our study, other hormones have been shown to alter plasma homocysteine in humans. Several studies have reported that men have higher homocysteine levels than women of childbearing years (Blom *et al.*, 1988; Boers *et al.*, 1983). Following menopause, when estrogen production has ceased, women have higher homocysteine levels than before menopause (Boers *et al.*, 1983; Brattstrom *et al.*, 1985). This increased plasma homocysteine was shown to be normalized by estrogen replacement therapy (Mijatovic *et al.*, 1998, Giri *et al.*, 1998). In an interesting study in the Netherlands, Giltay *et al.* (1998) found decreased homocysteine concentrations in male-to-female transsexuals who received estrogen and antiandrogen treatment. Taken together, the human studies suggest that sex hormones influence plasma homocysteine, however, the mechanism for such regulation has yet to be established. Preliminary studies using male rats have shown

that administration of estradiol or cortisol reduces plasma homocysteine (Kim *et al.*, 1997). Since these hormones influence each other's receptor system (Bunone *et al.*, 1996) and male rats lack female sex organs, one could postulate that these steroid hormones regulate plasma homocysteine by a similar mechanism. We have shown that glucocorticoids induce cystathionine β -synthase in rat hepatoma cells. It is possible that stimulation of the transsulfuration pathway may explain the sterol-induced decrease in plasma homocysteine. Further study is needed to determine if hepatic CBS is influenced by *in vivo* perturbations of glucocorticoid levels. Similarly, the role of estrogen in regulating homocysteine catabolism requires further study.

Many clinical studies have shown a positive correlation between insulin and plasma homocysteine levels (Gallistl *et al.*, 2000; Robillon *et al.*, 1994; Meigs *et al.*, 2001). In agreement with the assumption that insulin raises homocysteine levels, we have demonstrated an increased activity of hepatic transsulfuration enzymes and concurrent decreased homocysteine levels in the streptozotocin-induced diabetic rat. This effect was reversed by insulin treatment. The *in vivo* effect of insulin was, at least in part, due to suppression of hepatic cystathionine β -synthase gene expression. To support our *in vivo* data, we showed, for the first time, the direct stimulation of CBS gene transcription by glucocorticoids and cAMP (a second messenger of glucagon) in rat hepatoma cells. The finding that insulin antagonized this stimulation of CBS is consistent with the fact that the actions of glucocorticoids and glucagon in the regulation of protein, carbohydrate, and lipid metabolism are commonly opposed

by insulin (O'Brien *et al.*, 1991; Hauner *et al.*, 1987; Nebes and Morris, 1988). A role of insulin in regulating human CBS has recently been shown in human hepatoma cells. Insulin treatment reduced CBS activity by 70% in HepG2 cells (Ratnam *et al.*, 2002). The mechanism of insulin's action was investigated by fusing the CBS gene-promoter to a luciferase-reporter gene. Treatment of transfected cells with insulin resulted in a massive decrease in luciferase activity thereby showing that insulin decreases CBS activity through insulin sensitive sequences on the CBS gene. These results provide evidence that the hormonal regulation of CBS is similar in human and rodents (Ratnam *et al.*, 2002).

The role of cystathionine β -synthase in regulating plasma homocysteine levels is evident in mice and humans. Watanabe *et al.* (1995) have shown that transgenic mice, heterozygous for a mutant cystathionine β -synthase, have only half the normal activity of this enzyme and twice the normal plasma homocysteine level, while Down's syndrome patients, who have an extra allele of the cystathionine β -synthase gene, tend to have very low plasma homocysteine concentrations (Brattstrom *et al.*, 1989). Furthermore, the results from our glucagon-treated rat have provided evidence for the role of hepatic CBS in regulating plasma homocysteine. Glucagon administration increased hepatic CBS mRNA, activity and flux resulting in decreased plasma homocysteine concentrations in the rat.

From our *in vivo* and *in vitro* experiments we can postulate a physiological role for insulin and glucagon in regulating homocysteine metabolism. It seems that methionine is conserved during insulin excess. The extra methionine may be required

as a substrate for protein synthesis, which is stimulated by elevated insulin levels. In contrast, when methionine is in excess, such as after a high protein meal, there is an increase in hepatic transsulfuration flux (Finkelstein and Martin, 1986). This is relevant since plasma glucagon levels are elevated following a high protein meal (Linn *et al.*, 2000). It is not inconceivable that the combined actions of insulin and glucagon provide the underlying mechanism for altered hepatic transsulfuration flux in response to dietary changes. It is also likely that the transsulfuration pathway is activated in parallel with gluconeogenesis since the product of the transsulfuration pathway, pyruvate, is an important gluconeogenic precursor. In addition, the hormonal regulation of cystathionine β -synthase is similar to that of phosphoenolpyruvate carboxykinase, the rate-limiting step in gluconeogenesis (Sasaki *et al.*, 1984). Further evidence arises from diabetic (Hargrove *et al.*, 1989) and hyperglucagonemic rats (**Chapter 5**). Both models have elevated flux through the transsulfuration and gluconeogenic pathways (Noguchi *et al.*, 1993).

The role of glucocorticoids in regulating sulfur amino acid metabolism may be related to stress or trauma. It has been shown that whole body methionine catabolism and glutathione production was increased in the septic rat (Malmezat *et al.*, 2000). In hepatoma cells, glutathione production was increased during oxidative stress with 50% of the glutathione's cysteine derived from homocysteine (Mosharov *et al.*, 2000). It is possible that glucocorticoids, which are elevated during sepsis, stimulate methionine catabolism, through actions on MAT (Gil *et al.*, 1997) and CBS, in order to increase production of cysteine (an important substrate in glutathione

production). In such situations maintenance of the glutathione pool may be vital to limiting oxidative damage.

6.2 Conclusions

The central theme of this thesis is the hormonal regulation of homocysteine metabolism. Using *in vivo* and *in vitro* techniques we made the following key observations and conclusions:

1. Total plasma homocysteine is reduced in two rat models of hypothyroidism. This decrease in plasma homocysteine was associated with increased hepatic activity of the transsulfuration enzymes as compared to their respective controls. In addition, thyroid replacement therapy to thyroidectomized rats normalized both plasma homocysteine and hepatic enzyme activities. These results suggest that alterations in the hepatic transsulfuration may contribute to plasma homocysteine levels in the hypothyroid rat. In our studies the rat did not mimic the human situation and therefore cannot be used as a model to study homocysteine metabolism in the hypothyroid patient.

(Jacobs RL, Stead LM, Brosnan ME, and Brosnan JT (2000) Can J Phys and Pharm. 78, 565-570)

2. The streptozotocin-induced diabetic rat exhibit decreased plasma homocysteine as does the human type 1 diabetic patient. This decrease in plasma homocysteine was reversed (partially or completely) by insulin-treatment. The hepatic activities of enzymes responsible for converting methionine to cysteine were increased in the untreated-diabetic rat. These changes were normalized by insulin-treatment. Therefore, it is likely that increased hepatic catabolism of homocysteine is responsible for the decrease in plasma homocysteine observed in the untreated-diabetic rat.

(Jacobs RL, House JD, Brosnan ME, and Brosnan JT. (1998) Diabetes. 47:1967-1970)

3. Increased renal homocysteine catabolism cannot be responsible for the decreased plasma homocysteine in the diabetic rat. This conclusion is supported by two lines of evidence: first, the renal activities of the transsulfuration enzymes were not elevated in the untreated-diabetic rat and second, the proportion of plasma homocysteine metabolized by the kidney *in vivo* was not altered by streptozotocin-administration.

(Jacobs RL, House JD, Brosnan ME, and Brosnan JT. (1999) Diabetes. 47:1967-1970 and unpublished data)

4. Hepatic cystathionine β -synthase mRNA levels were elevated in the untreated-diabetic rat. This induction was reversed by insulin within 1 day of treatment. Studies using H4IIE cells provide convincing evidence for the direct suppression of CBS gene transcription by insulin and its induction by counter-regulatory effectors (such as glucocorticoids and c-AMP). By measuring CBS activity, protein concentrations and mRNA levels in rat hepatoma cells we showed that the action of insulin is dominant over the other hormones.

(Ratnam S, Mclean K, Jacobs RJ, Brosnan, ME Kraus J, and Brosnan JT. (2002) J Biol Chem. ePub Aug 26.)

6. Hyperglucagonemic rats exhibited decreased total plasma homocysteine levels. This was the first report to show a role for glucagon in regulating plasma homocysteine concentration.

7. We have provided a detailed explanation how glucagon stimulates hepatic sulfur-amino acid catabolism resulting in decreased plasma homocysteine levels. Hepatic activities of the transsulfuration enzymes were elevated by glucagon treatment. When incubated with methionine, hepatocytes isolated from glucagon-treated animals exported less homocysteine and converted more methionine to cystathionine than did control cells. This increased flux through CBS may be explained by increased hepatic transport of methionine, which raised the hepatic levels of important allosteric

regulators of CBS (S-adenosylmethionine and S-adenosylhomocysteine). These results indicate that glucagon can regulate plasma homocysteine through the effects on the hepatic transsulfuration pathway.

(Jacobs RL, Stead LM, Brosnan ME, and Brosnan JT (2001) J Biol Chem 276:43740-43747)

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