IMPACT OF VITAMIN B_6 DEFICIENCY ON TRANSSULPHURATION IN EARLY WEANED PIGS

A Thesis

Submitted to the Faculty

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By

ZHENBIN ZHANG

In Partial Fulfillment of the

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FACULTY OF GRADUATE STUDIES

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FOREWORD

This thesis was prepared following a manuscript format. There are four manuscripts. Manuscripts I and II were submitted to ANIMAL Journal and has been accepted. Manuscript III is a pilot study. Manuscript IV is under preparation. All manuscripts are formatted to meet the guidelines for the Journal of Animal Science.

ABSTRACT

Transsulphuration is a metabolic pathway in which homocysteine is metabolized to give rise to cysteine, a precursor for endogenous synthesis of glutathione and taurine *in vivo*. The reduced form of glutathione plays a critical role in maintaining reducing intracellular red-ox environment. Pyridoxal 5'-phosphate (PLP), the major biological form of vitamin B_6, serves as a cofactor for key enzymes in the transsulphuration pathway and one-carbon metabolism. Therefore, studies examining the nutritional regulation of transsulphuration via vitamin B_6 deficiency are of importance to the health of humans and young animals. In the first study, changes in biochemical indices of sulphur amino acid metabolism were characterized in piglets subjected to dietary vitamin B_6 depletion. Despite the use of a pair-feeding design, growth and feed efficiency in B_6 deficient pigs were lower than control pigs starting from the fourth week of depletion. Pigs consuming a vitamin B_6 deficient semi-purified diet showed reduced plasma PLP concentrations throughout the 6-week experiment. Decreases in hepatic cystathionine beta synthase (CBS), cystathionine gamma lyase (CGL), serine hydroxymethyltransferase (SHMT) activities and hepatic and plasma cysteine concentrations due to vitamin B_6 deficiency were observed. By contrast, severe hyperhomocysteinemia was observed starting from the fourth week of depletion. At the end of the 6^{th} week of depletion, plasma methionine and serine concentrations were increased while glycine concentration was decreased by vitamin B_6 depletion. A depletion-repletion study was then conducted to further characterize biochemical indices of sulphur amino acid metabolism in response to graded levels of vitamin B_6 repletion (0.75, 1.5, 2.25, 3 mg/kg pyridoxine•HCl) in pigs deficient of vitamin B_6. Significant dose dependent increases in plasma PLP and cysteine, and decreases in homocysteine to

vitamin B_6 repletion were demonstrated, and these were sensitive to the duration of repletion. To have a better understanding of the quantitative impact of vitamin B_6 deficiency on transsulphuration, kinetic measures of cysteine metabolism were undertaken. In a pilot study, a stable isotope model using L-[2,3,3-d_3]serine and L-[3-^{13}C]cysteine to quantify the rate of transsulphuration (TS), was established. The model was preliminarily established with constant infusion doses for L-[3-^{13}C]cysteine and L-[2,3,3-d_3]serine at 7.5 μmol/(kg•h) and 60 μmol/(kg•h), respectively. This study was used to establish infusion doses for the measurement of serine and cysteine kinetics. Results from the pilot study were then incorporated into a study designed to examine the effect of vitamin B_6 deficiency on serine and cysteine kinetics. The results show that whole body serine and cysteine fluxes were reduced by vitamin B_6 deficiency. Due to the extensive dilution of hepatic serine label, transfer to cysteine was not measurable in either group, and therefore the rate of transsulphuration could not be calculated. However, reductions in enzyme activity support impairment in TS that may have led to a reduction in cysteine rate of appearance. In summary, the current research has led to the development and characterization of a model of vitamin B_6 deficiency in the pig that can be used to establish quantitative estimates of the impact of vitamin B_6 status, as well as other nutrients, on whole body sulphur amino acid metabolism.

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LIST OF ABBREVIATIONS

SAA	Sulphur amino acids
TS	Transsulphuration rate
PLP	Pyridoxal 5'-phosphate
CH_3THF	5-methyltetrahydrofolate
CH_2THF	5,10-methylenetetrahydrofolate
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
THF	Tetrahydrofolate
Betaine	Trimethylglycine
GLY	Glycine
SER	Serine
GSH	Reduced form of glutathione
CBS	Cystathionine beta synthase
CGL	Cystathionine gamma lyase
SHMT	Serine hydroxymethyltransferase
MS	Methionine synthase
BHMT	Betaine:homocysteine methyltransferase
MTHFR	5,10-methylenetetrahydrofolate reductase
MAT	Methionine adenosyltransferase
GNMT	Glycine *N*-methyltransferase
SPT/AGT	Hydroxy pyruvate via serine animotransferase

HPLC	High performance liquid chromatography
GCMS	Gas chromatography mass spectrometry
LC-MS/MS	Liquid Chromatography-Mass Spectrometry/Mass Spectrometry
Fcys	Cysteine flux
d_3	$[^2H_3]$
d_2	$[^2H_2]$
TCA	Tricarboxylic acid
TCEP	Tris-carboxyethylphosphine
DTT	Dithiothreitol
SBDF	7-Fluorobenzofurazan-4-sulfonic acid ammonium
ADFI	Average daily feed intake
ADG	Average daily gain
FCE	Feed conversion efficiency
BW	Body weight
ESRD	End-stage renal disease
KIC	α-ketoisocaproate
ATP	Adenosine tri-phosphate
NADH	Nicotinamide adenine dinucleotide
RCB	Randomised Complete Block
GLM	Generalized linear model

CHAPTER ONE

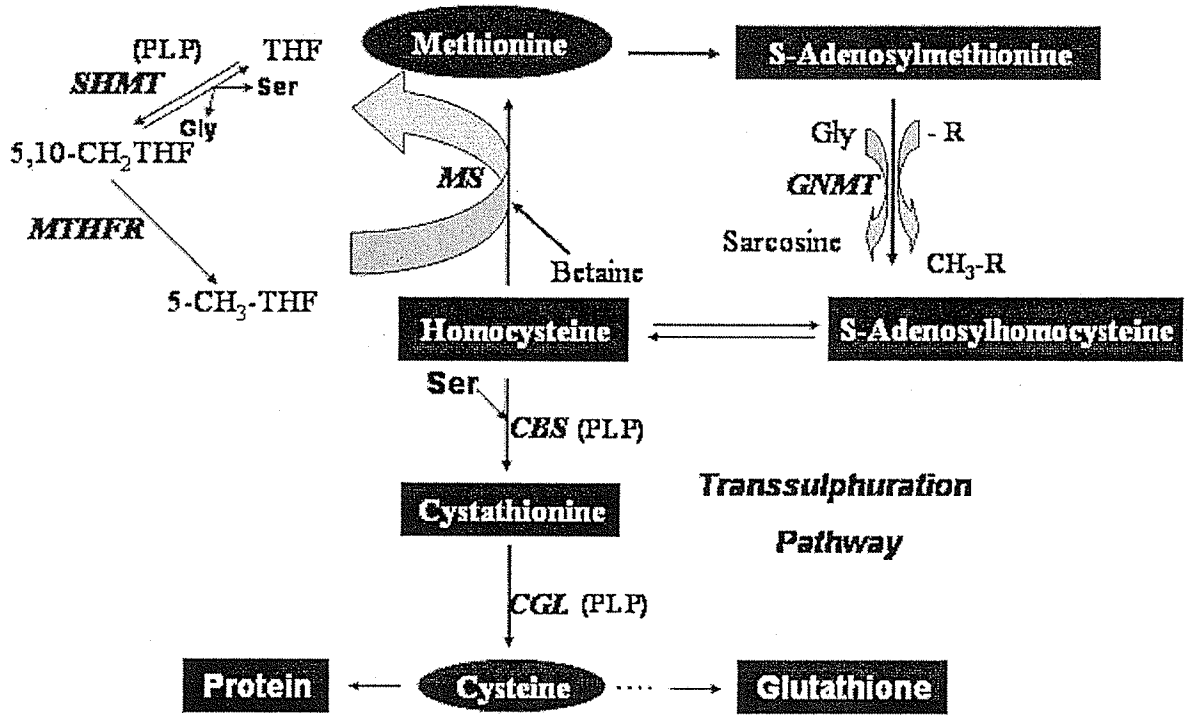
GENERAL INTRODUCTION

The sulphur amino acids (SAA) methionine, cysteine and homocysteine have been an important research topic in the last two decades due to the nutritional and physiological roles of SAA and their metabolism in relation to the maintenance of body function and health (Brosnan and Brosnan, 2006). Both methionine and cysteine serve as components of body protein. The former is well recognized as an essential amino acid in mammals and the latter is believed to be conditionally essential in preterm infants or under circumstances that immune system improvement needs to be addressed (Zlotkin and Anderson, 1982). As depicted in Figure 1, methionine is metabolized through the transmethylation, remethylation and transsulphuration pathways. Methylation reactions in the transmethylation pathway play an important role in the synthesis of small molecules such as, creatine, phosphatidylcholine, and epinephrine, modification of macromolecules (e.g. protein, DNA, RNA, lipid), detoxification of xenobiotics (e.g. thiols, arsenite) and inactivation of neurotransmitters (e.g. epinephrine, norepinephrine, dopamine) (Brosnan et al., 2007). The remethylation pathway is one of the major metabolic pathways that remove homocysteine, the non-protein producing thiol associated with chronic diseases including cardiovascular heart disease, neural disease and renal disease (Wilcken and Wilcken, 2001). The transsulphuration pathway is also an important catabolic route for homocysteine clearance. At the same time, it is an anabolic pathway for the endogenous production of cysteine, the precursor for

endogenous synthesis of glutathione and taurine. The transsulphuration pathway is therefore, of importance to the health of humans and animals due to its role in supplying cysteine for the synthesis of glutathione (GSH), a major regulator of a reduced cellular environment, and a mediator of many physiological reactions, such as, detoxification of xenobiotics (Franco et al., 2007).

Due to the significance of the transsulphuration pathway to nutrition and health, its regulation is of key importance. However, our understanding of the factors regulating the production of cysteine through the transsulphuration pathway is incomplete. The use of alternative models, such as the measurement of whole body rates of sulphur amino acid metabolism employing isotope dilution principles, provides opportunities to further understand the regulation of the transsulphuration pathway. The research presented in this thesis describes the development and characterization of a piglet model of impaired transsulphuration and its use to establish kinetic measures of the rates of sulphur amino acid metabolism *in vivo*.

Figure 1 Sulphur amino acid metabolism



Note: For abbreviations: Gly = Glycine, Ser = Serine, PLP = Pyridoxal 5'-phosphate, CBS = Cystathionine beta synthase, CGL = Cystathionine gamma lyase, MS = Methionine synthase, MTHFR = 5,10-methylenetetrahydrofolate reductase, SHMT = Serine hydroxymethyltransferase, GNMT = Glycine *N*-methyltransferase, THF = Tetrahydrofolate, CH_3THF = 5-methyltetrahydrofolate, CH_2THF = 5,10-methylenetetrahydrofolate

CHAPTER TWO

LITERATURE REVIEW

2.1 Sulphur Amino Acid Metabolism

2.1.1 General considerations

Sulphur amino acids (SAA) are amino acids containing sulphur atoms on the side chains. Methionine and cysteine are the two SAA, which are generally recognized as being incorporated into proteins (Nelson and Cox, 2000). The structures of these two amino acids are CH_3-S-CH_2-CH_2-$CHNH_2$-COOH and SH-CH_2-$CHNH_2$-COOH, respectively. In the early 1930s a new SAA sharing similar chemical properties to cysteine and cystine was discovered (Butz and du Vigneaud, 1932) and named "homocystine", an oxidized form of homocysteine. Three decades later homocysteine was detected in plasma of patients with homocystinuria (Carson et al., 1963). The structure of homocysteine is identified as SH-CH_2-CH_2-$CHNH_2$-COOH.

The SAA methionine and cysteine can be utilized to synthesize body proteins. At the same time, SAA and their metabolites play a significant role in maintaining biological and physiological body functions and health through a series of metabolic pathways termed transmethylation, remethylation, remethylation-related folate cycle and transsulphuration.

2.1.2 Transmethylation

As shown in Figure 1, methionine, derived from the diet, body protein breakdown or endogenous synthesis, is activated by ATP to generate S-adenosylmethionine (SAM),

which serves as a universal methyl donor. This reaction is catalyzed by methionine adenosyltransferase (MAT), which has three isozymes: I, II, III. MAT-I (α) is slightly inhibited by SAM and comprises of 15% of the total MAT activity; MAT-II (γ) is strongly inhibited by SAM and comprises of 5% of the total MAT activity; MAT-III (β) is favored by elevated SAM and comprises of the majority of the total MAT activity (Stipanuk, 1986). Transmethylation occurs through a reaction that gives rise to S-adenosylhomocysteine (SAH) and methylated compounds. The transmethylation reaction is actually catalyzed by a large number of methyltransferases, such as guanidinoacetate *N*-methyltransferase and phosphatidylethanolamine *N*-methyltransferase (Stead et al., 2006). Furthermore, when methionine intake is high, the enzyme glycine *N*-methyltransferase (GNMT) will be favoured, yielding the methylated product sarcosine (Mudd et al., 1980). GNMT activity is found in liver and pancreas with lower amounts in kidney (Yeo and Wagner, 1994). Most of the methyltransferases are inhibited by their product, SAH (Stipanuk, 1986). SAH is hydrolyzed to adenosine and homocysteine by SAH hydrolase. It is noted that this hydrolysis is a reversible reaction that is inhibited by the increased concentrations of either homocysteine or adenosine (Stipanuk, 1986). As a result this inhibition of SAH hydrolysis, SAH and SAM accumulate. Homocysteine may be used for the resynthesis of SAH, remethylated to methionine through the remethylation pathway, converted to cysteine by the transsulphuration pathway, or be exported from the cell. Each of these routes will be discussed further in the following sections.

The significance of transmethylation pathway lies in methylation reactions, which play critical roles in the synthesis of small molecules such as, creatine,

phosphatidylcholine, and epinephrine, modification of macromolecules (e.g. protein, DNA, RNA, lipid), detoxification of xenobiotics (e.g. thiols, arsenite) and inactivation of neurotransmitters (e.g. epinephrine, norepinephrine, dopamine) (Brosnan et al., 2007). Choline and betaine are "indirectly" sources of methyl groups for SAM. The transmethylation pathway is also an intermediary route for methionine metabolism, necessary for the removal of excessive methionine and the production of cysteine *de novo*. Disorders of methylation have been shown to cause health problems in humans. For example, in the case of mutations in guanidinoacetate methyltransferase in children, creatine synthesis is impaired, thus leading to mental retardation, speech delay and epilepsy (Stromberger et al., 2003).

2.1.3 Homocysteine

As mentioned above, the transmethylation pathway produces *homocysteine*, a non-protein producing amino acid. This compound has the chemical property of autooxidation with itself or other thiols such as cysteine to form mixed disulfides. As such, only a very small fraction of homocysteine exists as reduced form (≤ 1%) and the major proportion is protein-bound homocysteine in plasma (>70%) (Jacobsen, 2001). Homocysteine analysis has been thoroughly reviewed from the clinical perspective (eg: Refsum et al., 2004). Due to the challenges associated with the measurement of free homocysteine, total homocysteine has been widely determined and reported. The methods for homocysteine analysis include 1) immunoassays 2) capillary gas chromatography 3) capillary gas chromatography-mass spectrometry 4) capillary electrophoresis 5) liquid chromatography electrospray tandem mass spectrometry; and 6) high performance liquid chromatography (HPLC) with fluorescence, electrochemical or

colorimetric detection (Rasmussen and Møller, 2001). Among the above methodologies, HPLC is commonly used.

Hyperhomocysteinemia refers to a condition of increased circulating concentrations of total homocysteine, a value most commonly defined as higher than 15 μmol/L in humans. Epidemiological studies have shown that elevated plasma homocysteine levels were associated with an increased risk for certain diseases, including cardiovascular heart disease, Alzheimer's disease (Selhub, 1999; Stipanuk, 2004), hepatic steatosis (Namekata et al., 2004), renal disease (Guldener et al., 2001), neural tube defects (Selhub, 1999), ischemic and hemorrhagic stroke (Van Beynum et al., 1999; Hogeveen et al., 2002), inflammatory bowel disease (Danese et al., 2005) and female reproductive function (Guzmán et al., 2006). It follows that homocysteine is generally considered to be a "bad thiol".

While addressing the side effects of homocysteine on health, we should bear in mind that homocysteine has the following important biological functions: 1) it serves as a precursor for cystathionine, cysteine and further metabolites; 2) it functions in the methionine conversation cycle; 3) it serves as a methyl receptor in the betaine-homocysteine methyltransferase reaction, an obligatory step in choline catabolism; and 4) it is an essential substrate for the recycling of tissue folates (Finkelstein and Martin, 2000). Homocysteine is metabolized through the remethylation and transsulphuration pathways as detailed in the sections below.

2.1.4 Remethylation

The remethylation pathway is one of the two routes that remove homocysteine produced from methionine through the transmethylation pathway. Remethylation is a

process in which homocysteine accepts methyl groups from 5-methyltetrahydrofolate (CH_3THF) or betaine to generate methionine via methionine synthase (MS) or betaine:homocysteine methyltransferase (BHMT), respectively. MS, a ubiquitously distributed enzyme, utilizes CH_3THF and homocysteine to produce methionine and tetrahydrofolate (THF) with vitamin B_{12} as co-factor. This represents the folate-dependent remethylation pathway. CH_3THF is available from the folate cycle as discussed in the next section. BHMT, a Zn^{2+}-dependent thiomethytransferase, is mainly distributed in liver and kidney, with minor levels of expression in pancreas and lens (DelgadoReyes et al., 2001;Pajares and Pérez-Sala, 2006). This enzyme catalyzes the remethylation of homocysteine using betaine as the methyl donor. An *in vitro* model in rat liver provided evidence that the BHMT reaction processes 25% of the cellular homocysteine (Finkelstein and Martin, 1984). However, a study by Davis et al., employing stable isotope dilution techniques, showed that serine was the major methyl group donor while choline and betaine offered minor amounts of methyl groups for total body methylation (Davis et al., 2004). Betaine (trimethylglycine) is derived from dietary betaine and through choline via oxidation by choline dehydrogenase. The BHMT reaction is inhibited by its product, dimethylglycine (Finkelstein, et al., 1972; 1983; 1984).

In addition to the removal of homocysteine, remethylation conserves homocysteine as methionine, which can be used for either body protein synthesis or as a precursor of SAM that provides methyl groups for methylation reactions in the body. Furthermore, the remethylation pathway plays a role in shuttling CH_3THF. The metabolic phenomenon of "methyl trap" is a result of impairment of vitamin B_{12}-dependent remethylation (Herbert and Zalusky, 1962).

2.1.5 Folate cycle

The folate cycle is a biological process to provide CH_3THF, which is utilized for the remethylation of homocysteine. Folic acid from the diet is catalyzed by dihydrofolate reductase to THF (House et al., 2003), which then acquires a one-carbon unit from serine with the resultant production of 5,10-methylenetetrahydrofolate (CH_2THF) and glycine. This is a reversible reaction catalyzed by serine hydroxymethyltransferase (SHMT) requiring 5'-pyridoxal phosphate (PLP), the major biological vitamin B_6 in tissues, as co-factor. SHMT exists in both mitochondrial and cytosolic compartments in cells (Garrow et al., 1993). CH_2THF is further reduced by 5,10-methylenetetrahydrofolate reductase (MTHFR) to CH_3THF, using NADH as the electron donor. CH_3THF, the product of the folate cycle, serves as a methyl donor for homocysteine remethylation as mentioned above.

The biological significance of the folate cycle is directly associated with the following perspectives: 1) this cycle supplies one-carbon units, through one-carbon metabolism, for essential processes including amino acid metabolism, purine and pyrimidine synthesis, and the formation of the methylating agent SAM; 2) the folate cycle generates CH_3THF, a form of folate used in B_{12}- dependent remethylation (Bailey and Gregory, 2006).

2.1.6 Transsulphuration

The transsulphuration pathway is the other primary route to remove homocysteine, especially at concentrations of homocysteine that exceed the capacity of the homocysteine methyltransferases, and it represents the only route of irrevocable

homocysteine loss (Stipanuk, 2004). Two PLP dependent enzyme reactions are involved in this pathway. Cystathionine beta synthase (CBS), the first key enzyme in this pathway, condenses serine with homocysteine to form cystathionine. Cystathionine gamma lyase (CGL), the second enzyme, cleaves cystathionine, a 7-carbon thiol, into cysteine, ammonia and alpha-ketobutyrate. The latter goes to tricarboxylic acid (TCA) cycle at the level of succinyl-CoA after decarboxylation to give rise to CO_2. Cysteine, the end product of transsulphuration pathway, can either be used for protein synthesis or serve as a precursor for the generation of glutathione, taurine, and sulphate (Stipanuk, 2004). Transsulphuration enzymes are found in liver, kidney, intestine, pancreas (Brosnan et al., 2007), brain (Visvitsky et al., 2006), and eyes (Persa et al., 2004,2006).

As implied from the pathway, a major function of the transsulphuration pathway is to catabolize methionine and homocysteine. According to the review article by Stipanuk, perturbations of this pathway from substrates, cofactors, effectors or enzymes cause abnormal levels of homocysteine, SAM, SAH, cystathionine and cysteine (Stipanuk, 2004). It follows that transsulphuration pathway plays an inevitable role in maintaining appropriate homocysteine concentrations.

Beyond maintaining homocysteine concentrations, the transsulphuration pathway serves as a critical route for the endogenous production of cysteine. Cysteine serves as a precursor for generating glutathione, taurine and sulfate, which are involved in synthetic reactions, detoxification processes, osmotic regulation, nervous system function and antioxidative process/free radical defenses (Stipanuk et al., 2002). As such, in addition to homocysteine clearance, transsulphuration has significant importance in maintaining

body function and health through the provision of cysteine. Therefore, an understanding of the factors regulating transsulphuration is important for optimal animal health.

2.2 Regulation of transsulphuration

As discussed above, transsulphuration is a series of enzymatic reactions by which the sulphur atom from 5-C methionine is transferred to the serine carbon skeleton to give rise to 3-C cysteine. Therefore, any factors affecting enzymes, metabolites, or effectors in the metabolic pathways play critical roles in regulating transsulphuration.

2.2.1 Age

The rat study by Finkelstein and Mudd (1967) showed that hepatic and renal CBS and CGL activities were significantly higher in weanling rats compared to those in the suckling animals and were relatively constant in the older rats. This implies that the capacity for transsulphuration increases with age based on enzyme function. At the same time, as compared with the young mice, old mice demonstrated a higher magnitude of increases in homocysteine concentrations than cystathionine concentrations. This implies that age impacts the conversion of homocysteine to cystathionine as evident by less efficient conversion in older mice (Toroser and Rajindar, 2007). In other words, the CBS reaction was affected by age. In pigs, gradual increases in hepatic CBS activities were observed from birth through to 26 days of age. CGL activities, however, demonstrated a rapid spike in activity, followed by an immediate decline to a stable level in developing pigs (Ballance, 2004). Overall, developmental changes in transsulphuration enzymes demonstrated an increase in enzymatic capacity for transsulphuration that reaches mature levels within the first weeks after birth.

2.2.2 Regulation by redox status

Redox regulation of the transsulphuration pathway occurs at the level of CBS, which contains a heme that may serve as a sensor of oxidative environment (Banerjee and Zou, 2005). Reduction of the heme moiety has been associated with decreased CBS activity. By contrast, flux through CBS was increased under oxidizing conditions (e.g., H_2O_2 or t-butylhydroperoxide) (Mosharov et al., 2000; Zou and Banerjee, 2003). For instance, addition of 100 μM H_2O_2 or tertiary hydroperoxide to human hepatoma cells increased cystathionine production 1.6 and 2.1-fold, respectively (Mosharov et al., 2000). At the same time, homocysteine flux through the transsulphuration pathway exhibited a positive relationship with the oxidant concentrations.

2.2.3 Hormones

The investigation on the effects of different hormones on MAT, CBS and CGL activities in liver, kidney, pancreas, and brain in rats shows that there was lack of parallel changes in the three enzymes in one organ or in a single enzyme in all organs by hormones (Finkelstein, 1967). Specifically, hepatic CBS was increased by hydrocortisone, alloxan, and glucagon, decreased by insulin (Jacobs et al., 2001; Ratnam et al., 2002), but not altered by thyroxine, estradiol, progesterone, testosterone, and growth hormone. Hepatic CGL was enhanced by estradiol, progesterone and glucagons, but lowered by thyroxine (Finkelstein, 1967).

2.2.4 Regulation in pathological states

A genetic defect in CBS was first reported in 1964 (Mudd et al.). When there is a defect in CBS, transsulphuration is severely impaired. As a result of this perturbation of transsulphuration, methionine oxidation and the formation of transsulphuration products and the removal of homocysteine will be decreased. The biochemical consequences of a

CBS defect include hyperhomocysteinemia or homocystinuria, decreases in cystathionine and cysteine and increases in SAM, SAH and sarcosine. Homocystinuria was detected in patients with genetic defects in CBS, MS, and MTHFR (Finkelstein, 1974). Heterozygous disruption of CBS in rats lowered cysteine and glutathione concentrations (Visvitsky et al., 2004). However, the association between genetic variation in the CBS gene (699C-T, 1080C-T and 5697 (GT) STR) and increased homocysteine concentration was not detected (Lievers et al., 2003). Similarly the defect of CGL causes the accumulation of cystathionione in tissues, mild to moderate hyperhomocysteinemia, and a normal range of cysteine (Finkelstein, 2006).

The kidney plays a significant role in homocysteine metabolism mainly through the transsulphuration pathway (House et al., 1999). Diminished renal function, as indicated by reduced glomerular filtration rate, is inversely correlated with plasma homocysteine concentrations over almost the entire renal function range, which suggests a role for the kidney in homocysteine metabolism (Wollesen et al., 1999). The pathogenesis pertaining to the above relationship is not fully understood. A study using stable isotope methodology in patients with end-stage renal disease (ESRD) showed that homocysteine clearance by transsulphuration, as defined by the ratio of transsulphuration rate and homocysteine concentration, was considerably lower in ESRD subjects than those observed in healthy controls ($P<0.001$) (Stam et al., 2004). This indicates that an impairment of renal function decreases transsulphuration.

Elevated homocysteine concentrations in plasma have been linked to increased cardiovascular heart disease mortality, increased incidence of stroke and heart failure, increased incidence of dementia and Alzheimer's disease, and increased incidence of hip

fracture in older people (Selhub, 2006). While the nature of the association continues to be investigated, a potential link may be related to impairments in methylation reactions or in the supply of products through the transsulphuration pathway. In addition to the knowledge that the key enzymes in the transsulphuration pathway are involved in the clearance of homocysteine, the transsulphuration enzyme of CBS has been reported to be responsible for the production of hydrogen sulphide through an alternative pathway consuming homocysteine (Szabo, 2007).

2.2.5 Nutritional regulation

2.2.5.1 Amino acids

Methionine and cysteine: Evidence from studies of enzyme kinetics shows that the K_mS of transsulphuration enzymes for their substrates are higher than those enzymes in remethylation and transmethylation cycles (Stipanuk, 2004). Thus, remethylation is favoured over transsulphuration at low concentrations of homocysteine. In the case of low dietary methionine supply, transsulphuration is not favored due to the low SAM concentration, which is not able to activate CBS (Selhub, 1999). On the contrary, when dietary methionine is high, the resulting increase in intracellular SAM concentration will be involved in a) inhibition of MTHFR, which causes the reduction of CH_3THF and remethylation consequently; and b) activation of CBS, which enhances transsulphuration. As such, homocysteine transsulphuration is promoted over remethylation. Finkelstein (1967) reported the enhancing effect of increasing protein on hepatic MAT, CBS and CGL activities, which demonstrates the improved capacity to remove the excess methionine. High dose of methionine has been documented to be "toxic" in animals (Benevenga and Steele, 1984) but not serious in humans (Garlick, 2006). Interestingly,

homocysteine concentrations were found to be higher in rats consuming low casein diets (10%) versus those consuming high casein diets (30%), and CBS activity was reduced by low protein feeding (Okawa et al., 2006).

Cysteine: A growing volume of evidence shows that cysteine has a sparing effect on methionine requirement (Womack and Rose, 1941; Rose and Wixom, 1955; Reynolds et al., 1958; Clark et al., 1970). The proposed mechanism for this response relates to the effect of dietary cysteine on decreasing transsulphuration through changes in CBS messenger RNA transcription and thus enzyme activity (Finkelstein and Mudd, 1967; Finkelstein et al., 1988). As such, reductions in methionine catabolism occur. In their studies, di Buono et al. (2001a,b) determined the mean methionine requirement by indicator amino acid oxidation in the presence of dietary cysteine, and showed that it was 64% lower than that in the absence of excess dietary cysteine. However, studies using isotope dilution techniques in healthy adult men did not detect the sparing effect of cystine, the oxidized form of cysteine, on methionine requirements (Hiramatsu et al., 1994; Raguso et al., 1997). Okawa et al. (2007) recently reported a reduction in homocysteine concentrations in rats by supplementing cysteine in low-casein diets, but not in high-casein diets. This observation demonstrates that the impact of cysteine on homocysteine concentration is dependent on dietary protein levels.

Serine and glycine: Both serine and glycine are sources of one carbon units for folate metabolism. Serine has been reported to be the major donor of one-carbon units. An *in vitro* study using primary incubation of kidney cortical tubules showed that changes in the clearance of homocysteine and the production of cystathionine and cysteine through the transsulphuration pathway were dependent on the concentration of

serine in the media (House et al., 1997). However, an *in vivo* kinetic study in sheep receiving intravenous infusions of serine at 10 g/d did not show any significant difference in endogenous cysteine synthesis or in the ratio of transsulphuration to cysteine flux due to serine administration (Liu et al., 2000). It follows that the role serine plays in transsulphuration lacks consistency depending on the models and conditions of the experiments.

2.2.5.2 Micronutrients

2.2.5.2.1 Minerals

Selenium deficiency was observed to decrease plasma homocysteine and cysteine concentrations in a rat model, but also led to decreased BHMT activity (Uthus et al., 2002). The mechanism leading to the observed changes remains unclear, but may be related to changes in redox status caused by selenium deficiency. One of the major selenium pools is glutathione peroxidase, which catalyzes the oxidation of reduced glutathione to its oxidized form (NAS, 2000). Therefore, selenium and redox status are associated, which may lead to impacts on the rate of transsulphuration.

An *in vitro* rat study using perfused livers demonstrated that transsulphuration was enhanced by zinc deficiency. Evidence for the effect was found in increased alpha-ketobutyrate excretion from the livers (Duerre and Wallwork, 1986).

Cystathionine beta synthase (CBS) is a heme containing enzyme. The heme has both the ferric and ferrous states. Heme becomes relatively inert to ligand exchange with exogenous molecules in the ferric state (Banerjee and Zou, 2005). Therefore, iron may regulate CBS as well.

2.2.5.2.2 Vitamins

a) Choline and betaine

Choline and betaine are well documented to serve as substrates for homocysteine remethylation through BHMT. Experimental results show that hepatic BHMT activity increases in rats fed semi-purified diets with supplementations of 0.2% betaine or choline for three days (Finkelstein et al., 1983). Similar increases in hepatic BHMT activity by choline and betaine supplementation were also detected in a chick model using a methionine-deficient basal diet (Emmert et al., 1996). However, a kinetic study by Storch et al. (1991) found that transmethylation and transsulphuration increased with betaine supplementation ($P<0.05$), but not remethylation ($P=0.14$). Further research is needed to validate or clarify the role of betaine and changes in remethylation activity in the regulation of flux through the transsulphuration pathway.

b) Folate deficiency

When there is a folate deficiency or a defect in MTHFR, the synthesis of methionine will be reduced as a result of decreased remethylation. In an *in vitro* study with the Caco-2 human colon carcinoma cell line, total remethylation and folate-dependent remethylation were reduced by 32% and 63%, respectively, in folate-restricted cell cultures (Townsend et al., 2004). The mechanism of reduced remethylation might be due to a decrease in the expression and activity of MS by folate deprivation, as previously shown in a mouse model (Tchantchou et al., 2006). As mentioned above, reduced methionine synthesis from folate deficiency will result in low intracellular SAM concentration. In the meanwhile, due to the inhibition role of folate as the form of CH_3THF in GNMT reaction utilizing SAM, the lowered folate status will lead to the alleviation of the inhibting effect of folate on GNMT function, which relatively

accelerates GNMT reaction and the utilization of SAM consequently (Selhub and Miller, 1992). As a result of low SAM status from folate deficiency, CBS is not activated properly and transsulphuration pathway becomes ineffective.

c) B_{12} deficiency

When there is a deficiency of vitamin B_{12}, the cofactor of enzyme MS activity, an inhibition of remethylation occurs as a result of low enzyme activity. CH_3THF is therefore conserved due to the remethylation inhibition and the irreversible reaction of MTHFR. This is called a "methyl trap" (Shane, 1985). By contrast to folate deficiency, CH_3THF accumulation occurs in the case of B_{12} deficiency, which conserves SAM through the inhibition of GNMT. Transsulphuration as such, will be enhanced due to CBS activation from SAM.

d) B_6 deficiency

The role that vitamin B_6 deficiency plays in SAA metabolism is likely through its role in serving as a co-factor for key enzymes. Studies, albeit limited, show that in the case of dietary vitamin B_6 restriction, the metabolic pathways of remethylation and transsulphuration are disrupted (Martinez et al., 2000). In particular, vitamin B_6 is the exclusive cofactor in the transsulphuration pathway. Knowledge pertaining to the degree that vitamin B_6 deficiency impacts transsulphuration is critical in furthering our understanding of the nutritional regulation of SAA metabolism. This topic will be addressed in detail below.

2.3 Importance of vitamin B_6 to transsulphuration

2.3.1 General review of vitamin B_6

Vitamin B_6 is a collective term of six related compounds: pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), and their respective 5'-phosphates (PLP, PNP, and PMP). PLP and PMP are the major forms of vitamin B_6 in animal tissues (NAS, 1999). The major vitamin B_6 pool in animals is in muscle, where PLP is bound to glycogen phosphorylase. The main indices for assessing vitamin B_6 status are plasma PLP, erythrocyte and total blood PLP, urinary pyridoxic acid and xanthurenic acid, a tryptophan catabolite, and plasma homocysteine (NAS, 1999). Plasma PLP is the most commonly used criterion for assessing vitamin B_6 status. In humans, plasma PLP levels of 20 to 25 nmol/L are considered adequate (Bailey et al., 1999). The metabolism of vitamin B_6 occurs principally in the liver, where vitamin B_6 is taken in and converted to functional PLP with the participation of pyridoxal kinase and pyridoxine 5'-phosphate oxidase. The measures from this study (Chapter 5) show that hepatic PLP concentration is on ppm level, but plasma PLP concentration is on ppb level. This is a very good example of the central role of the liver in vitamin B_6 metabolism. Additionally, vitamin B_6 is catabolized to form 4-pyridoxic acid and 5-pyridoxic acid (McCormick, 2006).

2.3.2 Functions of Vitamin B_6

PLP has been well known to serve as a cofactor for over one hundred enzymes involved in amino acid metabolism including aminotransferases, decarboxylases, racemases, and dehydratases (NAS, 1999). This vitamin is therefore needed in the metabolic processes of decarboxylation, deamination, transamination, and transsulphuration (Lheureux et al., 2005). Because of the role of vitamin B_6 in protein catabolism, plasma PLP and urinary 4-pyridoxic acid concentratons were inversely correlated to protein intake in humans (Miller et al., 1985). This indicates that as the level

of dietary protein increases, more vitamin B_6 is retained in the body to supply PLP for the PLP-dependent enzymes involved in the catabolism of excess amino acid and less B_6 is available for conversion to 4-pyridoxic acid and plasma PLP. Vitamin B_6 is also needed for lipid metabolism that involves several aspects of PLP function (Birch, 1938; McCormick, 2006).

PLP is involved in the transamination of amino acids to keto acids which serve as precursors for gluconeogenesis. PLP is also required for decarboxylation of L-amino acids to generate amines. The latter play a role in neurotransmitters, hormones or as biogenic amines. Specifically, PLP is involved in the decarboxylation of 3,4-dihydroxyphenylalanine (DOPA) to dopamine, the conversion of tryptophan to both nitric acid and serotonin, and the conversion of glutamic acid to alpha-aminobutyric-acid (GABA) (Spinneker et al., 2007).

Vitamin B_6 is reported to be linked to immune function and cancer through its metabolic role in one-carbon metabolism and transsulphuration. According to the review by Spinneker et al., deficiency of vitamin B_6 causes thymic atrophy, lymphocyte depletion in lymph nodes and spleen in animals and decreased lymphocyte and interleukin (IL)-2 production in humans (Spinneker et al., 2007). Vitamin B_6 status is negatively correlated to the prevalence of cancers as well.

The enzymes requiring vitamin B_6 as a cofactor and involved in SAA metabolism directly or indirectly are cystathionine beta synthase (CBS) and cystathionine gamma lyase (CGL) in the transsulphuration pathway and serine hydroxymethyltransferase (SHMT) in the folate cycle and one-carbon metabolism. Evidence has accumulated to show that the activities of the above enzymes are lowered when there is vitamin B_6

deficiency (Smolin and Benevenga, 1984; Martinez et al., 2000; Davis et al., 2005). The methyl groups shuttled by SHMT are used for biological process of methylation. The transsulphuration pathway produces cysteine, a precursor for glutathione, which is the most important antioxidant functioning in the maintenance of reducing celluar environment and detoxification. Therefore, vitamin B_6 is associated with pathophysiology as mentioned above.

2.3.3 Significance of the study on vitamin B_6 nutrition in early weaned pig

Due to the similarity of human physiology to that in pigs, a study examining the impact of vitamin B_6 deficiency on SAA metabolism in piglets, especially for enzyme activities in tissues, may be pertinent to human clinical studies. Vitamin B_6 supplementation in early-weaned pigs is important due to the following perspectives. First, sow milk, the major source of nutrients for sucklings, is low in vitamin B_6 (Benedikt et al., 1996). This implies that vitamin B_6 status in pigs may be low upon weaning. Second, the demand for vitamin B_6 is high after weaning since the diets for early-weaned pigs are high in protein, which consumes more vitamin B_6 for catabolism (NAS, 1999). Therefore, the requirement of vitamin B_6 in early-weaned pigs should be high enough to meet the specific physiology and metabolism of early-weaned pigs. Therefore, a study with regard to the impact of vitamin B_6 status on early-weaned pigs is of great importance.

2.3.4 Role of vitamin B_6 deficiency in transsulphuration

As mentioned above, both CBS and CGL in the transsulphuration pathway require PLP as cofactor. The function of these enzymes is compromised as a result of low status of vitamin B_6, and transsulphuration is presumed to be reduced accordingly. The study

by Martinez et al. (2000) provided the evidence that vitamin B_6 deficiency in rats leads to decreased rate of transsulphuration, measured as the movement of deuterium label from $^2H_3$-serine into $^2H_3$-cysteine. Static pictures of elevated homocysteine and depressed cysteine concentrations induced by vitamin B_6 deficiency have also been observed in rats (Smolin and Benevenga, 1982; 1984; Lima et al., 2006). The response of CBS activity to vitamin B_6 deficiency is variable (Smolin and Benevenga, 1984; Mosharov et al., 2000). A study with a pig model by Smolin et al. (1983) demonstrated significant responses of homocysteine and cysteine to vitamin B_6 depletion. However, in a study with healthy adults, vitamin B_6 restriction did not alter homocysteine or cysteine concentrations, and had no impact on cysteine flux, but did lead to increased plasma cystathionine and glutathione concentrations (Davis et al., 2006). The different responses observed in biochemical indices to vitamin B_6 deficiency might be due to the differences in depletion diets, experimental models utilized and the degree of vitamin B_6 deficiency applied to the subjects. In particular, the use of *ad libitum* feeding design in all the studies mentioned above may lead to confounding effects from reduced intake of nutrients including amino acids and vitamins in the vitamin B_6 deficiency groups. These confounding effects may impact the interpretation of observed biochemical indices including homocysteine and cysteine. In addition, changes in vitamin B_6 status, enzymatic activities associated with sulphur amino acid metabolism and kinetics in pigs subjected to dietary vitamin B_6 depletion have yet to be reported. With respect to the quantitative impact of vitamin B_6 status on transsulphuration, the current literature base is inconsistent and likely dependent on the nature of the measured indices and experimental models. A sensitive isotopic

model with vitamin B_6 deficient pigs may enhance our understanding of the effect of vitamin B_6 deficiency on quantitative indices of transsulphuration rate.

2.4 Models for measuring transsulphuration rate

2.4.1 Definition of transsulphuration rate

The rate of transsulphuration (TS) in nutritional biochemistry refers to the amount of cysteine produced endogenously through the transsulphuration pathway per unit time. Specifically, the transsulphuration rate provides a kinetic measurement of endogenous cysteine synthesis which, however, cannot be achieved by static measures of cysteine concentration. The measurement of transsulphuration rate *in vivo* is most commonly determined through isotope dilution techniques.

2.4.2 Models to measure transsulphuration rate

On the basis of the tracers employed, the method for measuring transsulphuration rate can be classified into one of two groups: direct or indirect methods. For the direct method, two approaches are possible. In the first approach, radioactive isotope [^{35}S] labeled methionine is utilized as a tracer to look at the conversion of [^{35}S] cysteine from [^{35}S] methionine. In the second approach, isotopically labeled cysteine and serine are used as tracers to monitor the production of labeled cysteine derived from labeled serine. For the indirect method, usually [1-^{13}C] labeled methionine or homocysteine is used to trace the production rate of ^{13}C labeled carbon dioxide. Both the indirect and direct methods will be discussed further.

2.4.2.1 Indirect model

The indirect method usually refers to a model in which $^{13}CO_2$ production from

^{13}C-methionine is monitored as a surrogate for flux through the transsulphuration pathway. This model was introduced by Storch-Young et al. (1988). In this classical experiment, stable isotope double-labeled methionine (dual labeling or separate labeling) was exploited to quantify the rates of transmethylation, transsulphuration, remethylation, and methionine incorporation into and release from body proteins in humans. Specifically, the mechanism for measuring transsulphuration involves the measurement of the flow of the C-1 atom in methionine as it becomes C-1 in homocysteine, which becomes C-1 of the molecule α-ketobutyrate. The latter is then decarboxylated to $^{13}CO_2$. The measurement of $^{13}CO_2$ enrichment in breath together with the measurements of ^{13}C methionine in the infusate and blood, are used to calculate transsulphuration rate. The formula to calculate transsulphuration rate (TS) is $TS = VCO_2 \times (1/[^{13}C]$ methionine pool enrichment $- 1/[^{13}C]$ methionine tracer enrichment), where VCO_2 is the rate of $^{13}CO_2$ appearance. In the original model, [methyl-$^2H_3$] and [1-^{13}C] methionine were infused as primed-constant infusion via a venous catheter. $NaH^{13}CO_3$ was administered concurrently to prime CO_2 pool and to facilitate reaching CO_2 plateau. Samples of expired breath were collected for the analysis of $^{13}CO_2$ on a dual collector isotope ratio mass spectrometer. The enrichments of the methionine carboxyl and methyl groups were measured by gas chromatography-mass spectrometry. The authors obtained the mean value of transsulphuration under postabsorptive and fed status to be 3.99±0.44 and 8.25±0.56 $\mu mol \cdot kg^{-1} \cdot h^{-1}$, respectively (Storch et al., 1988). As per the formula, there is no need to use double-labeled tracers if one is only interested in modeling transsulphuration rate. Following the same methodology, di Buono et al. (2003) determined the transsulphuration rate in five healthy men. Furthermore, these trials validated the

application of the dual tracer model to the measurement of transsulphuration rate under steady state.

Raguso et al. (2000) updated the above $^{13}CO_2$ method using the following formula: $TS= (VCO_2 \times E_{13CO2} \times 1/R) \times 1/[E_1+(E_4\times 0.8)]$, where VCO_2 is the rate of carbon dioxide production, E_{13CO2} is the enrichment of ^{13}C in expired air, and R is the bicarbonate recovery factor (the factor R used to correct ^{13}C enrichment data in breath samples for the calculations of methionine and cysteine oxidation was 0.7 and 0.82 for fasting and fed conditions, respectively), E_1 and E_4 are the plateau plasma enrichments of methionine at m+1 ([1-^{13}C]methionine) and m+4 ([1-^{13}C, methyl-$^{2}H_3$]methionine), respectively, where m is the nominal integer mass of the tracee ion. The term $(E_1+E_4\times 0.8)$ is the total enrichment of methionine when assuming that the intracellular enrichment of the parent tracer is 80% of the plasma enrichment because of the fact that intracellular environment is the true precursor pool for SAA metabolism. In the latter method, [1-^{13}C]methionine and [1-^{13}C,methyl-$^{2}H_3$] methionine were intravenously administered. Blood and breath samples were collected every 15 min during the last hour of each metabolic phase (fasting and fed states). Isotopic enrichments were measured by using a gas chromatograph and mass spectrometer. They measured methionine transsulphuration rate for fasting and fed conditions to be 1.7 and 1.8 $\mu mol\cdot kg^{-1}\cdot h^{-1}$, respectively. The extrapolated 24-h rate of methionine transsulphuration was 41 $\mu mol\cdot kg^{-1}\cdot d^{-1}$. When compared with the Storch et al. (1988) method, the advantage of this method lies not only in the calculation of transsulphuration rate in which the bicarbonate recovery factor R is clearly quantified in the formula for specific states, but also through the use of

the factor of 0.8 to account for the fact that the metabolism of SAA happens intracellularly. Although the same tracer (methionine) was employed, the manner by which the isotope tracers were administered differed. In the Storch-Young et al. method (1988), the authors used [methyl-$^{2}H_3$] and [1-^{13}C] methionine as separate tracers, while in the method by Raguso et al. (2000) a combined [1-^{13}C; methyl-$^{2}H_3$] methionine dual labeled tracer was primed and constantly infused instead.

A challenge that exists with all dilution models relates to the choice of precursor pool enrichment (Reed and Davis, 1999). To address this, MacCoss et al (2001) introduced [^{13}C]homocysteine as a marker of intracellular enrichment to measure transsulphuration rate in humans using the formula $TS=F^{13}CO_2/E13_{C\text{-}Hcy}$, Where, $F^{13}CO_2$ is the rate of $^{13}CO_2$ excretion in the breath after being adjusted by 0.81 for bicarbonate retention; $E13_{C\text{-}Hcy}$ is the plasma [^{13}C] homocysteine enrichment. This method of calculation assumes that 1) all of the $^{13}CO_2$ is liberated via the transsulphuration pathway, and 2) plasma [^{13}C] homocysteine enrichment stands for the intracellular [^{13}C] methionine enrichment. The tracers and procedures administered are in accordance with that in the Storch-Young method (1988) except parameters used to calculate transsulphuration rate.

To summarize, the aforementioned studies employing stable isotopes, and breath CO_2 collection, can minimize the risk to subjects and researchers from the use of radiolabelled substrates in comparison with the model utilizing radioactive isotopes. In addition, only one tracer (methionine) is administered in these studies, and this may lead to reduced costs and errors arising from working with multiple isotopes. However, methionine transsulphuration is determined indirectly by methionine oxidation measured

from the rate of $^{13}CO_2$ appearance. This method can overestimate the transsulphuration rate, because some $^{13}CO_2$ will arise from sequestration of S-adenosylmethionine for polyamine synthesis. In this process, methionine is decarboxylated before its methyl group is transferred. The end product is S-aminopropylthiol. Fortunately, best estimates based on enzyme activity measurements and polyamine production suggested that less than 5% of the S-adenosylmethionine goes toward polyamine synthesis. Similarly, underestimation of transsulphuration might also occur due to oxidation of threonine, as this amino acid also yields alpha-ketobutyrate, which will lead to an intracellular dilution of label and affect the calculation of $^{13}CO_2$ production rates (Darling et al., 2000; House et al., 2001). The assumption in the indirect method by $^{13}CO_2$ production is that transsulphuration is the only pathway contributing to the production of CO_2 in which carboxyl group from methionine is transmited to alpha-ketobutyrate and further metabolized to CO_2. Therefore, the indirect method might give rise to values that are not quantitatively valid under all conditions. In addition, the bicarbonate recovery factor (R) in the formula to calculate transsulphuration varies in different states. Furthermore, this model requires not only the enrichment measurements for infusate and plasma, but also the collection and enrichment measurement of breath $^{13}CO_2$ processes, which can be technically infeasible for a pig model. Therefore, while providing a measure of transsulphuration, the $^{13}CO_2$ method itself has pitfalls and requires validation prior to use in conditions thought to perturb transsulphuration.

2.4.2.2 Direct model

a) Radioactive isotope model using [^{35}S]methionine

In this model [^{35}S] methionine is applied together with the use of radioactive- or stable isotope-labeled cysteine. The latter is used for calculating flux of cysteine. The former is used for tracing the conversion of radioactivity from methionine to cysteine. In a rat study (Malmezat, et al. 2000), the transsulphuration rate was measured by the use of radioactive isotope of ^{35}S methionine and stable isotope of ^{15}N cysteine. The authors determined the infusion rate of [^{15}N] cysteine (I_{Cys}), the enrichment of the cysteine tracer (E_{tr}), and the enrichment of cysteine in plasma (E_{pl}) in order to calculate the cysteine flux (F_{Cys}). The specific activity of [^{35}S] cysteine (SA_{Cys}) and the specific activity of [^{35}S] methionine (SA_{Met}) were also quantified. Transsulphuration rate (TS) was finally calculated using the formula: $TS = F_{Cys} \times 100 \times SA_{Cys} / SA_{Met}$, which was 0.25±0.08 and 0.68±0.19 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ for pair-fed and infected rats, respectively. Likewise, Williams et al. (1988) exploited radioactive isotopes of [^{35}S] methionine and [3,3-$^{3}H_2$] cysteine to estimate transsulphuration rate in pregnant Merino ewes. The labeled [^{35}S] methionine and [3,3-$^{3}H_2$]cysteine were abomasal administered in 750 mL solution with the dose of organic sulfur of 0, 0.5, and 1.0 g, respectively. The rate of administration of each solution was estimated from the difference in weights of the syringes at the start and end of each infusion of 6 hours. Weighed aliquots of the infusion solutions were assayed for ^{3}H and ^{35}S radioactivity by liquid scintillation spectrometry.

The common characteristic of the above two studies lies in the following two points: First, the radioactive isotope [^{35}S] was used as a tracer in both studies. Second, methionine and cysteine served as tracees. However, the moiety labeled in cysteine differs with radioactive isotope in one and stable isotope in the other. In both

experiments transsulphuration was measured directly from the rate of synthesis of [^{35}S] cysteine. The radioactive isotope model is relatively easy and convenient. However, concerns with the use of radioisotopes, including potential health risks, may be a major limitation for its application in human and animal studies.

b) Stable isotope model using serine and cysteine

A classic study using stable isotope labeled serine and cysteine as tracers to quantify transsulphuration rate was done in a lamb model (Liu et al., 2000). In this study L-[3-^{13}C]Cysteine and L-[2,3,3-d_3]Serine were employed to measure transsulphuration of methionine. The basic principle to quantify transsulphuration rate in this trial was to determine cysteine flux and the proportion of plasma enrichment of cysteine (m+3) derived from [2,3,3-d_3]serine to plasma enrichment of serine (m+3), respectively. In the former, cysteine flux (irreversible loss rate, ILR) is obtained by measuring enrichments of [3-^{13}C] cysteine (m+1) in the infusate and plasma, and infusion rate using an infusion pump. In the latter case, it is based on the pathway of transsulphuration in which serine accepts the sulphur atom from homocysteine to form cysteine. This was achieved by labeling serine with deuterium and determining the formation of cysteine labeled with deuterium. Employing this methodology, L-[3-^{13}C] cysteine and L-[2,3,3-d_3]serine were primed and constantly infused for 8 hours via a femoral vein catheter. Blood samples were drawn at regular intervals by jugular vein catheter throughout the infusion period. The enrichments of isotope in the infusate and plasma were measured by GC/MS. Transsulphuration rate was calculated based on flux value of cysteine and the plateau enrichment of cysteine (m+3) and serine (m+3), respectively. Transsulphuration rate was quantified as 3.25 to 4.92 mmol/d among treatments. Applying the same principle,

transsulphuration rate or cysteine synthesis rate from serine in children was determined to be 8.4 μmoL/kg/h using [3,3-d_2] cysteine and U-[$^{13}C_3$] serine (Jahoor et al., 2006). In the meanwhile, Gregory et al. (2000) detected [$^2H_3$]cystathionine enrichment in plasma of healthy man after primary and constant infusion of [$^2H_3$] serine at the dose of 4.63 μmoL/kg/h. However, transsulphuration rate was not reported due to the unavailability of cysteine (m+3) enrichment.

The technique using stable isotope labeled serine is safe and ethical to subjects and environments and no adverse effect on biological metabolism as well. In the meanwhile, it is a direct measurement, which reflected the true oxidation rate of methionine and therefore, avoided the systematical error derived from the transamination-decarboxylation route or dilution from threonine oxidation as discussed above. Furthermore, the analytical procedures using the Liu (2000) model are simplified. L-[3-^{13}C]cysteine, L-[2,3,3-d_3]serine and L-[2,3,3-d_3] cysteine are analysed by GC/MS from the same injection of the same sample, which cannot be accomplished in the analysis of radioactive tracers. However, a technical challenge of using this model in swine is that serine tracer is greatly diluted in the precursor pool of liver. A previous isotope dilution study in our laboratory shows that specific radioactivity of [^{3}H]serine in the liver was only 16% of the level in plasma (Ludke, 2004). Therefore, the infusion dose for serine has to be greatly increased in a swine model to allow for the detection of moiety of cysteine coming from labeled serine. The large infusion dose of serine imposes two concerns depending on the isotopes selected. First, if choosing radioactive isotope labeled serine, e.g. [^{3}H]serine, radiation risk to the environment and the potential side effect of high level of radiation on biological metabolism might be a limitation.

Second, when selecting stable isotope, the very high cost consumption of tracers is a barrier to the sample size. There is no study to our knowledge that has used [3-^{13}C]cysteine and [2,3,3-d_3] serine to determine transsulphuration rate in a pig model. Additionally, when using [2,3,3-d_3] serine, both [d_1]serine and [d_2]serine will be yielded metabolically, which results in a loss of some of the [2,3,3-d_3]serine tracer. This, to some degree, is also a factor that contributes to the use of a large dose of [2,3,3-d_3] serine.

2.4.3 The caveat on enrichments --- Dilution in the precursor pool

Irrespective of whether a direct or an indirect isotopic model is utilized, amino acid tracers are infused into and measured from plasma. However, the metabolism to be targeted such as, transsulphuration, occurs inside cells (i.e. the precursor pool is not plasma, but intracellular). It follows that the meaurement of enrichment from plasma is not the true enrichment of tracers that can be utilized for kinetic calculation since there is intracellular dilution of the infused tracers largely from protein breakdown and *de novo* synthesis. As a result, the enrichment in plasma of the tracers is higher than that in the precursor pool. To be able to measure kinetics of amino acids in the precursor pool, a correction factor is used to account for intracellular dilution, or a marker of intracellular dilution is applied (MacCoss et al., 2001).

In the literature with respect to amino acid kinetics, correction of plasma enrichment was not specified in most of the papers. A pioneering study by Matthews et al. (1982) estimated the correction factor of plasma leucine enrichment to be 77% by measuring plasma L-[1-^{13}C] α-ketoisocaproate (KIC) and L-[1-^{13}C]leucine enrichments based on the assumption that the keto acid KIC enrichment in plasma is equal to intracellular enrichment of leucine under steady state conditions. For methionine, a study

by Young et al. reported 0.8 as the correction factor (Young et al., 1991), which was utilized by some studies on methionine and cysteine enrichment corrections (Hiramatsu et al., 1994; Raguso et al., 1997, 2000). However, the correction factor for methionine enrichment was examined to be 0.58 in humans based on the assumption that plasma homocysteine enrichment is equal to intracellular methionine enrichment under steady state (MacCoss et al., 2001). As for a correction factor for plasma serine enrichment for the calculation of serine kinetics, knowledge is very limited. Davis et al. (2004) used 0.4 as a coefficient to account for intracellular dilution in humans. In a piglet model, an intracellular dilution factor was determined to be 0.16 based on measurements of specific radioactivity of ^{3}H in liver and plasma in pigs infused with L-[^{3}H] serine in our laboratory (Ludke, 2004). This indicates that the intracellular dilution differs with different models used. This fact needs to be kept in mind, as interpretations of kinetic rates will differ depending on the choice of precursor pool. In studies where access to true precursor pools are not feasible or ethical, correction factors need to be applied.

2.4.4 Comparison of the rate of transsulphuration

Table 1 summarizes the results from studies where rates of transsulphuration were measured *in vivo*, employing either direct or indirect approaches. Rates of transsulphuration range from 1.8 to 8.4 μmoL/kg/h depending on the protocols and models used. Accordingly, the contribution of transsulphuration to cysteine flux ranges from 1-20%. The majority of transsulphuration rates have been measured by the indirect isotope model using dual labeled methionine. The average of transsulphuration rate measured by the indirect isotope model in humans from the 9 papers listed in table 1 is 4.7±2.1 μmoL/kg/h, which is very close to the transsulphuration rate (5 μmoL/kg/h)

determined by the direct isotope model using L-[3-^{13}C]cysteine and L-[2,3,3-d_3]serine in sheep model (Liu et al., 2000). However, transsulphuration rate was determined to be 26 μmoL/kg/h in a piglet model using the indirect model, which is higher than that in other models (Riedijk et al., 2007). The latter data agree with previous studies which have shown that amino acid flux in neonatal piglets is substantially higher than that reported for human neonates, due to the higher rates of protein deposition and growth in piglets (Wykes et al., 1994). The study employing the direct model by Davis et al. (2006) did not report transsulphuration rates due to challenges encountered with methodology. Rates of transsulphuration determined by the direct model in swine have yet to be reported. Therefore, the ability to compare transsulphuration rates measured by different isotopic models, using the same subjects, is not possible.

Table 1 Summary of the rate of transsulphuration

Reference	Tracers used	Cys flux (μmoL/kg/h)	TS (μmoL/kg/h)	Ratio of TS and Cyseine flux (%)	Model
Storch et al., 1990	L-[1-^{13}C;methyl-$^{2}H_3$]Met	NA	7.6	NA	Adult male
Young et al., 1991	L-[$^{2}H_3$ methyl-1-^{13}C]Met	NA	4.7	NA	Adult male
Hiramatsu et al., 1994	L-[$^{2}H_3$ methyl-1-^{13}C]Met; L-[3,3-$^{2}H_2$]Cys	46.9	3.1	6.6	Adult male
Fukagawa et al., 1996	L-[methyl-$^{2}H_3$;1-^{13}C]Met; L-[3,3-$^{2}H_2$]Cys	55.1	4	7.3	Adults
Raguso et al., 1997	L-[1-^{13}C;methyl-$^{2}H_3$]Met; L-[3,3-$^{2}H_2$]Cys	83.9	4.2	5	Adults
Malmezat et al., 2000	[^{15}N]cysteine; [^{35}S]Met	176	2.5	1.4	Rats
Fukagawa et al., 1998	L-[methyl-$^{2}H_3$;1-^{13}C]Met; L-[3,3-$^{2}H_2$]Cys	35.7	3.4	9.6	Adults
Liu et al., 2000	L-[3-^{13}C]Cys; L[2,3,3-D_3]Ser	32	5	16	Sheep
Raguso et al., 2000	L-[1-^{13}C;methyl-$^{2}H_3$]Met; L-[3,3-$^{2}H_2$]Cys	27.7	1.8	6.5	Adults
MacCoss et al., 2001	L-[1-^{13}C;methyl-$^{2}H_3$]Met	NA	5.4	NA	Adults
Jahoor et al., 2006	L-[3,3-$^{2}H_2$]Cys; [U-$^{13}C_3$]Cys; [U-$^{13}C_3$]Ser	41.1	8.4	20	Children
Riedijk et al., 2007	L-[1-^{13}C]Met; L-[$^{2}H_3$]Met	NA	26	NA	Piglet

Note: For abbreviations: Met = methionine, Cys = cysteine, Ser = serine, TS = transsulphuration rate, NA = not available

2.4.5 Key techniques used in amino acid kinetic measurements

There are several key techniques involved in the study of amino acid kinetics. First, in order to provide isotopic tracers to the animal and take blood samples at specific intervals for extended intervals, venous catheters need to be installed at different sites prior to infusion. Previous work in primed-constant infusion experiments (Wolfe, 1984) demonstrated the optimal sampling protocol to be sampled upstream from the site of infusion. In the studies by Ludke (2004) in which [^{14}C] and [^{3}H] serine were employed to determine [^{14}C] and [^{3}H] cysteine, sites for infusing isotope and drawing blood samples were the femoral and jugular veins in piglets, respectively, and they were demonstrated to be feasible for the study of amino acid metabolism. Second, for the infusion technique, constant infusion accomplished by a peristaltic pump is shown to be adequate to establish plateau in enrichment of the amino acid conveniently and economically (Waterlow et al., 1978; Wolfe, 1984). The appropriate constant infusion rate is determined by the rate of appearance of the amino acid being traced and the desired enrichment. Finally, with respect to the use of a priming dose, this technique only affects the time it takes to attain final equilibrium, not the equilibrium value (Wolfe, 1984), so the priming dose technique is usually employed to shorten the time to reach an isotopic equilibrium. The priming dose is equal to the turnover time times the hourly rate of constant tracer administration.

The appropriate priming dose and constant infusion rate are critical for the measurement of transsulphuration rate by the use of stable isotopic models. Because the analytical sensitivity of mass spectrometry is less than the ideal sensitivity possible for radioisotopes, stable isotopic tracer quantities must be greater than the "massless" amount

of material used in radiotracer work (Yergey, 1994). Cuskelly et al. carried out an isotope dilution study in humans in which the following tracers were used: [$^{2}H_3$]serine (priming dose: 9.26 μmol/kg; constant infusion rate: 9.26 μmol/kg/h), [$^{2}H_3$]leucine (priming dose: 1.91 μmol/kg; constant infusion rate: 1.91 μmol/kg/h), [1-^{13}C]methionine (priming dose: 0.25 μmol/kg; constant infusion rate: 0.25 μmol/kg/h). However, isotope labeled cysteine was not detected (Cuskelly et al., 2001). This might be due to analytical technique as per the authors' explanation or not enough infusion dose of serine since there is large free precursor pool of serine to dilute the tracer as discussed above. Therefore, a pilot study in combination with the reference is necessary to understand the appropriate infusion dose for an isotope dilution study using stable isotopes.

2.5 Summary

Sulphur amino acids have significant nutritional importance for body protein synthesis. In particular, methionine is an essential amino acid in mammals. Additionally, sulphur amino acids play a role in many physiological processes. One of the major roles of methionine metabolism is to provide a source of methyl groups for methylation reactions, including the methylation of DNA and RNA. Another critical role of sulphur amino acid metabolism relates to the endogenous provision of cysteine, which contributes to animal health through the production of glutathione and taurine.

Transsulphuration, a catabolic pathway of methionine metabolism, is driven by two vitamin B_6 dependent enzymes of CBS and CGL. Manipulation of transsulphuration through nutrition provides a means for us to enhance body health and function. Among those nutritional factors affecting transsulphuration, vitamin B_6 has been deemed to be a key nutrient because of the cofactor role it plays in the pathway. Theoretically, vitamin

B_6 deficiency inhibits transsulphuration in terms of the reduction of enzyme activities in this pathway. However, based on literature search, the response of biochemical indices in the transsulphuration pathway to vitamin B_6 deficiency lacks consistency, and depends on the diet used, the experimental models and degree of depletion. Kinetic measures of transsulphuration in animal or human models deficient of vitamin B_6 is limited. At present, there are no reports on the impact of vitamin B_6 deficiency on transsulphuration rates in a piglet model. Therefore, the study of the effect of vitamin B_6 deficiency on transsulphuration will present not only a static picture of changes in biochemical indices but also kinetic data by which the nutritional regulation of transsulphuration by vitamin B_6 can be quantified.

CHAPTER THREE

HYPOTHESIS AND OBJECTIVES

3.1 Hypotheses

Null Hypothesis: Vitamin B_6 deficiency does not disrupt transsulphuration.

Alternative Hypothesis: Vitamin B_6 deficiency disrupts transsulphuration.

3.2 Objectives

The main purpose of this research was to investigate the impact of vitamin B_6 deficiency on transsulphuration. This will be achieved through the following objectives:

i) To characterize changes in biochemical indices of sulphur amino acid metabolism in pigs deficient of dietary vitamin B_6.

ii) To examine the response of sulphur amino acid metabolism to graded levels of vitamin B_6 repletion in vitamin B_6 deficient pigs.

iii) To establish a stable isotope model using L-[2,3,3-d_3] serine and L-[3-^{13}C]cysteine to determine transsulphuration rate.

iv) To quantify changes in kinetic measures using isotope dilution technique in a pig model deficient of vitamin B_6.

CHAPTER FOUR

MANUSCRIPT I

Characterization of Biochemical Indices of Sulphur Amino Acid Metabolism in the Vitamin B_6-Deficient Piglet

4.1 ABSTRACT

The objective of this study was to characterize the temporal changes in indices of sulphur amino acid metabolism in vitamin B_6-deficient piglets. Piglets (5.3 kg; n=6 per group) were fed a semi-purified diet containing either 0 (deficiency group) or 3 mg (control group) pyridoxine·HCl/kg diet. Animals were pair fed for six weeks. Piglets consuming vitamin B_6-deficient diets exhibited decreased average daily gains on the fourth week and feed conversion efficiency from the fourth week until the end of trial ($P<0.05$). Plasma pyridoxal 5'-phosphate concentrations in pigs consuming vitamin B_6-deficient diets were significantly lower than controls throughout the experiment ($P<0.01$), reaching a nadir of 14% of the control animals' value by trial's end. The apo- and total- activities of hepatic cystathionine beta synthase, cystathionine gamma lyase and serine hydroxymethyltransferase were decreased by vitamin B_6 deficiency ($P<0.05$). Vitamin B_6 deficiency decreased hepatic cysteine ($P<0.05$) but increased hepatic homocysteine concentrations ($P<0.01$) at trial's end. An examination of plasma total homocysteine and cysteine concentrations revealed significant ($P<0.05$) differences between treatments, with evidence of an abrupt shift in concentrations at three weeks post-initiation of dietary treatments (>25 fold increase in homocysteine; halving of cysteine values). At the end of the trial, vitamin B_6 deficiency significantly increased plasma methionine and serine levels, but decreased plasma glycine concentrations ($P<0.05$). The current data establishes temporal patterns in plasma indices of vitamin B_6 status and sulphur amino acid metabolism in the face of a vitamin B_6 deficiency in piglets.

Key words: pig, vitamin B_6, sulphur amino acid, homocysteine, cysteine

4.2 INTRODUCTION

As depicted in Figure 1, the sulphur amino acid homocysteine stands at the intersection between the remethylation and transsulphuration pathways of methionine metabolism (Selhub, 1999). Pyridoxal 5'-phosphate (PLP), a major biological form of vitamin B_6 in animal tissues, is a well established co-factor for the three enzymes directly involved in homocysteine disposition: Cystathionine beta synthase (CBS) and cystathionine gamma lyase (CGL) are key PLP-dependent enzymes in the transsulphuration pathway, and serine hydroxymethyltransferase (SHMT) is a PLP-dependent enzyme important for the folate cycle and one carbon metabolism (House et al., 1999). Vitamin B_6 status and, therefore, nutritional supply are thus implicated in the regulation of homocysteine flux and overall sulphur amino acid metabolism.

Over the last decade, interest in homocysteine metabolism has grown, due to the purported link between increased plasma concentrations and the risk for developing cardiovascular disease and stroke (Wilcken and Wilcken, 2001). While a clearer picture develops of the role that elevated homocysteine concentrations play in the risk for chronic disease development in humans, it is important not to lose sight of the fundamental importance of this amino acid in serving as the sole source of sulphur for the endogenous synthesis of cysteine (Wilcken and Wilcken, 2001). The *de novo* synthesis of cysteine, through the transsulphuration pathway, represents the primary route for the oxidation of the carbon skeleton of methionine, and is therefore an important component in the control of plasma and tissue methionine levels (House et al., 1999). As well, cysteine synthesis, besides being important for providing a substrate for protein synthesis, is crucial for the regulation of the availability of the biological antioxidant glutathione, as

well as other sulphur-containing compounds (ie: taurine) (Mosharov et al., 2000). Due to the central importance of PLP as a co-factor for the enzymes of the transsulphuration, the impact that vitamin B_6 nutrition has on homocysteine and cysteine metabolism is warranted. Increased homocysteine and decreased cysteine concentrations induced by vitamin B_6 deficiency have been documented in rat and pig models (Smolin and Benevenga, 1982,1984; Smolin et al., 1983; Martinez et al., 2000). However, measures of vitamin B_6 status and activities of CBS and CGL in pigs in response to a vitamin B_6 deficiency have yet to be investigated. To this end, an experiment was conducted to characterize the temporal changes in vitamin B_6 status and biochemical measures of SAA metabolism during the development of vitamin B_6 deficiency in the weanling pig.

4.3 MATERIAL AND METHODS

4.3.1 Animals and feeding

Twelve 14-d-old weaned cross-bred piglets were delivered from The Glenlea Swine Research Unit, University of Manitoba to the T.K. Cheung Centre for Animal Science Research, Fort Garry Campus, University of Manitoba. Pigs were assigned to two dietary treatments (n=6 per treatment): 1) basal diet + 3mg pyridoxine·HCl kg^{-1} diet (Control) and 2) basal diet + 0 mg pyridoxine·HCl kg^{-1} diet (Deficient). The semi-purified basal diet was referenced to the requirements for 5-10 kg pigs (NRC, 1998) except vitamin B_6 and is presented in Table 2.

Table 2 Composition of the basal diet used to assess the impact of B_6 deficiency on indices of sulphur amino acid metabolism in pigs.

Ingredients	Composition (%)	Nutrient Content (Calculated)	
Corn starch[1]	40.15	Metabolizable energy (kcal/kg)	3474
Casein (vitamin free)[1]	20.50	Crude protein (%)[5]	18.90
Lactose[1]	20.00	Total lysine (%)[4]	1.51
Corn oil	5.50	Total methionine (%)[4]	0.55
Cellulose[1] (Alphacel)	3.50	Total threonine (%)[4]	0.81
Cystine[2]	0.35	Total tryptophan (%)[4]	0.23
Vitamin & mineral premix[3]	10.00	Calcium (%)[4]	1.07
		Total phosphorus (%)[4]	0.76
		Vitamin B_6 (mg/kg)[5]	<0.02
Total	100		

Note:
[1]Ingredients purchased from Harlan Teklad, U.S.A.
[2]Purchased from Sigma®.
[3]Provided (per kg diet): Sodium, 2.4 g; chloride, 3.6 g; magnesium, 0.6 g; potassium, 4.2 g; copper, 9 mg; iron, 150 mg; manganese, 6 mg; zinc, 150 mg; iodine, 0.21 mg; selenium, 0.45 mg; vitamin A, 4400 IU; vitamin D_3, 440 IU; vitamin E, 32 IU; vitamin K, 1 mg; biotin, 0.1 mg; choline, 1g; folacin, 0.6 mg; niacin, 30 mg; pantothenic acid, 20 mg; riboflavin, 7 mg; thiamin, 2 mg; vitamin B_{12}, 35 μg. When added, pyridoxine·HCl was used as a source of dietary vitamin B_6.
[4]Calculated nutrient composition on the basis of the composition of purified ingredients.
[5]Analyzed nutrient composition: crude protein via LECO analysis; vitamin B_6 via microbial analysis, Medallion Laboratories, Minneapolis, MN, USA.

The powdered diet was mixed with water at the ratio of approximate 3 parts diet to 1 part water prior to feeding. A pair-feeding design was employed for this study to account for the potential confounding effect of feed intake on biochemical measures of sulphur amino acid metabolism. Water was offered freely. In order to avoid the confounding effect of weaning on initial feed intake, pigs were first adapted to the control diet (11 days) before implementation of the deficiency protocol, when pigs had reached 26 days of age. Pigs were maintained on the treatments for a total of 42 days. This experiment was approved by the University of Manitoba Institutional Protocol Management and Review Committee.

4.3.2 Experimental procedure

Feed intake was recorded daily. At the end of each seven-day period, feed was withdrawn 16 hours before weighing on the next morning. Fasting blood was taken via jugular vein into EDTA coated evacuated tubes and placed on the ice covered with aluminum foil. Blood was centrifuged at 9500 ×g to harvest plasma. Following blood collection on day 42, animals were anesthetized through exposure to a mixture of oxygen and isofluorane (2.5%) by way of a facemask. Once a surgical plane of anesthesia was obtained, a midline incision was made. Liver tissue samples were collected and put into liquid nitrogen. All the plasma and tissue samples were stored in -80°C until analysis.

4.3.3 Analytical methods

Plasma PLP was measured using a commercial radioenzymatic assay (Vitamin B_6 PLP ^{3}H-REA, ALPCO). Because PLP is light-sensitive, measurement was performed under protection from exposure to direct light.

Plasma total homocysteine and cysteine concentrations were analyzed according to the reverse phase-HPLC method of Araki and Sako (Araki and Sako, 1987), with modifications by using a newer phosphine reagent, tris (2-carboxylethyl) phosphine (TCEP) (Gilfix et al., 1997). Briefly, plasma samples were incubated with TCEP (Fisher Scientific, Nepean, ON), to reduce protein-bound and oxidized forms of homocysteine, followed by derivatization with 7-fluorobenzofurazan-4-sulfonic acid ammonium salt (SBD-F; Sigma Chemical Co., Oakville, ON). The fluorescent thiol derivatives were separated on a Waters C-18 column (5 μM, 4.5 x 250 mm; Waters Canada, Mississauga, ON), using isocratic elution (98% 0.1 M acetate, pH 5.5: 2% methanol) by means of a Shimadzu HPLC system (Man-Tech Associates, Guelph, ON) complete with auto-injector and fluorescence detector (excitation λ = 385 nm; emission λ = 515 nm). For liver thiol concentrations, extracts were first prepared as previously described (Farris and Reed, 1987). Briefly, one gram liver samples were combined with 10 volumes of 0.05M cold potassium phosphate buffer (pH 7.2), homogenized on ice for 90 seconds, and then centrifuged at 13 000 ×g, 4 °C for 30 min. Supernatant samples were reduced by mixing with equal volumes of 5% TCEP for 30 minutes at room temperature and deproteinized using 10% perchloric acid (5 volumes per volume of tissue supernatant). Samples were adjusted to alkaline pH using 4M KOH, vortexed, and the mixture centrifuged at 13,000 ×g for 10 min. Finally, 100 μL of supernatant was removed and treated as in the procedure for plasma samples. Concentrations of total homocysteine and cysteine were determined through the use of an external standard curve, and the inter- and intra-assay coefficients of variation were < 2%. Plasma free methionine, serine and glycine were measured via ion-exchange chromatography (LKB 4151 Alpha-Plus Amino Acid

Analyzer), with post-column ninhydrin derivitization, using methods as previously described (Blom and Huijmans, 1985).

Hepatic apo- and total CBS activities were determined as previously described, using radioisotopic techniques with modifications (Mudd et al., 1965;Taoka et al., 1998). One gram of tissue sample was homogenized with 4 mL of 0.05 M potassium phosphate buffer (pH 6.9) and centrifuged as mentioned above. The incubation mixture included 0.15 mM L-cystathionine, 41.67 mM DL-homocysteine, 0.42 mM PLP (total enzyme only), 0.32 mM S-adenosylmethionine, 2.08 mM DL-propargylglycine, 125 mM Tris, 2.08 mM EDTA, 25 mM L-[U-^{14}C]serine (~74 000 dpm/μmol) and 150 μL of homogenate supernatant with 25% dilution in a total volume of 600 μL. The reaction mixture, with the exception of [^{14}C]serine, was pre-incubated for 5 min at 37 °C. The reaction was started by adding [^{14}C]serine and was terminated after 60 minutes using 300 μL of 15% ice-cold trichloroacetic acid. Five hundred microliter of sample supernatant were loaded onto a column with AG 50W-X8 resin (Bio-Rad) and the column washed with 2×4 mL of water, 6×4 mL of 1N HCl, and 4×4 mL of water in sequence. The ^{14}C-cystathionine was eluted with 5 mL of 3N ammonium hydroxide. One milliliter of the collected solvent was mixed with the same fraction of scintillation fluid (ScintiSafe Plus^{TM}). Radioactivity was counted by a liquid scintillation counter. Protein concentration and incubation time were tested to be within the linear range. Protein concentration for tissue homogenate was determined using a Bicinchoninic Acid Protein Assay Reagent kit (Pierce, Product No. 23225).

Hepatic CGL activity was quantified by spectrophotometric assay (Stipanuk, 1979). The reaction mixture consisted of 0.1 M potassium phosphate buffer, pH 7.5, 4.0

mM L-cystathionine, 0.125 mM PLP (total enzyme only), 0.32 mM NADH, and 1.5 units of lactate dehydrogenase/mL. Protein concentration was optimized for both apo and total enzymes. The kinetics of NADH absorbance was measured by DU 800 Spectrophotometer at 37°C.

Hepatic SHMT was analyzed by a binding assay using radioactive isotope L-[^{14}C(U)]-Serine (Geller and Kotb, 1989; Lewis et al., 1998). The reaction mixture contained 0.4 mM serine, 2.0 mM THF, 2.5 mM EDTA, 1.0 mM 2-mercaptoethanol, 0.25 mM PLP (total enzyme only). This mixture was incubated for 10 min with the temperature of 37°C. The reaction was stopped by streaking a 25 μL aliquot onto a labeled 3×3 cm square of DE-81 filter paper (Whatman®). The paper was then washed with running distilled water at 2-3 liters/min for 20 min. After drying the paper was cut into squares. The radioactivity of the separate paper square was counted.

4.3.4 Statistical analysis

To adequately account for within-subject correlation over time and handle the missing data, the mixed model was applied to analyze the parameters with repeated observations including growth performance and indicators from plasma samples (Littell et al., 1996; Wang and Goonewardene, 2004). The diet effect and interaction effect between diet and week were partitioned in the model. Because of the homogenous variance over time in the control group and heterogeneous variances over time in the deficiency group, a common variance for control group and individual variance for deficiency group throughout the trial were employed. Since hepatic CBS, plasma methionine and glycine were not normally distributed, log transformations of these data were performed prior to statistical analysis. For tissue sample data, the PROC GLM was

used, and a Randomised Complete Block (RCB) design was applied in the model. Animals were treated as block. Diet and block effects were partitioned in the model.

4.4 RESULTS

One pig from the control group was put down at the end of the first week of experimentation due to failure to thrive.

4.4.1 Growth performance

Vitamin B_6 deficiency did not affect average daily feed intake (ADFI) (Table 3), as a result of the pair feeding design ($P>0.05$). However, average daily gain (ADG) was decreased on the fourth week and feed conversion efficiency (FCE) was decreased (Table 3) from the fourth week until the sixth week ($P<0.05$). In the latter three weeks, ADG was reduced by 29-33%, while FCE was lowered by 33-50% due to vitamin B_6 deficiency.

Table 3 Effect of vitamin B_6 deficiency on growth performance in piglets

(Manuscript I)

Week	ADFI (g/d)			ADG (g/d)			FCE		
	$+B_6$	$-B_6$	P value	$+B_6$	$-B_6$	P value	$+B_6$	$-B_6$	P value
1	202±38[a]	214±35[a]	0.8200	146±36[a]	161±33[a]	0.7564	0.722±0.052	0.749±0.048[a]	0.7114
2	374±38[b]	368±35[b]	0.8997	310±36[b]	300 ±33[b]	0.8435	0.827±0.052	0.813±0.048[a]	0.8464
3	418±38[b]	405±35[b]	0.8057	334±36[b]	296±33[b]	0.4474	0.797±0.052	0.730±0.048[a]	0.3502
4	417±38[b]	393±35[b]	0.6500	323±38[b]	218±34[ab]	**0.0470**	0.766±0.058	0.540±0.052[b]	**0.0059**
5	396±38[b]	372±35[b]	0.6513	270±36[b]	205±33[a]	0.1948	0.682±0.052	0.547±0.048[b]	**0.0316**
6	393±39[b]	374±37[b]	0.7239	297±39[b]	211±38[ab]	0.1163	0.733±0.058	0.547±0.058[b]	**0.0291**

Notes:
[1] Data are presented as least square mean ± standard error.
[2] *P* values are derived from difference of main effect of diet. *P* values < 0.05 are considered significant.
[3] The same shoulder letters in each column mean non-significant ($P>0.05$).
4 ADFI represents average daily feed intake; ADG represents average daily gain; FCE represents feed conversion efficiency.

4.4.2 Plasma PLP concentrations

With the exception of the baseline data, plasma PLP concentrations in vitamin B_6 deficient pigs were significantly lower in comparison with those observed in control animals ($P<0.01$) (Table 4). In the control pigs, there was no change in plasma PLP concentration over time ($P>0.05$). However, plasma PLP concentrations in vitamin B_6 deficient pigs were decreased by 63% on the first week ($P<0.05$), and were further reduced during later weeks ($P<0.05$). Plasma PLP concentrations in the vitamin B_6 deficient pigs were similar on the third, fourth and sixth week ($P>0.05$) but were all lower than other time points ($P<0.05$). Plasma PLP concentrations on the sixth week in the deficient group were only 13% and 14% of baseline and control groups, respectively.

Table 4 Effect of vitamin B_6 deficiency on plasma PLP, total homocysteine, and cysteine concentrations in piglets (Manuscript I)

Week	PLP (nmol/L)			Homocysteine (μmol/L)			Cysteine (μmol/L)		
	$+B_6$	$-B_6$	P value	$+B_6$	$-B_6$	P value	$+B_6$	$-B_6$	P value
0	37.3±4.3	38.3±3.0^{a}	0.8529	28.4±1.9^{a}	25.9±3.7^{c}	0.5728	226.0±14.1^{a}	232.1±12.6^{a}	0.7501
1	37.2±3.9	14.3±0.9^{b}	<0.0001	26.2±1.7^{a}	22.8±1.1^{c}	0.0932	190.6±12.6^{ab}	182.2±11.5^{b}	0.6239
2	45.7± 4.9	8.5±0.4^{c}	<0.0001	17.7±1.7^{b}	23.9±2.1^{c}	0.0361	170.7±12.6^{b}	183.3±11.5^{b}	0.4663
3	43.2±3.9	6.0±1.0^{d}	<0.0001	17.7±1.7^{b}	336.5±14.9^{b}	0.0002	192.6±12.6^{ab}	97.5±14.1^{c}	<0.0001
4	41.5±3.9	4.0±0.6^{d}	<0.0001	13.5±1.7^{b}	411.7±30.7^{a}	0.0002	220.5±12.6^{a}	107.3±12.6^{c}	<0.0001
5	38.8±4.3	8.7±0.5^{c}	<0.0001	11.5±1.7^{c}	342.0±39.9^{b}	0.0011	200.5±12.6^{ab}	101.2±12.6^{c}	<0.0001
6	36.2±4.3	5.0±0.4^{d}	<0.0001	13.3±1.7^{b}	490.2±33.5^{a}	0.0001	224.7±12.6^{a}	94.6±12.6^{c}	<0.0001

Notes:

[1] Data are presented as least square means ± standard error.

[2] *P* values are derived from difference of main effect of diet. *P* values < 0.05 are considered significant.

[3] The same shoulder letters in each column mean non-significant ($P>0.05$).

4.4.3 Hepatic enzyme activities

Vitamin B_6 deficiency decreased hepatic CBS activity to 10 and 33% of control values for the apo- and total-enzyme, respectively (P <0.01) (Table 5). Both hepatic apo-CGL and total-CGL activities were reduced (P <0.05) by vitamin B_6 deficiency. Significant reductions in hepatic apo- and total-SHMT were also observed after 6 weeks of dietary vitamin B_6 depletion (P <0.05).

Table 5 Effect of vitamin B_6 deficiency on hepatic enzymatic activities in piglets subjected to six-week dietary vitamin B_6 depletion (Manuscript I)

Enzymes	PLP in Incubation Media	$+B_6$	$-B_6$	P value
CBS	-	218±18	19±18	0.0067
nmoL/(h·mg protein)	+	297±26	78±26	0.0099
CGL	-	1.43±0.10	0.65±0.09	0.0050
nmoL/(min·mg protein)	+	2.31±0.12	1.69±0.12	0.0268
SHMT	-	0.70±0.03	0.24±0.04	0.0007
nmoL/(min·mg protein)	+	1.57±0.08	1.06±0.08	0.0130

Notes:

[1] Data are presented as least square mean ± standard error.

[2] *P* values are derived from difference of main effect of diet. *P* values < 0.05 are considered significant.

[3] -PLP refers to measurement of apo-enzyme activity.

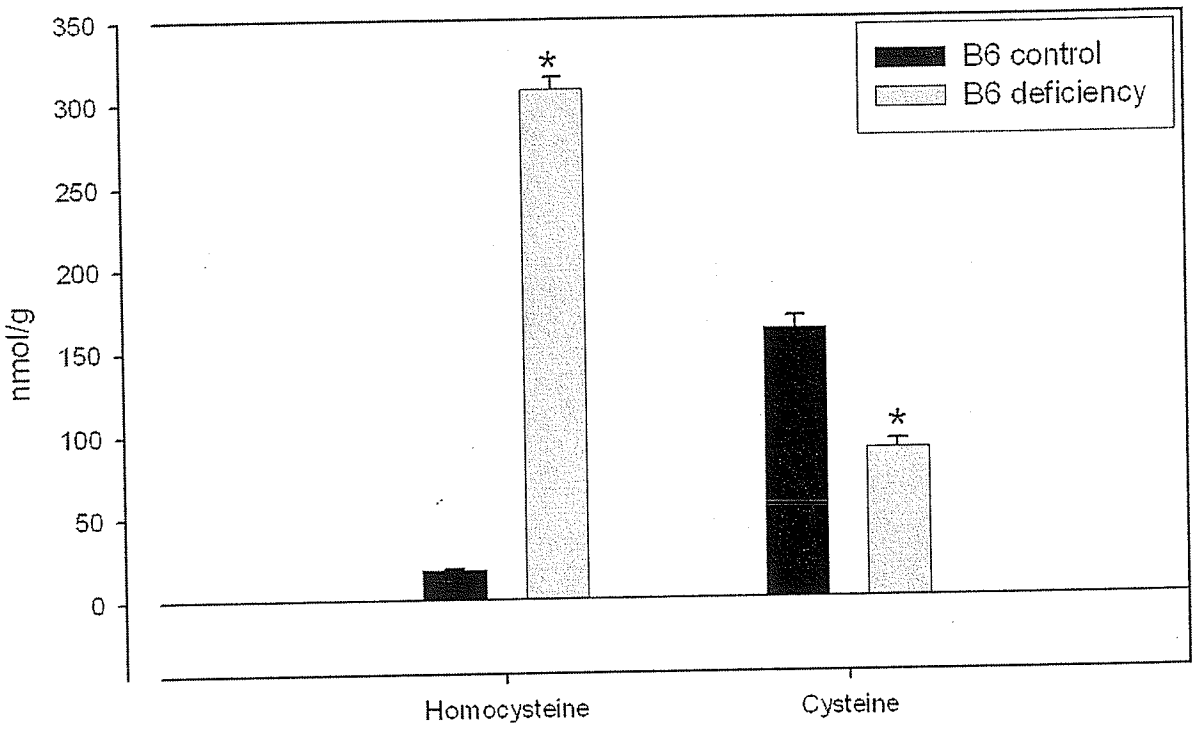
[4] +PLP refers to measurement of total enzyme activity.

4.4.4 Plasma and hepatic total homocysteine and cysteine concentrations

Vitamin B_6 deficiency significantly increased plasma total homocysteine concentration from the second week until the sixth week (P <0.05; Table 4). In particular, severe hyperhomocysteinemia in vitamin B_6 deficient pigs occurred from the third week until the sixth week. During this period, the concentration of plasma total homocysteine reached 336.5~490.2 μmol/L, which was approximately 19~37 times that in the control group. In the control group, plasma total homocysteine concentration decreased as the age increased. By contrast, significant decreases in plasma total cysteine were detected from the third week (P <0.01).

As in the plasma, a significant increase in hepatic total homocysteine (P <0.01) and a decrease in hepatic total cysteine (P <0.05) as a result of vitamin B_6 deficiency were observed (Figure 2).

Figure 2 Effect of vitamin B_6 deficiency on hepatic homocysteine and cysteine concentrations. Asterisk indicates that values for vitamin B_6 deficient pigs are significantly different ($P<0.05$) from corresponding control values.



4.4.5 Plasma amino acid concentration

Vitamin B_6 deficiency had no effect on plasma methionine, serine and glycine concentrations on the third week ($P > 0.05$) (see appendix I). At the end of the sixth week, plasma glycine concentration in deficiency group (724±64) was 55% of control (1319±335) ($P < 0.05$). By contrast, vitamin B_6 deficiency increased plasma methionine (71±13 vs. 836±413) and serine (119±18 vs. 179±16) concentrations by 10-fold and 50%, respectively ($P < 0.05$).

4.5 DISCUSSION

Vitamin B_6 serves a critical role as a cofactor in the metabolisms of amino acids, one-carbon units, lipids, glucose and neurotransmitter biosynthesis (Mackey et al., 2006). As such, this vitamin plays a central role in intermediary metabolism. In this study the adverse metabolic effects of vitamin B_6 deficiency are likely responsible for the observed reductions in weight gain and feed utilization efficiency even though feed intakes between treatments were not different (Parker and Linkswiler, 1972; Audet and Lupien, 1974; Okada and Suzuki, 1974; Wolfson et al., 1986; Wolfson and Kopple, 1987). This is consistent with a rat study in which depressed growth was reported despite the use of a pair feeding design (Wolfson and Kopple, 1987).

Plasma PLP concentration is the most commonly used measure of vitamin B_6 status. To our knowledge, the current study provides the first temporal characterization of changes in B_6 status, as measured by plasma PLP, in pigs. Plasma PLP concentrations in vitamin B_6 deficient pigs were decreased by 38% on the first week of diet treatment. Further reductions in plasma PLP concentrations were observed during subsequent weeks, with a nadir of 4-6 nmol/L reached by the end of the third week. Reduced plasma PLP

concentrations induced by vitamin B_6 restriction have been reported in humans (Davis et al., 2005) and rats (Martinez et al., 2000; Scheer et al., 2005) but data in pigs is limited. In general, previous studies have been limited to end point measures of PLP concentrations. The strength of the current data lies in the temporal characterization of additional biochemical measures, including plasma homocysteine, cysteine, and other amino acid concentrations, in response to vitamin B_6 deficiency in the pig.

As illustrated in Figure 1, the remethylation and transsulphuration pathways are the primary routes for homocysteine removal, with the transsulphuration pathway representing the sole route for *de novo* cysteine synthesis. In the current study, decreases in both the apo (without exogenous PLP) and total (with exogenous PLP) activities of hepatic CBS, CGL and SHMT were observed after six weeks of B_6 depletion, consistent with observations from rat studies (Smolin and Benevenga, 1984;Lima et al., 2006), although the magnitude of changes differed from published reports. In particular, the activities of hepatic apo-CBS and CGL were 9% and 45% of control animals, respectively, in the current trial. Previous authors have reported reductions of apo-CGL activities in rats receiving a semi-purified diet without addition of pyridoxine to 30% of control animal values, where rats were receiving 2 mg/kg pyridoxine, with no change in apo-CBS activities (Lima et al., 2006). Therefore, the current data support a greater sensitivity of CBS relative to CGL in response to a six-week vitamin B_6 depletion period in a pig model.

Based on enzyme activity results, an impaired transsulphuration pathway was present in pigs as a result of the six-week vitamin B_6 depletion. While enzyme activity levels were not determined at all time points, the accumulation of homocysteine and

reduction of cysteine in plasma and liver support such an impairment. Plasma homocysteine concentrations in vitamin B_6 depleted piglets in the current study did not change within the first two weeks of B_6 depletion. Interestingly, dramatic increases in plasma homocysteine concentrations were observed at the end of the third week, and these were maintained through the sixth week of the depletion period, with levels approaching 500 μmol/L. The abrupt increase in plasma homocysteine concentrations coincided with the halving of plasma cysteine concentrations at the end of three weeks on vitamin B_6 devoid diets. Previous research has shown that CBS has a strong affinity for PLP (Kery et al., 1999), potentially explaining the delay in the observed hyperhomocysteinemia and hypocysteinemia. The hyperhomocysteinemia induced by vitamin B_6 deficiency in this study is consistent with other reports in pigs (Smolin et al., 1983) and rats (Smolin and Benevenga, 1982,1984;Martinez et al., 2000), but not in healthy humans (Davis et al., 2005) with vitamin B_6 restriction treatment. The discrepancy with the latter observation likely reflects the severity of B_6 restriction: Pigs with depressed plasma PLP concentrations are able to maintain plasma homocysteine concentrations within normal limits, however this is limited to the first two weeks of depletion. It is reported that vitamin B_6 turnover is slow because this vitamin is sequestered by glycogen phosphorylase and released only when glycogen is depleted (Matte et al., 2001). Since the current study employed a pair-feeding design, cysteine intake was kept constant for the two treatments. Therefore, the observed reductions in plasma cysteine concentrations in B_6 deficient pigs provide strong evidence that *de novo* synthesis of cysteine is a major contributor to circulating cysteine and supply to peripheral tissues in adequately nourished pigs.

While the current data support an impaired flux through the transsulphuration pathway in B_6 deficient pigs, the use of *in vivo* isotope dilution techniques is required for the quantification of the impairment. Research from Gregory's lab has provided evidence that B_6 deficiency in rats, as supported by reduced plasma and hepatic PLP and increased homocysteine concentrations, leads to decreased rates of both transsulphuration, measured as the movement of deuterium label from $^2H_3$-serine into $^2H_3$-cysteine, and remethylation, measured as the formation of [$^2H_2$] methionine from a methyl group derived from [$^2H_3$]serine (Martinez et al., 2000). Impairments in both the remethylation and transsulphuration pathways would impact the primary routes of homocysteine disposal and could have resulted in the severe hyperhomocysteineima observed in the current study.

Previous research has shown that hepatic methionine concentration in rats (Scheer et al., 2005) and plasma methionine concentration in humans (Davis et al., 2005) are not affected by vitamin B_6 restriction. In the current study, increases in fasting plasma methionine concentrations in B_6 deficient pigs were observed at the end of 6 weeks, relative to those of the control pigs. Increased plasma methionine may reflect a reduction in the utilization of methionine for protein synthesis, as supported by the markedly depressed feed conversion efficiency in the deficient pigs. To date, little research (Martinez et al., 2000) has been performed to examine the impact of B_6 deficiency on protein kinetics, despite the recognized importance of this vitamin in protein and amino acid metabolism. An inhibition of transmethylation in vitamin B_6 deficient animals can also contribute to increased methionine concentrations, although previous researchers did not observe reductions in transmethylation rates in B_6 deficient rats (Martinez et al.,

2000). Elevated homocysteine levels are linked to increased S-adenosylhomocysteine concentrations (Yi et al., 2000), an inhibitor of methylation reactions. It is likely that the severity of B_6 restriction in the current study was greater than that observed in rats, as supported by the depressed CBS and CGL activities, and the magnitude of the changes in plasma and hepatic thiol concentrations, and this could have induced a reduction in overall transmethylation rates, but this remains to be determined.

The B_6-dependent enzyme SHMT catalyzes the interconversion of glycine and serine, and exists in both cytosolic and mitochondrial forms (Garrow et al., 1993). In the current study, total hepatic apo-SHMT activity was reduced to 34% of control values after 6 weeks of B_6 deficiency. Concurrent to the reduction in SHMT activity, plasma serine concentrations were increased and plasma glycine concentrations decreased as a result of B_6 deficiency. While this data support a reduced net flux of serine to glycine through SHMT, perturbations in the concentrations of these amino acids may have resulted from alterations in other metabolic processes. Depressed flux through the transsulphuration pathway could lead to an increased supply of serine. Glycine metabolism is governed by (1) SHMT reaction, (2) glycine n-methyltransferase (GNMT) reaction, (3) folate-dependent glycine cleavage system (GCS) and (4) sarcosine dehydrogenase reaction (House et al., 2003). A reduced flux through SHMT may limit the supply of 5-methyltetrahydrofolate, lead to a reduced flux through the remethylation pathway, and reduce the inhibitory effect of 5-methyltetrahydrofolate on GNMT activity (Wagner et al., 1985). This would then lead to a further decrease in glycine concentrations. Stable isotopic studies in the B_6 deficient pigs would help to further characterize the impact of B_6 status on sulphur amino acid and one-carbon metabolism.

To summarize this study, the following characterization was achieved as a result of dietary vitamin B_6 depletion: 1) vitamin B_6 status as measured by plasma PLP concentrations was reduced; 3) hepatic enzyme activities of SHMT, CBS and CGL were decreased; 4) hepatic and plasma homocysteine concentrations increased while cysteine concentrations, by contrast, decreased; 5) specific amino acids changed as shown in increased methionine and serine and decreased glycine concentrations.

Therefore, the current study has provided a temporal characterization of the impact of vitamin B_6 deficiency on indices of sulphur amino acid metabolism in the young pig. The results shed light on the sensitivity of plasma thiol concentrations to the degree of B_6 deficiency and may help aid in the interpretation of the linkage between B_6 status and plasma homocysteine concentrations in clinical settings. The use of the B_6 deficient pig model offers an opportunity to implement stable isotope studies to quantify the net impact of B_6 status on sulphur amino acid, one-carbon and overall protein kinetics.

CHAPTER FIVE

MANUSCRIPT II

Changes in Indices of Sulphur Amino Acid Metabolism in Response to Graded Levels of Vitamin B_6 Repletion in Vitamin B_6-Depleted Piglets

5.1 ABSTRACT

This study was designed to characterize changes in indices of sulphur amino acid metabolism in response to graded levels of vitamin B_6 repletion in the vitamin B_6 deficient piglet. Twenty 14-d-old pigs were subjected to 4-week vitamin B_6 depletion. Following the sacrifice of four pigs to serve as repletion baselines, the remaining animals were re-assigned to one of four dietary vitamin B_6 repletion treatments: 0.75, 1.5, 2.25 and 3 mg/kg diet as pyridoxine·HCl (n=4). Fasting blood was taken on day 3, 7, 10, and 14 after repletion, respectively. At D14 after repletion all the animals were sacrificed to end the trial. Vitamin B_6 repletion increased average daily gain (ADG) and feed efficiency on the first week of repletion ($P<0.05$), but had no effect on average daily feed intake (ADFI) and all the growth parameters on the second week of repletion ($P>0.05$). Dose-dependent response of plasma pyridoxal 5'-phosphate (PLP) concentration to vitamin B_6 repletion was observed throughout the experiment ($P<0.01$). In the end plasma PLP concentration in the treatment of 2.25 mg/kg was significantly higher than those in the lower repletion treatments ($P<0.05$) but was not different to that in the repletion level of 3 mg/kg ($P>0.05$). Plasma homocysteine concentrations in each level of vitamin B_6 repletion decreased over repletion from baseline (510.9±33.9 μM). On the first week of repletion plasma homocysteine concentrations in the treatment of 0.75 mg/kg were significantly higher than those in the other treatments ($P<0.05$). At the end of repletion, plasma homocysteine concentrations in the treatments of 2.25 and 3 mg/kg were still lower than those in treatments of 0.75 and 1.5 mg/kg ($P<0.05$). There was a linear correlation between plasma PLP and homocysteine concentrations within 10 days after repletion. Plasma cysteine concentrations however, were lower on the first week of

repletion (P<0.05). Plasma PLP and homocysteine data support increasing vitamin B_6 requirement to 2.25 mg/kg as pyridoxine·HCl. At the end of repletion vitamin B_6 repletion increased hepatic cystathione gamma lyase (P<0.01) but had no effect on hepatic cystathionine beta synthase and serine hydroxymethyltransferase activities (P>0.05). In conclusion, plasma PLP and homocysteine showed dose-dependent response to vitamin B_6 repletion and therefore, serve as good indicators for vitamin B_6 status. The observed patterns of these two parameters are supportive of an inclusion level of 2.25 mg/kg diet as pyridoxine·HCl, in diets for young pigs.

Key words: piglets, vitamin B_6, homocysteine, repletion, requirement

5.2 INTRODUCTION

As shown in Figure 1, cystathionine beta synthase (CBS), the first enzyme in the transsulphuration pathway in sulphur amino acid (SAA) metabolism, utilizes homocysteine and serine to produce cystathionine. The latter is then catalyzed by cystathionine gamma lyase (CGL) to give rise to cysteine and α-ketobutyrate. Both these reactions require pyridoxal 5'-phosphate (PLP), the major biologically active form of vitamin B_6 in animal tissues as a cofactor. Serine hydroxymethyltransferase (SHMT) is also a PLP-dependent enzyme, which catalyzes the reversible inter-conversion of serine and tetrahydrofolate (THF) to glycine and 5,10-methylenetetrahydrofoate. The latter is a precursor of 5-methyltrahydrofoate, a co-substrate for homocysteine remethylation via methionine synthase. Therefore, vitamin B_6 plays a critical role in the metabolism of homocysteine, cysteine and one-carbon metabolisms as well. Metabolites including homocysteine and cysteine involved in enzymatic reactions dependent on vitamin B_6 in SAA metabolism have been found to be sensitive estimates of deficiency of vitamin B_6. A static picture of temporal changes in indices of SAA metabolism in piglets subjected to vitamin B_6 depletion has been established in the previous study. Increased homocysteine and decreased cysteine concentrations induced by vitamin B_6 depletion were reported in rodent and pig models (Smolin et al., 1983; Martinez et al., 2000). Inhibition of enzymatic reactions of CBS, CGL, and SHMT as well as changes in thiol concentrations by vitamin B_6 restriction were examined in rats (Smolin and Benevenga, 1984;Lima et al., 2006) and pigs in our laboratory (Chapter 4). However, the response of indices of SAA metabolism to graded levels of vitamin B_6 repletion is not yet clear.

The current vitamin B_6 requirements are 1.5 mg/kg of diet for both 5-10 and 10-20 kg pigs (NRC, 1998). These estimates were mainly based on growth rate, feed efficiency, blood physiological parameters of hemoglobin, red blood cell and lymphocyte counts, and biochemical indice of urinary xanthurenic acid (Miller et al., 1957; Sewell et al., 1964). The limitation of the above studies first lies in that growth performance, the major response criteria used for estimating requirements, could be affected by the environment (Sewell et al., 1964). Second, the estimates differ with the criteria used (Miller et al., 1957). Furthermore, these studies were carried out four decades ago. The development of genetic potential, production system and nutrition over generations together with the very few reports on vitamin B_6 requirements call for a review of the current recommendation of vitamin B_6. The study by Woodworth et al. (2000) shows that adding 3.3 mg/kg of pyridoxine to commercial ingredients based diet improved growth performance in pigs within 14 days post-weaning. Matte et al. reported that dietary supplementation of 50 mg/kg B_6 saturated erythrocyte PLP in pigs within 2 weeks post-weaning (Matte et al., 2005). This research demonstrates that vitamin B_6 requirements of pigs may need to be increased. Additionally, a depletion/repletion study has been shown to be more sensitive in the detection of small differences in biological parameters (Peo et al., 1957a,b). It is hypothesized that vitamin B_6 and biochemical indices of SAA metabolism are responsive to graded levels of vitamin B_6 repletion in pigs depleted in vitamin B_6. Therefore, the current depletion/repletion experiment was designed to examine temporal changes in vitamin B_6 status and biochemical indices of SAA metabolism in response to graded levels of vitamin B_6 repletion in the vitamin B_6 depleted piglet.

5.3 MATERIALS AND METHODS

5.3.1 Animals

This experiment was approved by the University of Manitoba Institutional Protocol Management and Review Committee. Twenty 14-d-old weaned cross-bred male piglets were delivered from The Glenlea Swine Research Unit, University of Manitoba to the T.K. Cheung Centre for Animal Science Research, Fort Garry Campus, University of Manitoba. Pigs were individually housed in an environmentally controlled room. There were little holes in each divider between two pens for the visual interaction of pigs. Metal chains and plastic balls were provided in each pen for enrichment. All the animals in the experiment were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993).

5.3.2 Diet and design

Depletion Protocol. A semi-purified basal diet without added crystalline vitamin B_6 was utilized. All the ingredients in the basal diet were purchased from Harlan Teklad, U.S.A. All the pigs were subjected to vitamin B_6 depletion using the vitamin B_6-devoid basal diet. Except for pyridoxine, the vitamin B_6 devoid diet, as shown in Table 2 was formulated to meet or exceed the requirements for 5-10 kg pigs. The powdered diet was mixed with water at the ratio of approximate 3 parts diet to 1 part water prior to feeding. Water and feed were offered freely. In order to avoid the confounding effect of weaning on initial feed intake, pigs were first adapted to a diet supplemented vitamin B_6 at the level of 3 mg/kg pyridoxine·HCl for three days before implementation of depletion protocol. The average initial body weight at the beginning of depletion was 5.0 kg.

Based on the former study (Manuscript I), a four-week depletion period was applied in the current experiment.

Repletion Protocol. At the end of depletion, 4 pigs were sacrificed, by the use of Euthanyl through jugular vein, in order to collect liver samples to serve as baselines for the repletion protocol. The remaining animals were reassigned to one of the four dietary treatments of vitamin B_6 repletion with 4 replicates per treatment. The four vitamin B_6 repletion diets were formulated by adding synthetic pyridoxine·HCL at 0.75, 1.5 (NRC requirement, 1998), 2.25 or 3.0 mg/kg diet to the vitamin B_6 devoid diet. The duration of the dietary repletion was two weeks. Pigs were fed *ad libitum*.

5.3.3 Data Collection

Feed intake was recorded daily. Body weight was measured weekly after 16 hours of feed withdrawal. Average daily feed intake (ADFI), average daily gain (ADG) and feed conversion efficiency (FCE) were calculated.

5.3.4 Blood and Tissue Collection

Fasting blood was taken before the start of depletion and at the end of depletion period. During the repletion period, fasting blood was sampled on day 3, 7, 10, and 14 after dietary repletion, respectively. Blood was collected via jugular vein into EDTA coated evacuated tubes, placed on ice, and covered with aluminum foil. Blood was centrifuged at 9300 ×g to harvest plasma. Following blood collection at the end of the repletion period, all the animals were sacrificed using Euthanyl as mentioned above and liver samples harvested and put immediately into liquid nitrogen. All the plasma and tissue samples were stored at -80°C until analysis.

5.3.5 Analyses

Plasma PLP was measured using a commercial radioenzymatic assay (Vitamin B_6 PLP ^{3}H-REA, ALPCO). Hepatic PLP was determined by reverse phase-HPLC method with fluorescence detection (Gregory, 1980;Ubbink et al, 1985). Briefly, 0.5 gram of liver sample was homogenized with 8 volumes of 10% trichloroacetic acid. After centrifuge, 1 mL of the supernatant was taken and incubated with 50 μL of 0.5 M semicarbizade for 30 minutes at 40 °C. Following extraction with 3 mL diethyl ether and 3 mL dichloromethane, around 100 μL of the supernatant was harvested for loading onto the HPLC. The PLP semicarbazone derivative was separated on a Waters C-18 column (5 μM, 4.5 x 250 mm; Waters Canada, Mississauga, ON), using isocratic elution (phosphate buffer with 3% acetonitrile, pH 2.9) by means of a Shimadzu HPLC system (Man-Tech Associates, Guelph, ON) and measured by fluorescence detector (excitation λ = 350 nm; emission λ = 478 nm). The mobile phase flow rate was 1.1 mL/min.

Plasma and hepatic total homocysteine and cysteine concentrations were analyzed according to the reverse phase-HPLC method of Araki and Sako (1987), with modifications as suggested by Gilfix et al., (1997).

Hepatic apo and holo CBS activities were determined using radioisotopic techniques with modifications (Mudd et al., 1965; Taoka et al., 1998). One gram of tissue sample was homogenized with 4 mL of 0.05 M potassium phosphate buffer (pH 6.9) and centrifuged as mentioned above. The incubation mixture included 0.15 mM L-cystathionine, 41.67 mM DL-homocysteine, 0.42 mM PLP (holo enzyme only), 0.32 mM S-adenosylmethionine, 2.08 mM DL-propargylglycine, 125 mM Tris, 2.08 mM EDTA, 25 mM L-[U-^{14}C]serine (~74 000 dpm/μmol) and 150 μL of homogenate supernatant with 25% dilution in a total volume of 600 μL. The reaction mixture, with the exception

of [^{14}C]serine, was pre-incubated for 5 min at 37 °C. The reaction was started by adding [^{14}C]serine and was terminated after 60 minutes using 300 μL of 15% ice-cold trichloroacetic acid. Five hundred μL of sample supernatant were loaded onto a column with AG 50W-X8 resin (Bio-Rad) and the column washed with 2×4 mL of water, 6×4 mL of 1N HCl, and 4×4 mL of water in sequence. The ^{14}C-cystathionine was eluted with 5 mL of 3N ammonium hydroxide. One mL of the collected solvent was mixed with the same fraction of scintillation fluid (ScintiSafe Plus^{TM}). Radioactivity was counted by a liquid scintillation counter. Protein concentration and incubation time were tested to be within the linear range.

Hepatic CGL activity was quantified by spectrophotometric assay (Stipanuk, 1979). The reaction mixture consisted of 0.1 M potassium phosphate buffer, pH 7.5, 4.0 mM L-cystathionine, 0.125 mM PLP (holo enzyme only), 0.32 mM NADH, and 1.5 units of lactate dehydrogenase/mL. Protein concentration was optimized for both apo and holo enzymes. The kinetics of NADH absorbance was measured by DU 800 Spectrophotometer at 37°C.

Hepatic SHMT was analyzed by a binding assay using radio isotope L-[^{14}C(U)]-Serine (Geller and Kotb, 1989;Lewis, et al., 1998). The reaction mixture contained 0.4 mM serine, 2.0 mM tetrahydrofolate, 2.5 mM EDTA, 1.0 mM 2-mercaptoethanol, 0.25 mM PLP (holo enzyme only). This mixture was incubated for 10 min with the temperature of 37°C. The reaction was stopped by streaking a 25 μL aliquot onto a labeled 3×3 cm square of DE-81 filter paper (Whatman®). The paper was then washed

with running distilled water at 2-3 liters/min for 20 minutes. After drying the paper was cut into squares. The radioactivity of the separate paper square was counted.

Protein concentration for tissue homogenate was determined using a Bicinchoninic Acid Protein Assay Reagent kit (Pierce, Product No. 23225).

5.3.6 Statistical Analysis

To adequately account for within-subject correlation over time and handle the missing data, the mixed model was applied to analyze the parameters with repeated observations including growth performance and indicators from plasma samples (Littell *et al.,* 1996; Wang and Goonewardene, 2004). The diet effect and interaction effect between diet and week were partitioned in the model. Because of the homogenous variance over time in the control group and heterogeneous variances over time in the deficiency group, a common variance for control group and individual variance for deficiency group throughout the trial were employed. For tissue sample data, the PROC GLM was used, and a Randomised Complete Block design was applied in the model (SAS Inst., Inc., Gary, NC, U.S.A.). Animals were treated as block. And diet and block effects were partitioned in the model. For the data before diet treatment and at the end of depletion, average and standard error values were obtained using PROC MEANS (SAS Inst., Inc., Gary, NC).

A generalized function, the Richards equation (Thornley and France, 2007, pp. 155-157) was used to test whether the plasma PLP concentrations (PLP) vs. repletion time (D) profile could be better described using a sigmoid function. The functional form used was:

$$PLP = \frac{PLP_0 PLP_f}{[PLP_0^n + (PLP_f^n - PLP_0^n)e^{-kD}]^{1/n}}$$

where PLP_0 is PLP at 0 repletion time, PLP_f is PLP at final repletion time, D is repletion time (D), k (/d) is a positive rate parameter and $n \geq -1$. The Richards equation was chosen because it encompasses the logistic, Gompertz, and monomolecular (diminishing returns) when its additional parameter (n) has a value of 1, 0 and -1, respectively. Fitting the Richards equation to the composite data showed that n was not significantly different from 1. Therefore, the logistic equation was used. The logistic function was fitted to the data set using the nonlinear procedure (PROC NLIN; SAS Inst. Inc., Cary, NC). The function was fitted with a common PLP_0 value but PLP_f and k were allowed to vary. The parameter estimates with their standard errors and the required repletion time at 90% of the final plateau PLP concentration (PLP_{90f}) were obtained.

5.4 RESULTS

5.4.1 Growth performance

Growth performance data is shown in Table 6. Average ADFI, ADG, and FCE during depletion period were 390±15 g/d, 291±14 g/d, 0.742±0.013, respectively. During the repletion period, ADFI was not affected by vitamin B_6 repletion ($P>0.05$). However, significant increases in ADG by vitamin B_6 repletion were observed in the first week of repletion ($P<0.05$) during which ADG in pigs receiving 3 mg/kg pyridoxine·HCl was higher than that in pigs receiving 0.75 mg/kg pyridoxine·HCl ($P<0.05$). However, the impact of repletion level on ADG was not observed on the second week of repletion ($P>0.05$). Similarly, a significant effect of vitamin B_6 repletion on FCE was detected on the first week after repletion as well ($P<0.05$).

Table 6 Effect of vitamin B_6 repletion on average daily feed intake (ADFI, g/d), average weight gain (ADG, g/d), and feed conversion efficiency (FCE) in vitamin B_6 depleted piglets (Manuscript II)

	Treatment[1]					
Item	0.75 mg/kg	1.5 mg/kg	2.25 mg/kg	3 mg/kg	SEM	*P*-value
ADFI[2]						
Week 1[3]	727	739	786	794	40	0.5681
Week 2[3]	996	1002	1059	1049	40	0.6101
ADG[2]						
Week 1[3]	511[b]	592[ab]	562[ab]	673[a]	38	0.0306
Week 2[3]	708	686	724	640	38	0.4571
FCE[2]						
Week 1[3]	0.708[b]	0.806[ab]	0.716[b]	0.850[a]	0.039	0.0497
Week 2[3]	0.713	0.686	0.683	0.611	0.039	0.3323

[a-b]Within a row, means without a common superscript letter differ ($P<0.05$).

[1]Vitamin B_6 was supplemented as crystalline pyridoxine·HCl.

[2]Average ADFI, ADG, and FCE throughout depletion period were 390±15 g/d, 291±14 g/d, and 0.742±0.013, respectively (n=20).

[3]Week1 = the first week after starting vitamin B_6 repletion; Week2 = the second week after starting vitamin B_6 repletion.

5.4.2 Plasma and hepatic PLP concentrations

As shown in Table 7, plasma PLP concentrations before dietary depletion and at the end of depletion were 24.93±1.58 (n=20), and 3.18±0.22 nmol/L (n=20), respectively. Plasma PLP concentrations increased with increasing dietary vitamin B_6 repletion levels throughout the repletion period ($P<0.01$). In the 0.75 mg/kg pyridoxine·HCl dietary treatment, plasma PLP concentrations increased significantly over repletion until day 10 after repletion. In dietary treatments of 1.5 and 3.0 mg/kg pyridoxine·HCl, plasma PLP concentrations on day 7 were higher than those on day 3 after repletion ($P<0.01$), but not different with those at later repletion time points ($P>0.05$). In 2.25 mg/kg pyridoxine·HCl dietary treatment, plasma PLP concentrations on the second week after repletion were higher than those on the first week after repletion ($P<0.05$). Nonlinear statistical analysis showed that the k value, the parameter reflecting repletion speed, was much higher in the highest repletion level of 3 mg/kg pyridoxine·HCl than that in the lowest repletion level of 0.75 mg/kg pyridoxine·HCl (0.72±0.09 vs. 0.21±0.04). Similarly, the plateau PLP (PLP_f) concentraton was 25.8±1.2 and 14.1±3.0 nmol/L for 3.0 mg/kg and 0.75 mg/kg repletion levels, respectively (see appendix II and III).

As seen in Table 8, hepatic PLP concentration in depleted piglets was 2.88±1.38 nmol/g. This concentration was elevated to the level between 4.11 and 11.17 nmol/g. Hepatic PLP concentration at the end of repletion in pigs receiving 0.75 mg/kg pyridoxine·HCl was lower than those in all the other treatments ($P<0.05$). There were no differences in hepatic PLP concentrations in pigs receiving 1.5, 2.25, and 3 mg/kg vitamin B_6 as pyridoxine·HCl, respectively ($P>0.05$).

Table 7 Effect of vitamin B_6 repletion on plasma pyridoxal 5'-phosphate (PLP), homocysteine and cysteine concentrations in vitamin B_6 depleted piglets (Manuscript II)

PLP[1] (n*M*)						
Day after repletion	0.75 mg/kg	1.5 mg/kg	2.25 mg/kg	3mg/kg	SEM	*P*-value
Day 3	3.35^{bC}	4.66^{bB}	9.43^{aB}	11.93^{aB}	0.80	<0.0001
Day 7	5.85^{cB}	15.15^{bA}	15.33^{bB}	25.47^{aA}	1.76	<0.0001
Day 10	10.62^{cA}	18.17^{bA}	21.43^{bA}	26.85^{aA}	2.71	0.0028
Day 14	10.91^{cA}	19.49^{bA}	27.84^{aA}	23.76^{aA}	2.07	0.0025
Polynomial contrast	Linear	Cubic	Cubic	Quadratic		
Homocysteine[2] (μ*M*)						
Day 3	393.3^{aA}	100.8^{bA}	67.2^{bA}	51.2^{bA}	55.7	0.0028
Day 7	168.6^{aB}	42.5^{bB}	34.8^{bB}	37.1^{bB}	30.3	0.0224
Day 10	40.1^{B}	36.6^{B}	32.0^{B}	30.8^{B}	3.7	0.3124
Day 14	45.2^{aB}	42.4^{aB}	33.4^{bB}	30.9^{bB}	2.9	0.0136
Polynomial contrast	Cubic	NA	Cubic	NA		
Cysteine[3] (μ*M*)						
Day 3	122.4^{bC}	156.7^{abB}	172.7^{a}	174.6^{a}	12.8	0.0479
Day 7	136.3^{bC}	191.3^{aAB}	184.2^{a}	204.1^{a}	13.3	0.0183
Day 10	219.3^{B}	210.5^{A}	208.7	210.9	14.0	0.9501
Day 14	183.9^{A}	188.8^{AB}	186.6	184.6	9.1	0.9801
Polynomial contrast	Linear	NA	NA	NA		

$^{a-c}$Within a row, means without a common superscript lowercase letter differ ($P<0.05$).
$^{A-C}$Within a column, means without a common superscript uppercase letter differ ($P<0.05$).
[1]Average PLP concentrations before and at the end of depletion were 24.93±1.58, and 3.18±0.22 n*M*, respectively (n=20); [2]Average homocysteine concentrations before and at the end of depletion were 32.3±2.2 and 510.9±33.9 μ*M*, respectively (n=20); [3]Average cysteine concentrations before and at the end of depletion were 198.7±7.1 and 80.3±6.2 μ*M*, respectively (n=20).
NA means polynomial contrast is not applicable to linear, quadradic, or cubic.

Table 8 Effect of vitamin B_6 repletion on hepatic PLP, homocysteine, cysteine concentrations and hepatic apo-enzymatic activities (nmol/g) in vitamin B_6 depleted piglets (Manuscript II)

	Treatment[1]					
Item	0.75 mg/kg	1.5 mg/kg	2.25mg/kg	3 mg/kg	SEM	*P*-value
PLP[2] (nmol/g)	4.11[b]	9.47[ab]	11.17[a]	9.88[a]	1.21	**0.0156**
Thiols[2] (nmol/g)						
Homocysteine	125	156	176	159	16	0.1917
Cysteine	2006[ab]	2323[a]	1957[ab]	1800[b]	121	0.0578
Enzymes[3] (nmol/(min·mg protein))						
CBS	3.50	5.86	5.39	4.94	0.61	0.2436
CGL	0.42[b]	0.58[ab]	0.75[a]	0.78[a]	0.06	**0.0045**
SHMT	0.78	0.77	0.82	0.88	0.05	0.4640

[a-b]Within a row, means without a common superscript lowercase letter differ ($P<0.05$).

[1]Vitamin B_6 was supplemented as crystalline pyridoxine ·HCl.

[2]Average hepatic PLP, homocysteine and cysteine concentrations at the end of depletion were 1.51±0.31, 392±63 and 1716±77 nmol/g, respectively (n=4);

[3]Apo enzyme means without adding exogenous pyridoxal 5'-phosphate (PLP) to the reaction system for analysis; CBS represents cystathionine beta synthase; CGL represents cystathionine gamma lyase; SHMT represents serine hydroxymethyltransferase; Average hepatic apo CBS, CGL, and SHMT activities at the end of depletion were 1.65±0.14, 0.14±0.01, and 0.30±0.07 nmol/(min·mg protein), respectively (n=4).

5.4.3 Plasma and hepatic homocysteine and cysteine concentrations

Plasma Homocysteine. Plasma homocysteine concentrations (Table 7) before and after depletion were 32.3±2.2 and 510.9±33.9 μ*M*, respectively (n=20). Except day 10 after repletion, plasma homocysteine concentrations were significantly affected by vitamin B_6 repletion ($P<0.05$). Within the first week of repletion, plasma homocysteine concentrations in pigs receiving 0.75 mg/kg pyridoxine·HCl were higher than those in pigs receiving higher repletion levels of vitamin B_6 ($P<0.05$). At the end of repletion, plasma homocysteine concentrations in pigs receiving 2.25 and 3 mg/kg pyridoxine·HCl were lower than those in pigs receiving 0.75 and 1.5 mg/kg pyridoxine·HCl ($P<0.05$). Plasma homocysteine concentrations in each level of vitamin B_6 repletion decreased over the repletion period.

Plasma Cysteine. Plasma cysteine concentrations (Table 7) before and after depletion were 198.7±7.1 and 80.3±6.2 μ*M*, respectively. The effect of graded levels of vitamin B_6 repletion on plasma cysteine was significant only in the first week after repletion ($P<0.05$).

Hepatic Homocysteine and Cysteine. Hepatic homocysteine and cysteine concentrations (Table 8) at the end of depletion were 392±63 and 1716±77 nmol/g, respectively. Hepatic homocysteine concentration was resumed to the level between 125±16 and 176 ±16 nmol/g at the end of repletion. However, there were no differences in hepatic homocysteine concentrations among repletion levels. Similarly, hepatic cysteine concentration was restored to the level between 1800±121 and 2323 ±121 nmol/g at the end of repletion. B_6 repletion levels had no effect on hepatic cysteine concentrations at the end of repletion ($P>0.05$).

5.4.4 Hepatic enzyme activities

Hepatic enzyme activities are shown in Table 8. Hepatic apo CBS, CGL, and SHMT activities at the end of depletion were 1.65±0.14, 0.14±0.01, and 0.30±0.07 nmol/(min·mg protein), respectively. Hepatic apo forms of CBS and SHMT at the end of repletion were not different among vitamin B_6 repletion levels ($P>0.05$). Hepatic apo-CGL was significantly affected by vitamin B_6 repletion level ($P<0.01$). Apo-CGL activities in pigs receiving 0.75 mg/kg pyridoxine·HCl were lower than those in pigs receiving other three levels of vitamin B_6 ($P<0.05$). There were no differences in hepatic apo-CGL activities among treatments of 1.5, 2.25 and 3.0 mg/kg pyridoxine·HCl ($P>0.05$).

5.5 DISCUSSION

To our knowledge, this is the first study to characterize changes in indices of SAA metabolism to graded levels of vitamin B_6 repletion in vitamin B_6 depleted pigs. ADFI (345g/d) in 5~13.3 kg pigs during depletion period in this experiment was even lower than that in 5~10 kg pigs in NRC (1998). The lower feed intake by vitamin B_6 depletion is consistent with previous work in our laboratory. Feed intake was not different throughout repletion period. Depression of appetite and food intake by vitamin B_6 deficiency has been well documented (NRC, 1998). It is postulated that when starting repletion of vitamin B_6, PLP status in all pigs was enough to prevent the loss of appetite. Therefore, there was no difference in feed intake among different repletion levels ($P>0.05$). On the first week of vitamin B_6 repletion, ADG and FCE were significantly affected by increasing repletion levels of vitamin B_6 ($P<0.05$). However, these responses did not show a dose-dependent phenomenon. The observation that ADG in pigs receiving 0.75 mg/kg pyridoxine·HCl was lower than that in pigs from the other higher vitamin B_6 repletion levels implies that the amount of vitamin B_6 added at 0.75 mg/kg pyridoxine·HCl was still limiting as also evidenced from observations of hyperhomocysteinemia and low levels of plasma PLP and cysteine at this time point. The lower weight gain in pigs receiving 0.75 mg/kg pyridoxine·HCl might be due to the metabolic effect of vitamin B_6 (Mackey, et al., 2006). The lower weight gain therefore, led to decreased feed conversion efficiency since feed intake was not changed. As vitamin B_6 repletion progressed, the repletion effect of vitamin B_6 on growth promotion disappeared on the second week while the effects on plasma homocysteine and PLP concentrations were still evident. Therefore, growth, the net result of numerous metabolic

events over a longer period is not a sensitive indicator of short-term changes in biochemical metabolites as reported in another study (House et al., 2003).

PLP serves as a direct measure of vitamin B_6 status *in vivo*. PLP at the beginning of the depletion phase was 24.9 nmol/L and declined to 3.2 nmol/L, consistent with observations from Manuscript I. The patterns of response in plasma PLP to pyridoxine repletion reflect both dose and time dependency. Martinez et al. (2000) provided evidence of a dose dependency of plasma PLP to increasing dietary B_6 from 0 to 2 mg/kg diet in rats. With respect to the temporal dependency of plasma PLP to dietary B_6 repletion, both repletion rate and plateau PLP concentration differ with repletion doses as shown in the k values and PLP_f values. The fact that the repletion rate in the treatment of 3 mg/kg diet was much faster than the lowest repletion level of 0.75 mg/kg diet implies that it takes more time to reach plateau level for animals at lower repletion levels. However, the reason why the animals on the lower PLP repletion doses exhibited lower plateau PLP concentration remains unclear. With respect to the temporal dependency of plasma PLP to dietary vitamin B_6 repletion, at the lowest level of dietary B_6 repletion (0.75 mg/kg), plasma PLP was still increasing into the second week of repletion ($P<0.05$). By day 14, only those pigs receiving the highest levels of dietary B_6 (2.25 and 3.0 mg/kg diet) exhibited maximal PLP responses and these were consistent with the observed pre-depletion levels. A similar pattern was observed for hepatic PLP concentrations during the repletion phase. This implies that plasma or hepatic PLP is a sensitive indicator to vitamin B_6 status. While the aforementioned results allow for the characterization of vitamin B_6 status through the use of direct markers of plasma and hepatic PLP

concentrations, the primary intent of the current study included the examination of the sensitivity of sulphur amino acid metabolism to dietary vitamin B_6 supply.

As mentioned above, metabolism of homocysteine through the transsulphuration pathway is undertaken by the PLP-dependent enzymes CBS and CGL. Likewise, the endogenous production of 5-methyltetrahydrofolate, a substrate for homocysteine remethylation, requires PLP (Figure 1). All of the three enzyme activities of CBS, CGL and SHMT after a 4-week depletion period were depressed relative to pre-depletion levels. A two-week repletion period resulted in substantial restoration of activity levels ($P<0.05$). This might be due to the fact that when vitamin B_6 is repleted, PLP status is improved, with resultant impacts on the structure of B_6 dependent-enzymes (reviewed in House et al., 1999). At the end of repletion, the effect of graded levels of vitamin B_6 repletion on enzyme activities was significant in CGL ($P<0.01$), but not in CBS and SHMT ($P>0.05$). This implies that these enzymes respond differently to vitamin B_6 status temporarily. It is deduced that CGL has a higher affinity than CBS for PLP in pigs based on the observation in our previous study that there was one week lag of occurrence of significant decrease in cysteine than the increase in homocysteine by vitamin B_6 depletion (Chapter 4). The Km values of CBS, SHMT and CGL for PLP were reported to be 700 n*M*, 850 n*M*, and 1400 n*M*, respectively by *in vitro* measurements (Oh and Churchich, 1973; Perry et al., 2007). This indicates that CGL has a lower affinity than CBS and SHMT for PLP, which means that it takes longer time for CGL to reach its full capacity to combine PLP. We found that CGL activities at the end of repletion in the current experiment is only 29%~55% of that in control piglets receiving the same basal diet supplementing 3 mg/kg pyridoxine·HCl in our previous study. As such, the duration

of two-week repletion is enough for CBS and SHMT, but not enough for CGL to fully recover due to that the affinity of CGL for PLP is almost doubled that of CBS and SHMT as discussed above. The enzyme response of SHMT to graded levels of vitamin B_6 repletion in pigs in this experiment is not consistent with that in a study by Scheer et al. (2005) in which apo-SHMT activities in rats receiving 5 weeks of graded levels of pyridoxine declined with reduction of dietary pyridoxine ($P<0.05$). In the meantime, Lima et al. (2006) did not detect differences in CGL activities in rats receiving five weeks of semi-purified diet with supplementation of 0, 0.1, 0.5, 1 and 2 mg pyridoxine, respectively ($P>0.05$).

The novelty of this experiment lies in the use of homocysteine and cysteine as response criteria for vitamin B_6 status using a depletion-repletion protocol. Homocysteine and cysteine are well documented to be sensitive to vitamin B_6 deficiency (Smolin and Benevenga, 1982,1984; Smolin et al., 1983; Martinez et al., 2000). However, little is known concerning the response of homocysteine and cysteine to graded levels of vitamin B_6 repletion. At the end of depletion, severe hyperhomocysteinemia was developed due to the very low status of PLP *in vivo*. Consistent with the observed restoration in enzyme activity levels upon vitamin B_6 repletion, plasma total homocysteine and cysteine concentrations reverted back to control levels during repletion phase. The pattern of changes for both total homocysteine and cysteine mirrored the temporal and dose-dependent responses observed for plasma PLP. Nonlinear regression analysis shows the data set between plasma PLP and homocysteine fits inverse first order equation HCY=-37.2963+1387.2212/PLP ($P<0.0001$; $R^2=0.7163$). A depletion-repletion study in rats also illustrated a decrease in plasma homocysteine as the progress of

repletion in folate-depleted rats ($P<0.05$). In general, when plasma PLP concentrations exceeded 10 nmol/L, plasma total homocysteine and cysteine approximated concentrations observed in pigs receiving diets with adequate levels of vitamin B_6.

By contrast to homocysteine, plasma cysteine concentrations were increased by vitamin B_6 repletion within the first week of repletion. The repletion effect of vitamin B_6 on cysteine had disappeared in the second week of repletion ($P>0.05$). However, the lower CGL activity in pigs receiving 0.75 mg/kg pyridoxine·HCl than in those receiving 2.25 and 3 mg/kg pyridoxine·HCl ($P<0.05$) at the end of repletion would be expected to lead to changes in cysteine concentration since cysteine is the product of CGL catalyzed reaction. This inconsistency might be contributed by cysteine turnover, probably due to the downstream metabolism of cysteine to glutathione, taurine and sulphate. The positive correlation between plasma PLP and cysteine was maintained within the first week ($P<0.05$) as well. The fact that the significant impact of vitamin B_6 repletion on homocysteine was maintained longer than it was for cysteine implies that homocysteine may serve as a more sensitive indicator of vitamin B_6 status, as opposed to cysteine.

While not the primary focus of the present study, the results do provide additional insight into the adequacy of current dietary vitamin B_6 recommendations for young pigs. Plasma PLP and homocysteine concentrations are sensitive to vitamin B_6 status in repletion study. It follows that these two parameters can be utilized to serve as response criteria for estimating vitamin B_6 requirement. Plasma PLP and homocysteine concentrations in pigs receiving 2.25 mg/kg diet of pyridoxine·HCl were significantly different than those in the treatments of 0.75 and 1.5 mg/kg, but not different from those in pigs consuming 3.0 mg/kg by day 14 of the repletion period. The concentrations of

these two indicators in the treatment of 2.25 mg/kg recovered to the levels prior to depletion as well. As such, these data support increasing vitamin B_6 requirement to 2.25 mg/kg, as added pyridoxine·HCl, for pigs in the 10-20 kg weight class, reflecting an estimate that is 150% of the current NRC recommendation (NRC, 1998). These results support the recommendations of others to readjust vitamin B_6 recommendations upwards for young pigs (Woodworth et al., 2000; Matte et al., 2005).

In summary, graded levels of vitamin B_6 repletion resulted in dose-dependent responses of plasma PLP concentration and indices of SAA metabolism. As the level of dietary vitamin B_6 repletion increased, plasma PLP plateau concentration and repletion rate increased, and time to reach 90% of plateau shortened. Plasma homocyseine decreased as repletion level increased and demonstrated linear correlation with plasma PLP within 10 days after repletion. Changes in plasma PLP and homocysteine concentrations responsive to graded levels of vitamin B_6 repletion provided supports for the increase in vitamin B_6 requirement in 10-20 kg pigs to 2.25 mg/kg as pyridoxine·HCl.

CHAPTER SIX

MANUSCRIPT III

Establishment Of An *in vivo* Isotope Model Using L-[2,3,3-d_3]Serine and L-[3-^{13}C]Cyseine To Quantify The Rate Of Transsulphuration In The Early Weaned Pig — A Pilot Study.

6.1 ABSTRACT

The objectives of this pilot study were to establish priming and constant infusion doses for the measurement of L-[3-^{13}C]cysteine and L-[2,3,3-d_3]serine kinetics, based on plasma precursor pool enrichment. Additionally, the study was undertaken to determine whether we could measure the rate of transsulphuration, thus establish a stable isotope model based on these two stable isotope tracers to quantify transsulphuration rate in piglets. Piglets were freely fed the same semi-purified diet as the control diet used in manuscript I and II. Pigs were surgically installed with a jugular and femoral catheters. The priming infusion dose for L-[3-^{13}C]cysteine was 7.5 μmol/kg and constant infusion rate was 7.5 μmol/(kg•h). The priming infusion dose for L-[2,3,3-d_3]serine was 60 μmol/kg and constant infusion dose was 60 μmol/(kg•h). After three days recovery, an eight-hour isotope infusion was initiated. Blood was sampled hourly during the infusion period. Isotopic enrichments were measured by GC-MS. Cysteine (m+3) enrichments from serine (m+3) were low, but permitted a calculation of the transsulphuration rate as 1.1 μmol/(kg•h), which was 5% of cysteine flux of 22.5 μmol/(kg•h). Serine flux was determined to be 204 μmol/(kg•h). The stable isotope model using L-[3-^{13}C]cysteine and L-[2,3,3-d_3]serine to determine transsulphuration rate was preliminarily established based on the observation of significant and measurable enrichments.

Key words: Stable isotope model, serine, cysteine, pig, transsulphuration rate

6.2 INTRODUCTION

Pyridoxal 5'-phosphate (PLP), the major biological form of vitamin B_6, serves as a cofactor for cystathionine beta synthase (CBS) and cystathionine gamma lyase (CGL) in the transsulphuration pathway and serine hydroxymethyltransferase (SHMT) in 1-carbon unit metabolism. Therefore, the status of vitamin B_6 is associated with sulphur amino acid metabolism and 1-carbon metabolism as well. A large volume of research shows the role of vitamin B_6 deficiency in perturbing the metabolism of serine and sulphur amino acids. Overall, a consistent conclusion is that vitamin B_6 deficiency results in decreases in hepatic and plasma PLP concentrations, and hepatic SHMT, CGL activities. However, the results of hepatic CBS activity, plasma homocysteine and cysteine concentrations show a lack of consistency (Smolin and Benevenga, 1982,1984; Smolin et al., 1983; Martinez et al., 2000; Scheer et al., 2005; Davis et al., 2005,2006; Lima et al., 2006), and are dependent on the degree of deficiency and the models used in the experiments. The experiments from our laboratory in early weaned pigs showed increases in plasma homocysteine and serine concentrations and decreases in plasma PLP, cysteine, glycine concentrations, and hepatic CBS, CGL, and SHMT activities, which were resulted from dietary vitamin B_6 depletion (Manuscripts I & II). It follows that in combination with other studies, the given research (Manuscripts I & II) confirmed the role that vitamin B_6 deficiency plays in inhibiting transsulphuration and one-carbon unit metabolism in a pig model. As such, kinetic measurements pertaining to the effect of vitamin B_6 deficiency on transsulphuration are critical to quantify the net impact of B_6 deficiency on the synthesis of cysteine for use by the body.

As discussed in Chapter 2, the methodology for determining transsulphuration rate can be grouped into one of two general methods - indirect or direct. With the indirect method, ^{13}C labeled methionine oxidation is followed as an indirect measure of transsulphuration rate, and this method is typically known as Storch-Young model (Storch et al., 1988). The mechanism for measuring transsulphuration rate using this method is that the labeled 1-^{13}C moiety can be finally metabolized to yield $^{13}CO_2$. The measurement of $^{13}CO_2$ enrichment in breath together with measurement of ^{13}C methionine in the infusate and blood, are used to calculate transsulphuration rate. This method is widely used in human studies to quantify the kinetics of transsulphuration, remethylation, and transmethylation (Storch et al., 1988,1990; Young et al., 1991; Fukagawa et al., 1996,1998; Raguso et al., 1997,2000; MacCoss et al., 2001; Jahoor et al., 2006). However, the disadvantage of this method lies in overestimation from production of $^{13}CO_2$ derived from sequestration of S-adenosylmethionine for polyamine synthesis. The other method for transsulphuration rate quantification is direct method using L-[2,3,3-d_3] serine and stable isotope labeled cysteine, such as L-[3-^{13}C]cysteine. In this method, the sulphur atom from homocysteine is transferred to serine skeleton to yield cysteine, which explains how the term "transsulphuration" is derived. Cysteine, the product of transsulphuration pathway, serves as a precursor for the endogenous synthesis of glutathione and taurine *in vivo*.

Because of the advantage of avoiding overestimation from transamination of methionine, the direct method has been attracting the attention of researchers. In particular, this method is applicable to the study of piglets because it does not require collecting breath samples. Determination of transsulphuration rate using L-[2,3,3-d_3]

serine and L-[3-^{13}C]cysteine has been reported to be successful in sheep (Liu et al., 2000), but not in rats (Davis et al., 2006; Jahoor et al., 2006). Measurement of transsulphuration rate in the whole-body level has not been reported in a pig model. One challenge from using this method in pigs comes from the considerable dilution of infused serine tracer. A study in piglets infused with [^{3}H] serine for 4 hours in our laboratory showed that the regression relationship between plasma specific radioactivity (SRA) (x) and hepatic SRA (y) was $y=0.1602x+6.4009$ ($R^2=0.815$) (Ludke, 2004). This demonstrates that the hepatic serine pool was substantially diluted to 16% of that found in plasma. This fact likely necessitates the infusion of higher dose of serine tracer to allow us to detect labeled cysteine derived from labeled serine. In addition, the analysis of cysteine enrichment may be challenging since cysteine oxidation is easily to be occurred. Therefore, a pilot study was undertaken to establish a stable isotope model utilizing L-[2,3,3-d_3] serine and L-[3-^{13}C]cysteine, with the intent of determining the quantitative impact of deficient vitamin B_6 status on serine and cysteine kinetics, and the rate of transsulphuration in young pigs.

6.3 HYPOTHESIS

NULL HYPOTHESIS

The stable isotope model with L-[3-^{13}C]cysteine and L-[2,3,3-d_3]serine is not capable of determining transsulphuration rate.

ALTERNATIVE HYPOTHESIS

The stable isotope model with L-[3-^{13}C]cysteine and L-[2,3,3-d_3]serine is applicable to determining transsulphuration rate.

6.4 OBJECTIVES

1) To examine whether the selected priming doses and constant infusion rates of L-[3-^{13}C]cysteine and L-[2,3,3-d_3]serine allow for determining transsulphuration rate.

2) To set up a stable isotope model employing L-[3-^{13}C]cysteine and L-[2,3,3-d_3]serine to quantify transsulphuration rate in piglets.

6.5 METHODOLOGY

6.5.1 Animals and management

Four Male [(Yorkshire×Landrace)×Duroc] cross-bred piglets were obtained from the University of Manitoba's Glenlea Research Station swine barn. The pigs were weaned at 17-d-old and admitted to the University of Manitoba Animal Science Research Unit. The animals were housed individually in pens with plastic-covered expanded metal floors and fed the control semi-purified diet as used in Manuscript I and II.

6.5.2 Surgery

Isotope infusion and blood collection were performed through femoral and jugular venous catheters, respectively. These catheters were installed surgically. The general protocol for surgery followed those of Wykes et al., (1993). After an overnight fast, piglets were anesthetized with 4% halothane in oxygen. After induction of anesthesia, halothane concentration was reduced to approximately 2.5%. Piglets were placed on the operation table and thoroughly scrubbed with skin cleanser, followed by 70% isopropyl alcohol and finally a betadine solution, which was applied to the entire left side of the pig, neck and throat regions, and the inner left hind leg. A subcutaneous injection of Cronyxin (pain management; 1mL/45kg BW) and an intramuscular injection for Excenel (antibiotic; 1mL/17kg BW) were given.

The first incision was made on the upper back above the shoulder. The second incision was close to the central neck. The incisions were opened a little bit using hemostats. A one-inch hole was punctured left to the first incision using a cutting needle and a catheter was introduced. By the use of small straight hemostats, a grommet was threaded just under the first incision to fix the catheter. Using a trochar, the catheter was

tunneled subcutaneously to the incision at the side of the neck. This procedure was repeated for the femoral catheter except that the catheter was tunneled to the incision on the knee. Subcutaneous and skin sutures were then followed on the first incision. After re-positioning the animal, an incision was cut laterally and the jugular vein was dissected. A catheter was inserted into the vessel with the help of curved hemostats, forceps, a straight angled 22-gauge needle, and an introducer. Sutures of grommets, muscle and skin were followed. An adapter was attached and rinsed with heparinized saline. Antibiotic cream (Polysporin™) was applied on the incisions and a jacket with pocket was put on in which the outside end of the catheter was held.

Excenel injection and antibiotic cream were repeated for 3 days post-surgery to reduce the chance of infection. Body temperature was monitored for several days post-operation and catheters were checked daily for patency (Ludke, 2004).

6.5.3 Stable isotope tracers

The stable isotope tracers, L-[3-^{13}C] cysteine and L-[2,3,3-d_3] serine were purchased from Cambridge Isotope Laboratories, Inc (50 Frontage Road, Andover, MA, 01810, USA) with chemical purity of greater than 98% for both. L-[3-^{13}C] cysteine was chosen to quantify the flux of cysteine. L-[2,3,3-d_3] serine was chosen to serve as a substrate marker for transsulphuration. The net result of the transsulphuration pathway is that the sulphur atom is transferred to the skeleton of L-[2,3,3-d_3] serine to generate L-[2,3,3-d_3] cysteine.

6.5.4 Infusion doses

The appropriate priming dose and constant infusion rate are critical for the measurement of transsulphuration rate. If the rates for priming dose and infusion rate are

too low, it may not be possible to detect the cysteine coming from labeled tracers; If too high, isotope costs may become prohibitive. With respect to cysteine and serine infusions in swine, limited information exists with respect to the infusion of L-[3-^{13}C]cysteine and L-[2,3,3-d_3]serine to determine transsulphuration rate. In a sheep model constant infusion doses for L-[3-^{13}C]cysteine and L-[2,3,3-d_3]serine were 1.37 and 4.36 μmol/(kg•h), respectively (Liu et al., 2000). Based on the challenges from cysteine oxidation, and evidence from our laboratory related to the extensive dilution of hepatic serine label (Ludke, 2004), the constant infusion rate was set as 7.5 μmol/(kg•h) for L-[3-^{13}C]cysteine and 60 μmol/(kg•h) for L-[2,3,3-d_3]serine. Accordingly, the priming dose was 7.5 μmol/kg for L-[3-^{13}C]cysteine and 60 μmol/kg for L-[2,3,3-d_3]serine.

At 6:00 AM on the morning of infusion, 180 mL of sterile tracer solution were made based on body weight, infusion rate (20 mL/hr) and target infusion doses (serine: 60 μmol/(kg•h); cysteine: 7.5 μmol/(kg•h)). Tracers were dissolved with saline, filtered and transferred to a sterile bottle.

6.5.5 Isotope infusion and blood sampling

With respect to optimal sites for infusion and sampling, sites upstream from the site of infusion in primed-constant infusion experiments are preferred (Wolfe, 1984). For the current study, isotopes were infused into the femoral vein and sampled from the jugular vein, respectively.

After collecting baseline blood samples, a priming dose was infused for 12 minutes at the speed of 99 mL/h using an infusion pump. The infusion speed was switched to 20 mL/h at the end of 12 minutes of infusion. At the end of one hour after

infusion, 8 mL of blood sample was drawn using a heparinized syringe through jugular vein catheter and transferred into an EDTA coated vacuum tube. The constant infusion was kept until the end of 8 h after infusion. Blood was sampled at an hourly interval. The animal had free access to feed and water during infusion period. At the end of infusion, the pig was sacrificed right away using Euthanyl through jugular vein catheter and liver samples collected into liquid nitrogen right away.

6.5.6 Processing of harvested blood samples

Following collecting plasma, half of the plasma was stored in –80°C freezer. The other half was treated as the following procedure to prevent cysteine from oxidation: to each portion of 1 mL plain plasma, 200 μL of 100 mM dithiothreitol (DTT) and 20 μL of 5 mM nor-leucine (in 0.1 N HCl) were added and incubated at room temperature for 30 minutes. Following incubation, 1500 μL of acetone was added to deproteinize the plasma. The supernatant was harvested after centrifuge and added 100 μL of the above DTT. The processed samples were stored in -80°C until analysis.

6.5.7 Measurement of isotopic enrichments

Enrichment measurement of stable isotope labeled amino acids was based on the method by Wykes et al. (1996) with modifications. Plasma was de-proteinized by adding 800 μL of 1M glacial acetic acid to 200 μL plasma. Liver samples (200 mg) were homogenized in 5 mL of ice-cold de-ionized water and incubated for 10 minutes on ice after adding 500 μL of 10% Tris (2-carboxyethyl) phosphine hydrochloride (TCEP). The above samples were de-proteinized by adding 4.5 mL 22.2% trichloroacetic acid (TCA). The amino acids from the supernatant of plasma and liver samples were purified using

Dowex columns (Bio-Rad, AG-50W-X8 resin, 200-400 mesh, H^+) and dried using a Speedvac. Esterification using acetyl chloride and propanol (1:5) was followed and further derivatized with 100 μL hepatofluorobutyric anhydride. After dissolving with 300 μL ethyl acetate, the samples were loaded into an auto sampler microvial for analysis using negative chemical ionization GCMS. The natural isotopic abundance m was performed at m/z 519 and 535 for serine and cysteine, respectively. The isotopic enrichments of deuterium labeled serine m+1, m+2, m+3 were made at m/z 520, 521, 522, respectively. Cysteine labeling coming from serine was monitored at m/z 536, 537, 538, respectively.

6.5.8 Calculation of cysteine and serine kinetics

The calculation of metabolic kinetics was based on those used by Liu et al. (2000). The plasma irreversible loss rate (ILR, μmoL/(kg·h)) of serine or cysteine was calculated using the formula:

$$\mathbf{ILR=[(MPEi/MPEs)-1]\times I}$$

Where MPEi is the enrichment of the infusate and MPEs is the plateau enrichment of the amino acids in plasma. "I" stands for the rates of isotope infusion (60 and 7.5 μmoL/(kg·h) for serine and cysteine, respectively). Transsulphuration rate (TS), the amount of mass transferred from serine to cysteine, was calculated as

$$\mathbf{TS=ILRcys\times MPEcys(m+3)/MPEser(m+3)}$$

Where ILRcys is the ILR of cysteine, MPEser(m+3) is the plasma plateau enrichment of [2,3,3-D_3] serine, MPEcys(m+3) is the plasma plateau enrichment of [2,3,3-D_3] cysteine derived from [2,3,3-D_3] serine.

6.6 RESULTS AND DISCUSSION

6.6.1 Enrichments and fluxes

The objective of this pilot study was to establish a primed-continuous infusion dose for both serine and cysteine that would: a) lead to a plateau in amino acid enrichment; b) lead to the sensitive measurement of sufficient enrichment in the given isotopes; and c) provide for the measurement of transfer of label from serine to cysteine. Based on the results, the objectives were met with the use of a single pilot subject.

As shown in Figure 3, plasma serine (m+3) reached plateau starting from the end of the first hour after infusion, which was maintained until the end of eight hours of infusion. This demonstrates that the serine tracer infusion dose met the objectives for measuring serine flux. The flux value for serine was calculated as 204 μmoL/kg/h (Table 9). Similarly, cysteine flux was calculated as 22.5 μmoL/kg/h based on L-[3-^{13}C] cysteine infusion rate and the enrichments of cysteine (m+1) in plasma and infusate. However, cysteine (m+3) in plasma was not detected. This may be due to the limitations of GCMS analytical technique. Alternatively, the failure to detect measurable cysteine (m+3) enrichment may be related to the large dilution of serine in the liver as mentioned above. When greatly diluted in the liver, the production of cysteine (m+3) will be reduced, which will be further diluted once into blood stream. As shown in table 9, cysteine (m+3) was detected in the liver, but not in plasma. A solution to detecting cysteine (m+3) in plasma may be to further increase the serine (m+3) infusion dose.

6.6.2 Transsulphuration rate

Since transsulphuration happens intracellularly, hepatic cysteine (m+3) enrichment was employed to calculate transsulphuration rate (TS). TS was calculated as 1.1 μmoL/kg/h, and thus accounted for 5% of cysteine flux (Table 9).

Figure 3 Plasma serine (m+3) enrichment in piglet infused with L-[2,3,3-$^{2}H_3$] serine and L-[3-^{13}C]cysteine

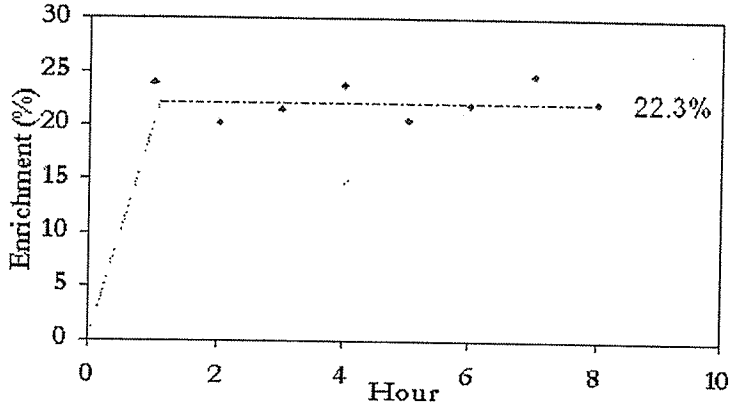


Table 9 Summary of pilot kinetic measurements (Manuscript III)

Items	Results
Cysteine (m+1) enrichment in plasma (%)	24.5
Cysteine (m+1) enrichment in infusate (%)	98
L-[3-^{13}C] cysteine infusion rate (μmoL/kg/h)	7.5
Cysteine flux, Q_{cys} (μmoL/kg/h)	22.5
Serine (m+3) enrichment in plasma (%)	22.3
Serine flux (μmoL/kg/h)	204
Cysteine (m+3) enrichment in liver (%)	1.1
Cysteine (m+3)/serine (m+3) (%)	4.9
Transsulphuration rate, TS (μmoL/kg/h)	1.1

Note:

1) Primed infusion rates were 60 and 7.5 μmoL/kg for L-[2,3,3-$^{2}H_3$] serine and L-[3-^{13}C]cysteine, respectively.

2) Constant infusion rates were 60 and 7.5 μmoL/kg/h for L-[2,3,3-$^{2}H_3$] serine and L-[3-^{13}C]cysteine, respectively.

3) Transsulphuration rate calculation was made based on the assumption that hepatic cysteine enrichment is equal to plasma cysteine plateau enrichment

4) 6.7 SUMMARY

The infusion rates for L-[2,3,3-d_3]serine and L-[3-^{13}C] cysteine allowed us to achieve measureable enrichments and transsulphuration rate. The stable isotope model using L-[2,3,3-d_3]serine and L-[3-^{13}C] cysteine for determining transsulphuration rate was preliminarily established. Based on this pilot study, the infusion dose for L-[2,3,3-d_3] serine could be further increased in next study in order to detect the enrichment of cysteine (m+3) in plasma.

CHAPTER SEVEN

MANUSCRIPT IV

Measurement Of Serine and Cysteine Kinetics In The Vitamin B_6 Deficient Pig

7.1 ABSTRACT

Vitamin B_6 status plays a significant role in 1-carbon and sulphur amino acid metabolism. We hypothesized that whole-body serine and cysteine kinetics, including transsulphuration rate (TS) will be decreased during vitamin B_6 deficiency in pigs. Ten (5/treatment) 14-d-old pigs were fed a semi-purified diet containing either 0 (deficient group) or 3 mg (control group) pyridoxine·HCl/kg diet, using a pair-feeding design. After three weeks of dietary vitamin B_6 depletion, kinetic measurements were undertaken through the use of primed-continuous infusion of L-[2,3,3-d_3]serine and L-[3-^{13}C]cysteine. The priming dose was 90 μmol/kg for L-[2,3,3-d_3]serine and 7.5 μmol/kg for L-[3-^{13}C]cysteine. The constant infusion rate was 90 and 7.5 μmol/(kg•h) for L-[2,3,3-d_3]serine and L-[3-^{13}C]cysteine, respectively. Blood samples were taken at baseline and hourly for a total of 6 hours. The results indicated that, among the performance indices, only feed efficiency at the third week was significantly reduced (P<0.05). As a result of dietary vitamin B_6 depletion, significant decreases in plasma and hepatic pyridoxal 5'-phosphate (PLP) concentrations, hepatic enzymatic activities of cystathionine beta synthase (CBS), cystathionine gamma lyase (CGL), and serine hydroxymethyltransferase (SHMT), as well as increases in homocysteine concentrations were observed (P<0.05). Whole-body plasma serine flux rate in vitamin B_6 deficient pigs was reduced by 30% (655.9±26.7 vs. 463.9±26.7 μmoL/kg/h, P<0.01). Plasma cysteine flux was 119.8±3.6 μmoL/kg/h for control and 97.8±3.6 μmoL/kg/h for B_6 deficient pigs (P<0.05). However, TS was not detected, likely due to the extent of intracellular dilution of the serine label. The turn over of one-carbon units was affected, as evidenced by the increased glycine (m+1) enrichment due to vitamin B_6 deficiency. In conclusion, the

results from this experiment demonstrate perturbation of metabolism of serine and cysteine in terms of kinetics and static measures of the biochemical indices by vitamin B_6 deficiency in a pig model.

Key Words: vitamin B_6, cysteine, serine, isotope dilution, pigs

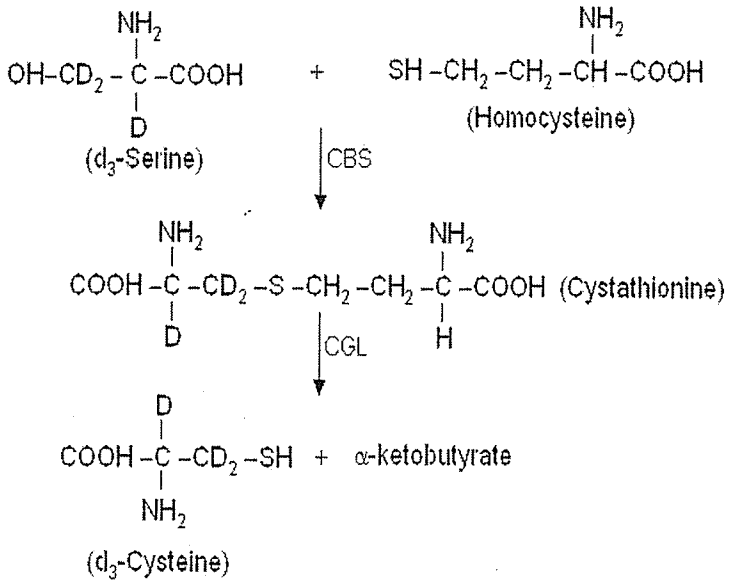
7.2 INTRODUCTION

Pyridoxal 5'-phosphate (PLP), the major biological form of vitamin B_6, serves as a cofactor for over 100 enzymes, including the enzymes cystathionine beta synthase (CBS) and cystathionine gamma lyase (CGL) in the transsulphuration pathway and serine hydroxymethyltransferase (SHMT) in the folic acid cycle. As such, the status of vitamin B_6 is associated with homocysteine remethylation, transsulphuration and *de novo* supply of cysteine, accordingly. A series of investigations demonstrated that dietary vitamin B_6 deficiency caused significant decreases in hepatic and plasma PLP concentrations, hepatic SHMT and CGL activities and increases in plasma glycine and cystathionine concentrations, but had no affect on hepatic CBS activity (Martinez et al., 2000; Scheer et al., 2005; Davis et al., 2005,2006; Lima et al., 2006). However, changes in homocysteine and cysteine concentrations demonstrated a lack of consistency, and likely varied due to the model employed and the degree of vitamin B_6 deficiency developed (Davis, et al., 2006; Lima et al., 2006).

Serine has been shown to be a major source of one-carbon units (Davis et al., 2004) and the substrate for transsulphuration as discussed below. The metabolism of serine requires vitamin B_6 as a cofactor (Nelson and Cox, 2000). The experiments presented in Chapters 4 & 5 provide strong evidence of consistent increases in plasma homocysteine and serine concentrations and decreases in plasma PLP, cysteine and glycine concentrations, and hepatic CBS, CGL, and SHMT activities by vitamin B_6 depletion in the young pig. These data confirm the role that vitamin B_6 deficiency plays in inhibiting transsulphuration and affecting the metabolism of one-carbon units.

Transsulphuration is a metabolic process in which the sulphur atom from homocysteine is transferred to the carbon skeleton of serine via enzymatic reactions of CBS and CGL (Figure 4). Cysteine, the product of the transsulphuration pathway, serves as a precursor for the endogenous synthesis of glutathione and taurine *in vivo*. Therefore, the transsulphuration pathway is important to the health of humans and animals, through the provision of the reduced form of glutathione (GSH), which plays a major role in maintaining a reduced cellular environment and as a mediator of many physiological reactions, including the metabolism of xenobiotics (Franco et al., 2007).

Figure 4 Proposed metabolic pathway for isotope labeling of d_3-cysteine via d_3-serine



Abbreviations: d_3, [$^2H_3$]; d_2, [$^2H_2$]; CBS, cystathionine beta synthase; CGL, cystathionine gamma lyase

However, the information with respect to kinetic measurements of sulphur amino acids in vitamin B_6 deficiency is limited (Davis et al., 2006). In particular, the rate of transsulphuration (TS), a terminology reflecting the rate of endogenous production of cysteine, at the whole-body level in pigs deficient of vitamin B_6 has not previously been reported. The measurement of TS, cysteine and serine flux in a pig model subjected to vitamin B_6 depletion is important in order to quantify the impact of vitamin B_6 deficiency on 1-carbon metabolism and transsulphuration.

For the current study, it was hypothesized that vitamin B_6 deficiency decreases serine and cysteine fluxes and TS through the inhibition of enzymes in transsulphuration pathway and folic acid cycle. The objective of this experiment was to determine kinetic measurements of transsulphuration and serine turnover using L-[2,3,3-D_3] serine and L-[3-^{13}C] cysteine, in addition to static measurement of metabolites in the transsulphuration pathway.

7.3 MATERIAL AND METHODS

7.3.1 Animals, dietary treatment and surgery

This experiment was approved by the University of Manitoba's Institutional Protocol Management and Review Committee. Ten 14-d-old cross-bred pigs were individually housed and assigned to receive one of two dietary treatments with five replicates each. The treatments consisted of a basal semi-purified diet supplemented with either 0 (deficient) or 3 (control) mg/kg vitamin B_6 as pyridoxine·HCl. The semi-purified basal diet was formulated to meet or exceed the requirements for 5-10 kg pigs (NRC, 1998). The composition of the basal diet included 40.15% corn starch, 20.5% "vitamin free" test casein (90% crude protein), 20% lactose, 5.5% corn oil, 3.5% cellulose (Alphacel), 0.35% cystine (Sigma®), 9% mineral premix and 1% vitamin premix. Details of the diet were presented in Chapter 4.

Pigs were pair-fed to avoid the confounding effects of anorexia associated with B_6 deficiency, and the corresponding perturbations in the intake of nutrients associated with sulphur amino acid metabolism such as, folate, vitamin B_{12} and methionine. Animals were staggered to start diet treatment by pairs. Diets were mixed with water (3 parts of diet vs. one part of water). Feed intake was recorded daily and body weights were collected at a weekly interval.

Based on our previous study using the same protocol (Chapter 4), the beginning of the fourth week after dietary treatment was chosen for the start of the isotopic tracer infusion, on the basis of changes in plasma and hepatic PLP and homocysteine concentrations as well as hepatic CBS, CGL, and SHMT activities due to vitamin B_6

depletion. Therefore, a recovery surgery for each animal was carried out to insert jugular and femoral venous catheters one week prior to isotope infusion, essentially as described in Chapter 6. After being fasted overnight, pigs were pre-medicated at the dose of 0.3 mL/kg with the mixture of ketamine and xylazine (10:1) and administered Cronyxin (1 mL/45kg) and Excenel (1mL/17kg) intramuscularly. Anesthesia was initiated with 2.5% isoflurane in oxygen. Catheters (Silastic® Laboratory Tubing) with size of 0.030 mm×0.065mm and 0.025 mm×0.047 mm were inserted into jugular and femoral veins, respectively. Catheters were subcutaneously tunneled and secured to the incisions. Antibiotic cream (Polysporin™) was applied to all incisions. Respiration rate, heart rate, percent of oxygen saturation, and body temperature were monitored during surgery. Together with applying antibiotic cream to incisions, intramuscular injections of Excenel were performed daily for three days post-surgery. Body temperature measurement and catheter flushing with heparinized saline were conducted daily.

7.3.2 Stable isotope labeled tracers and infusion protocol

The isotope tracers, L-[2,3,3-D_3] serine and L-[3-^{13}C] cysteine, were purchased from Cambridge Isotope Laboratories, Inc (50 Frontage Road, Andover, MA, 01810, USA).

L-[3-^{13}C] cysteine was used to determine the plasma flux or the irreversible loss rate of cysteine *in vivo*. L-[2,3,3-D_3] serine was chosen to measure transsulphuration kinetics directly because L-[2,3,3-D_3] serine (m+3) goes to the transsulphuration pathway to give rise to deuterium labeled cysteine (m+3) (Figure 4). Based on a pilot study (Chapter 6), the constant infusion doses for L-[3-^{13}C] cysteine and L-[2,3,3-D_3] serine

were defined as 7.5 and 90 μmoL/(kg·h), respectively. The primed infusions had the same doses as those in constant infusion, respectively. On the morning of each infusion day, 180 mL of sterile tracer solution was made individually for each pig based on body weight, infusion rate and target infusion dose. Tracers were dissolved in sterile saline, filtered and transferred to a sterile bottle.

On each infusion day, two pair-fed pigs as per the order of receiving dietary treatments were used for isotope infusion. After collecting baseline blood samples, a priming dose of 7.5 μmol/kg L-[3-^{13}C] cysteine and 90 μmol/kg L-[2,3,3-D_3] serine in a portion of 20 mL infusate was administered via femoral vein catheter. A constant infusion was followed for 6 hours using the same catheter. The flow rate of the infusate was kept as 20 mL/h using infusion pumps after calibration. At each hourly sampling time, 8 mL of blood sample was drawn using a heparinized syringe through jugular vein catheter and transferred into an EDTA coated vacuum tube hourly. Pigs were kept on pair-feeding during infusion and had free access to water. At the end of the sixth hour of infusion, animals were sacrificed using Euthanyl through jugular vein catheter immediately following the final blood sampling period. Livers were harvested immediately, weighed and collected into liquid nitrogen.

7.3.3 Processing of harvested blood samples

Following plasma collection, half of the plasma from baseline and infusion periods was stored at –80°C. The remaining fresh plasma was treated according to the following procedure: To each portion of 1 mL plain plasma, 200 μL of 100 mM DTT and 20 μL of 5 mM nor-leucine (in 0.1 N HCl) were added and incubated at room

temperature for 30 min. Following incubation, 1500 μL of acetone was added to deproteinize the plasma. The supernatant was harvested after centrifuge and added 100 μL of the above DTT. The processed samples were stored at -80°C until analyzed.

7.3.4 Metabolite analyses

Plasma PLP was measured using a commercial radioenzymatic assay (Vitamin B_6 PLP 3H-REA, ALPCO). Plasma and hepatic total homocysteine and cysteine concentrations were analyzed according to the reverse phase-HPLC method of Araki and Sako (1987), with modifications as suggested by Gilfix et al., (1997).

7.3.5 Hepatic enzyme activity analysis

Hepatic apo and total CBS activities were determined using radioisotopic techniques with modifications (Mudd et al., 1965; Taoka et al., 1998). Hepatic CGL activity was quantified by spectrophotometric assay (Stipanuk, 1979). Hepatic SHMT was analyzed by a binding assay using radioactive isotope L-[14C(U)]-Serine (Geller and Kotb, 1989; Lewis, et al., 1998). Protein concentration for tissue homogenate was determined using a Bicinchoninic Acid Protein Assay Reagent kit (Pierce, Product No. 23225).

7.3.6 Measurement of isotopic enrichments

7.3.6.1 Instrument and method

Enrichment measurement of isotopically labeled amino acids was conducted by LC-MS/MS as referenced to Rafii et al (2007). The equipment used for measuring enrichment was a benchtop triple quadrupole mass spectrometer API 4000 (Applied Biosystems/MDS Sciex, Foster City, CA, USA). This instrument was run in positive ionization mode with the TurboIonSpray ionization probe source. The instrument was

coupled to an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). Analyst NT software (version 1.4.1) was utilized to control system operation and data acquisition. Maximum sensitivity for target compounds including serine, cysteine, glycine, methionine and cystathionine, was obtained by measuring product ions (multiple reaction monitoring [MRM]) from the fragmentation of the protonated $[M + H]^+$ molecule in a positive ion mode. The maximum intensity of the $[M + H]^+$ precursor (parent) ions (m/z 104 for serine, m/z 120 for cysteine, m/z 221 for cystathionine, m/z 74 for glycine, m/z 148 for methionine) was achieved by optimizing the declustering and entrance potentials. By adjusting the collision energy potential and the collision exit potential, the signal for the most abundant product (daughter) ions (m/z 63 for serine, m/z 77 for cysteine, m/z 134 for cystathionine, m/z 31 for glycine, m/z 62 for methionine) was optimized. Nitrogen was served as the collision gas at a pressure of 5.0×10^{-3}mbar. The system was running with an isocratic gradient. The flow phase used was aqueous 30% methanol/0.1% formic acid running at 250 μL/min. A Waters Symmetry C_8 column (3.5 μm, 2.1×100 mm, Waters Canada, Mississauga, ON, Canada) was used for separation. The injection volume was 3 μL. Precision/accuracy of the LC-MS/MS assay, quantitative recoveries of the method and reproducibility of calibration curves were also tested to meet analytical requirements.

7.3.6.2 Sample preparation

Plasma was incubated with 500 mM DTT solution for 15 minutes. Then 300 μL of a 5:3 mixture of 0.1% formic acid and 0.025% trifluoroacetic acid in water/methanol was added to precipitate protein. After 5 minutes at room temperature, the mixture was centrifuged for 5 minutes at 13,000 *g*. The clear supernatant was transferred to auto

sampler vials. Liver samples were homogenized with ice-cold de-ionized water (1 gram liver vs. 4 mL water). After centrifuging at 10,000 *g* for 30 minutes, the clear supernatant was taken for analysis.

7.3.7 Kinetic calculations

Enrichment (tracer/tracee ratio) was obtained by subtracting the baseline enrichment from the collected samples after isotope tracer infusion. Plateau enrichments were determined by the average of three consecutive time points with less than 5% of coefficient variation.

The calculation of metabolic kinetics was based on those of Liu et al. (2000). The plasma irreversible loss rates (ILR, μmoL/(kg·h)) of serine and cysteine were calculated using the formula:

$$\mathbf{ILR=[(MPEi/MPEs)-1]\times I,}$$

Where MPEi is the enrichment of the infusate and MPEs is the plateau enrichment of the amino acids in plasma. Plateau enrichments were defined as the average of three consecutive time points of enrichments with coefficient of variation value no more than 5%. "I" stands for the rate of isotope infusion (90 and 7.5 μmoL/(kg·h) for serine and cysteine, respectively). Transsulphuration rate (TS), the amount of mass transferred from serine to cysteine, was calculated as

$$\mathbf{TS=ILRcys\times MPEcys(m+3)/MPEser(m+3)}$$

Where ILRcys is the ILR of cysteine, MPEser(m+3) is the plasma plateau enrichment of [2,3,3-d_3] serine, MPEcys(m+3) is the plasma plateau enrichment of [2,3,3-d_3] cysteine derived from [2,3,3-d_3] serine. To account for the impact of high dose of serine infusion,

corrected TS was calculated by multiplying a cofactor of Qserine/(Qserine+90) (Clarke and Bier, 1982).

7.3.8 Statistics

The mixed model was applied to analyze the parameters with repeated observations including growth performance and indicators from plasma samples (Littell et al., 1996; Wang and Goonewardene, 2004). The diet effect and interaction effect between diet and week were partitioned in the model. PROC GLM was used for tissue sample data including hepatic CBS, CGL and SHMT. A RCB design was applied in the model. Animals were treated as block. Diet and block effects were partitioned in the model.

7.4 RESULTS

7.4.1 Growth performance and biochemical indices

Consistent with the data from Chapter 4, vitamin B_6 deficiency had no effect on average daily feed intake (ADFI) due to the pair-feeding protocol ($P>0.05$). Average daily gain (ADG) was not changed within 3 weeks after depletion ($P>0.05$). Feed conversion efficiency (FCE) was reduced by 23% on the third week after depletion ($P<0.05$), but not changed on the first two weeks by vitamin B_6 deficiency ($P>0.05$) (Table 10).

Table 10 Effect of vitamin B_6 deficiency on growth performance in piglets (Manuscript IV)

Week	ADFI (g/d)			ADG (g/d)			FCE		
	$+B_6$	$-B_6$	P value	$+B_6$	$-B_6$	P value	$+B_6$	$-B_6$	P value
1	292 ± 27^{b}	335 ± 33^{a}	0.3206	265±30	311±30	0.2898	0.899 ± 0.035^{a}	0.856 ± 0.035^{a}	0.3931
2	451 ± 27^{a}	402 ± 33^{a}	0.2705	341±30	307 ±30	0.4291	0.755 ± 0.035^{b}	0.692 ± 0.035^{b}	0.2149
3	359 ± 27^{b}	316 ± 33^{b}	0.3283	283±30	230±30	0.2282	0.785 ± 0.035^{b}	0.656 ± 0.035^{b}	**0.0168**

Note: 1) Data are presented as least square mean ± standard error. 2) P values are derived from difference of main effect of diet. P values < 0.05 are considered significant. 3) The same shoulder letters in each column mean non-significant ($P>0.05$).

At the end of the third week of depletion, plasma and hepatic PLP concentrations were reduced by 80% (32.5 vs. 6.3 nmol/L) and 76% (8.8 vs. 2.1 nmol/g) due to dietary vitamin B_6 depletion ($P<0.001$) (Table 11, 12). As a result of vitamin B_6 deficiency, plasma and hepatic homocysteine concentrations were increased by 172% and 59% respectively ($P<0.05$), while hepatic and plasma cysteine concentrations were not changed ($P>0.05$) (Table 11, 12). Both apo and total isozymes of CBS, CGL and SHMT were significantly decreased by dietary vitamin B_6 treatment ($P<0.05$). The apo-enzyme activities of CBS, CGL and SHMT in B_6 deficient pigs were lowered by 40%, 63%, and 38%, respectively (Table 13).

Table 11 Effect of vitamin B_6 deficiency on plasma PLP, homocysteine, and cysteine concentrations in piglets (Manuscript IV)

Week	PLP (nmol/L)			Homocysteine (μmol/L)			Cysteine (μmol/L)		
	+B-6	–B-6	P value	+B-6	–B-6	*P* value	+B-6	–B-6	P value
0	35.2±4.4	37.4±3.6[a]	0.7024	24.8±1.9[a]	27.9±2.1	0.3168	194.4±6.5[a]	180.2±3.7	0.0825
3	32.5±4.4	6.3±0.3[b]	**0.0003**	11.5±1.9[b]	31.3±1.9	**<0.0001**	160.9±6.5[b]	153.1±10.1	0.5365

Note: 1) Data are presented as least square mean ± standard error. 2) P values are derived from difference of main effect of diet. P values < 0.05 are considered significant. 3) The same shoulder letters in each column mean non-significant (P>0.05).

Table 12 Effect of vitamin B_6 deficiency on hepatic PLP, homocysteine and cysteine concentrations in piglets (Manuscript IV)

Hepatic PLP (nmol/g)				Hepatic homocysteine (μmol/L)				Hepatic cysteine (μmol/L)			
+B-6	–B-6	SEM	P value	+B-6	–B-6	SEM	P value	+B-6	–B-6	SEM	P value
8.84	2.11	0.511	**0.0007**	121.1	192.8	14.90	**0.0270**	1192	1493	93.1	0.0850

Note: 1) P values are derived from difference of main effect of diet. P values < 0.05 are considered significant.

Table 13 Effect of vitamin B_6 deficiency on hepatic enzymatic activities in piglets (Manuscript IV)

Enzyme		**+B-6**	**–B-6**	***P* value**
CBS (nmoL/min/mg protein)	Apo-enzyme	2.582±0.155	1.558±0.155	**0.0096**
	Total-enzyme	2.357±0.082	1.733±0.082	**0.0059**
CGL (nmoL/min/mg protein)	Apo-enzyme	1.765±0.076	0.647±0.076	**0.0005**
	Total-enzyme	1.939±0.091	1.402±0.091	**0.0138**
SHMT (nmoL/min/mg protein)	Apo-enzyme	0.844±0.036	0.520±0.036	**0.0031**
	Total-enzyme	1.319±0.066	1.059±0.066	**0.0495**

Note: 1) P values are derived from difference of main effect of diet. P values < 0.05 are considered significant.

7.4.2 Kinetic measurements

7.4.2.1 Plasma serine enrichments and flux

As shown in Figure 5, serine (m+3) enrichment (tracer/tracee ratio) plateau in plasma was reached starting from the first hour of infusion and maintained until the end of infusion. The plasma serine (m+3) plateau enrichment (atoms % excess) was significantly increased by B_6 deficiency (12.02 vs. 16.29, MSE 0.55) ($P<0.01$). Accordingly, serine flux (μmoL/kg/h) was decreased by vitamin B_6 deficiency (655.85 vs. 463.93, MSE 26.77) ($P<0.01$) (Table 14). In this experiment serine (m+1) was detected as well, which, however, was not affected by vitamin B_6 deficiency ($P=0.3368$) (Table 14).

7.4.2.2 Hepatic serine (m+3) enrichment

Though low, hepatic serine (m+3) enrichment was increased by vitamin B_6 deficiency ($P<0.05$) (Table 14).

Figure 5 Time course of serine (m+3) enrichment in piglets

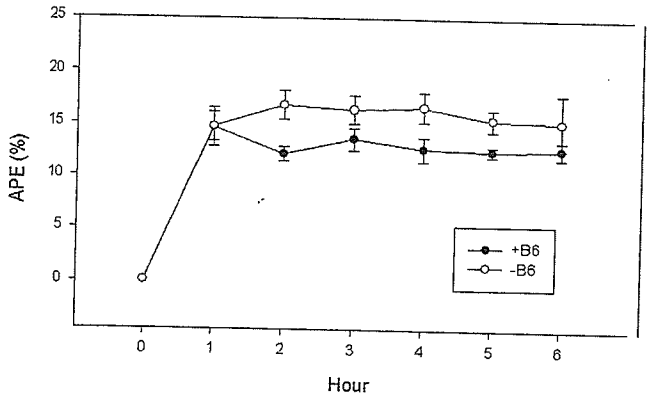


Table 14 Impact of vitamin B_6 deficiency on serine kinetics in piglets (Manuscript IV)

Kinetic parameters	**+B-6**	**–B-6**	**SE**	***P* value**
Serine (m+3) plateau enrichment (atoms % excess)	12.02	16.29	0.55	**0.0057**
Serine (m+1) plateau enrichment (atoms % excess)	1.373	1.134	0.155	0.3368
Serine flux in plasma (μmoL/kg/h)	655.85	463.93	26.77	**0.0071**
Hepatic serine (m+3) enrichment (atoms % excess)	0.2295	0.3886	0.0316	**0.0237**

Note: 1) P values are derived from difference of main effect of diet. P values < 0.05 are considered significant.

7.4.2.3 Plasma cysteine enrichment and flux

As shown in Figure 6, cysteine (m+1) plateau enrichment in plasma was reached at the end of the fourth hour and maintained until the end of infusion. Vitamin B_6 deficiency significantly increased plasma plateau cysteine (m+1) enrichment ($P<0.05$) and decreased cysteine flux (μmoL/kg/h) (119.80±3.61 vs. 97.84±3.61 μmoL/kg/h) ($P<0.05$) (Table 15). In this experiment cysteine (m+3) was not detected in either plasma or liver samples.

Figure 6 Time course of cysteine (m+1) enrichment in piglets

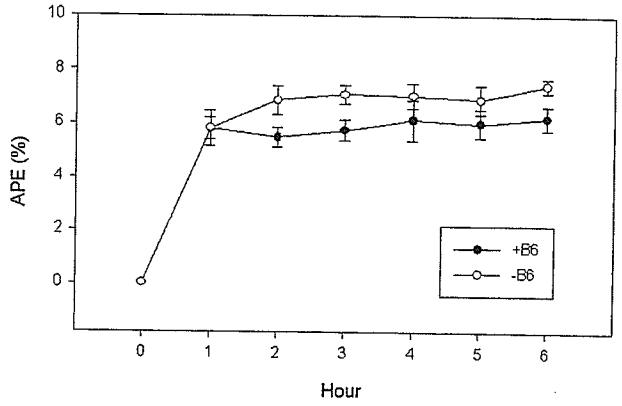


Table 15 Impact of vitamin B_6 deficiency on cysteine kinetics in piglet (Manuscript IV)

Kinetic parameter	+B-6	–B-6	SE	*P* value
Plasma cysteine(m+1) plateau enrichment (atoms % excess)	5.89	7.08	0.20	**0.0149**
Hepatic cysteine (m+1) enrichment (atoms % excess)	2.32	2.36	0.30	0.8935
Cysteine flux in plasma (μmoL/kg/h)	119.80	97.84	3.61	**0.0126**

Note: 1) P values are derived from difference of main effect of diet. P values < 0.05 are considered significant.

7.4.2.4 Transfer of label from serine

As seen in Table 16, glycine (m+1) enrichment was increased by vitamin B_6 deficiency ($P<0.05$). However, methionine (m+1) enrichment was not affected by vitamin B_6 deficiency ($P=0.2885$).

Table 16 Impact of vitamin B_6 deficiency on plasma glycine (m+1) and methionine (m+1) enrichments (Manuscript IV)

Enrichment	+B-6	–B-6	*P* value
Glycine (m+1) enrichment (atoms % excess)	1.882±0.159	2.746±0.159	**0.0185**
Methionine (m+1) enrichment (atoms % excess)	0.653±0.129	0.867±0.105	0.2885

Note: 1) P values are derived from difference of main effect of diet. P values < 0.05 are considered significant.

7.5 DISCUSSION

In this study ADFI and ADG were not significantly different between the control and vitamin B_6 deficiency groups, which is consistent with our previous observations. However, feed efficiency in this experiment was reduced by B_6 deficiency at the end of the third week after B_6 depletion, which is one week earlier than that in our previous studies (Chapter 4). This might be due to a shorter adaptation period used in the current study, as compared to those used previously (3 vs. 11 days). The pigs in the current trial were younger than those in our previous study (Chapter 4). Consequently, it might have taken longer for the older pigs to deplete vitamin B_6 stores in the body as evidenced in plasma PLP at the end of the third week in this experiment (6.3 μM), which was between 8.5 and 14.3 μM for the same age in the previous study (Chapter 4).

The observed significant decreases in hepatic apo-enzymes (without addition of exogenous PLP to the incubation media) of CBS, CGL and SHMT by B_6 depletion in this study ($P<0.01$) are in agreement with observations in other models (Smolin and Benevenga, 1984; Lima et al., 2006), but not in rats (Martinez et al., 2000). Hepatic CBS and CGL activities in B_6 depleted piglets were reduced by 91% and 55%, respectively, in our previous study (Chapter 4) as compared to controls at the end of 6 weeks of depletion. In the current experiment, however, hepatic CBS and CGL was reduced by 40% and 63%, respectively, which implies that CGL is more sensitive to B_6 depletion, which is in agreement with studies in which mild to moderate B_6 deficiencies were developed (Park and Linkswiler, 1969; Lima et al., 2006). Therefore, the degree of impairment of each aspect of sulphur amino acid metabolism may differ due to the degree of vitamin B_6 deficiency.

As discussed in Chapter 4 using the same vitamin B_6 deficiency protocol, the reduced enzymatic activities of CBS, CGL and SHMT by B_6 depletion resulted in the inhibition of transsulphuration or remethylation. Consequently, increases in hepatic ($P<0.05$) and plasma ($P<0.001$) homocysteine concentrations by B_6 depletion in this experiment were observed, which was consistent with our previous studies and other reports as well (Smolin et al., 1983; Martinez et al., 2000; Scheer et al., 2005), but not the human study in which homocysteine was not changed by mild vitamin B_6 deficiency (Davis et al., 2005). However, no significant impact of B_6 depletion on hepatic and plasma cysteine concentrations at the end of the third week after depletion were detected ($P>0.05$), which is not in agreement with significant decrease in cysteine concentrations in Chapter 4. Looking at plasma biochemical indices in Chapter 4, we found that there was a period of time between the second and the third week after B_6 depletion in which homocysteine (23.9~336.5 μM) and cysteine (183.3~97.5 μM) concentrations changed sharply upon plasma PLP concentration between 6.0 and 8.5 nM. In this case the end of the third week after depletion protocol might be on the metabolic status that the point leading to significant cysteine reduction and large magnitude of homocysteine increase due to B_6 deficiency was just missed. Plasma PLP concentration of 6.3 nM is also in coincidence between 6.0 and 8.5 nM in which biochemical indices sharply changed in Chapter 4.

The significance of this study lies in the kinetic measurements of serine and cysteine using isotope dilution technique, which allow us to have a better understanding of serine and cysteine availability and endogenous supply of cysteine *in vivo* in the whole-body level under nutritional regulation of vitamin B_6. In particular, endogenous

cysteine synthesis has been attracting the attention of researchers because cysteine serves as a substrate for the synthesis of glutathione and taurine, the compounds important in defending against oxidative stress (Meister and Anderson, 1983; Wright et al., 1986). Serine synthesis is important because of its role in intermediary metabolism as a substrate in the synthesis of glucose via pyruvate, protein, phosphatidylserine, cystathionine, and neuromodulators (de Koning et al., 2003). In addition, among the methyl groups of glycine, sarcosine, dimethylglycine, histidine, formate, and serine used by methionine synthase to convert homocysteine to methionine, the three carbon of serine has been shown to be the primary donor of one-carbon units for homocysteine remethylation (Appling, 1991; Cooke, 2001; Davis et al., 2004). Therefore, the information with respect to serine kinetics is important in evaluating sulphur amino acid metabolism under the physiological status of B_6 deficiency.

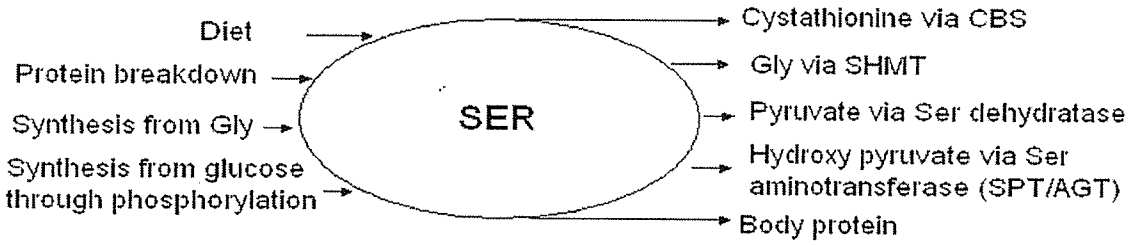
As shown in Figure 5, plasma d_3-serine (m+3) plateau enrichment in B_6 deficient pigs was higher than that in control pigs ($P<0.01$). This implies that the infused serine tracer was less diluted *in vivo* in B_6 deficient pigs than that in control pigs. As a result, serine flux, a kinetic measure of serine turnover rate in the whole body, was reduced by 29% in B_6 deficient pigs in comparison with control (655±27 vs. 463±27 μmoL/kg/h, $P<0.01$). The decreased serine flux also indicates that the supply of serine for transsulphuration, remethylation and 1-C metabolism is reduced when there is deficiency of vitamin B_6 in the body. At the same time, serine flux in control (655±27 μmoL/kg/h) was much higher than that determined in the pilot study (204 μmoL/kg/h). This is because the infusion rate of serine in the current study was 1.5 times of that in the pilot study. Liu et al. (2000) reported that serine flux in the treatment of serine infusion (10

grams of serine per day) was significantly higher than that in control with saline infusion only ($P<0.001$), which is consistent with the results in this study.

Under steady state, serine flux or turn-over is equal to the disappearance of serine, which includes the following components: 1) usage for cystathionine via CBS; 2) usage for glycine synthesis via SHMT; 3) usage for pyruvate via serine dehydratase; 4) usage for hydroxyl pyruvate via serine aminotransferase (SPT/AGT); and 5) usage for body protein (Figure 7). Serine is mainly degraded by B_6-dependent enzymes (Park and Linkswiler, 1971). All of the first four components mentioned above require PLP as co-factors (Ishikawa et al., 1996; Nelson and Cox, 2000). A kinetic study demonstrates that whole body protein turnover is reduced by B_6 deficiency as evidenced by greater isotopic enrichment of [$^2H_3$]leucine in rats depleted of dietary vitamin B_6 (Martinez et al., 2000). Applying this to the current study, serine usage for body protein should be lowered accordingly. Therefore, the decrease in each component above contributes to the observed reduction in serine flux under the circumstance of vitamin B_6 deficiency. The increase in plasma serine (m+3) enrichment and decrease in serine flux caused by vitamin B_6 deficiency in this study are in agreement with a report in rats (Martinez et al., 2000), but not a study with healthy adults in which serine enrichment and flux were not changed by B_6 deficiency (Davis et al., 2005). Additionally, an isotope dilution study by Davis et al. shows that when there was mild B_6 deficiency (plasma PLP 55 and 22.6 nmol/L for baseline and B_6 restricted subjects) in healthy young women and men, plasma PLP and *in vitro* lymphocyte SHMT were decreased, but plasma homocysteine and serine concentrations, serine isotopic enrichment and flux were not affected by B_6 deficiency (Davis, et al., 2005). The protocol in this study did not address remethylation kinetics.

Therefore, information regarding changes in B_{12}, BHMT dependent and total remethylation by B_6 deficiency in a piglet model will continue to be an important research question, despite the fact that only a very small fraction (2.8%) of the serine flux is used for remethylation (Davis et al., 2004).

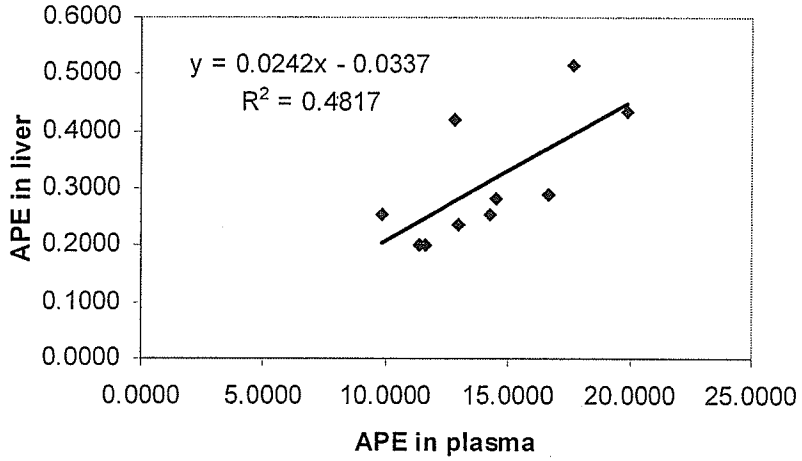
Figure 7 Free serine pool turnover



In this experiment, serine (m+1) enrichment was detected, which accounted for 11% and 7% of serine (m+3) enrichment in control and B_6 deficient pigs, respectively (Table 14). Gregory et al. (2000) also reported serine (m+1) in humans infused with L-[2,3,3-$^2H_3$]serine. Serine (m+1) is the result of folate-dependent one carbon metabolism. In the meanwhile, enzyme-catalyzed reactions producing isotope exchange may also lead to the production of serine (m+1) and serine (m+2) (Baggott, 2001). Serine (m+2) was not analyzed in the current experiment. The detection of serine (m+1) enrichment demonstrates the loss of label from serine (m+3), which is one of the reasons that prevented us from detecting labeling of cysteine from serine (m+3).

An important finding in this experiment is that a regression equation between serine (m+3) enrichments in plasma (x) and liver (y) was established (Figure 8) as $y=0.0242x-0.0337$ ($P=0.0260$). The slope value of 0.0242, which served as a correction factor for intracellular dilution, tells us that only 2% of infused serine (m+3) from plasma was left in liver, which indicates a great intracellular dilution in the model. The coefficient obtained in this experiment is even lower (0.02 vs. 0.16) than that from a previous pig study using radioactive isotope tracer L-[^{3}H] serine in our laboratory (Ludke, 2004) but similar to the pilot study using the same isotope model. In a human study by Davis et al. (2004), a value of 0.4 was used as coefficient to account for intracellular dilution. However, the authors were not able to detect labeling of cysteine from infused serine (Davis et al., 2004). The large magnitude of intracellular dilution might be one of the reasons that prevented us from detecting cysteine labeling metabolically derived from infused serine as discussed below.

Figure 8 Relationship between plasma serine (m+3) enrichment and liver serine (m+3) enrichment in pigs infused L-[2,3,3-d_3]serine and L-[3-^{13}C]cysteine

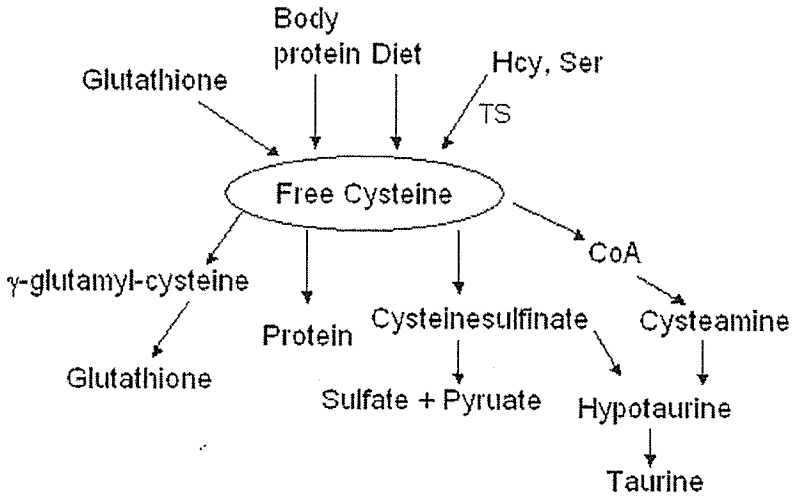


The inhibition of transsulphuration by vitamin B_6 deficiency has been documented (Park and Linkswiler, 1969; Smolin and Benevenga, 1982; Lima et al., 2006). The rate of cysteine turnover and the extent of homocysteine consumption for cysteine turnover have not been quantified in a pig model deficient of vitamin B_6. The present experiment, to our knowledge, is the first study to provide *in vivo* cysteine kinetics using isotope dilution technique in a pig model deficient of vitamin B_6. Cysteine flux, as determined from plasma, for control and B_6 deficient pigs was quantified to be 119.80 and 97.84 μmoL/kg/h, respectively ($P<0.05$). Cysteine flux in other models ranges 20~176 μmoL/kg/h (Hiramatsu et al., 1994; Fukagawa et al., 1996,1998; Raguso et al., 1997,2000; Liu et al., 2000; Malmezat et al., 2000; Davis et al., 2006). Cysteine flux was decreased by vitamin B_6 deficiency in this study. However, cysteine flux along with plasma homocysteine concentrations in healthy young men and women were not significantly affected by mild vitamin B_6 restriction (26.1 vs. 26.5 μmoL/kg/h) ($P>0.05$) (Davis et al., 2006). When looking at free cysteine pool, cysteine turnover or flux is rate of appearance of cysteine, which consists of cysteine from glutathione, body protein breakdown, intake of diets, and endogenous synthesis from homocysteine and serine through the transsulphuration pathway (Figure 9). In this case, endogenous production of cysteine through transsulphuration as reflected from transsulphuration rate, should not be higher in B_6 deficient pigs as the inhibition of transsulphuration, with respect to enzymatic activities, was observed. The intakes of dietary cysteine were similar according to the pair-feeding protocol. Origin of body protein breakdown in B_6 deficient pigs might be lower than that in control pigs as discussed above (Martinez et al., 2000). Source of cysteine from glutathione should not be increased in the case of B_6 deficiency,

a condition similar to oxidative stress under which glutathione production from cysteine is enhanced (Mosharov et al., 2000; Lima et al., 2006). Therefore, the decreased cysteine turn over might be attributed to body protein breakdown, glutathione conversion or transsulphuration rate.

Unlike serine that shows large intracellular dilution, hepatic cysteine (m+1) enrichment accounted for 39% and 33% of those in plasma, respectively. This indicates that cysteine can serve as a good tracer for kinetic measurement.

Figure 9 Free cysteine pool turnover



In this experiment determination of transsulphuration rate relies on measurements of cysteine flux, serine (m+3) enrichment, and cysteine (m+3) enrichment derived from serine (m+3). However, cysteine (m+3) was not detectable in this trial, which makes transsulphuration rate (TS) determination infeasible. Using the same isotopic model of L-[2,3,3-d_3] serine L-[3-^{13}C] cysteine, TS in control sheep of 27 kg body weight was determined to be 5.0 μmol/kg/h (Liu et al., 2000). Using ^{13}C-tracer technique with dual labeled L-[$^{2}H_3$-methyl-1-^{13}C] methionine, TS was determined to be in the range of 2.1~9.4 μmol/kg/h in humans depending on the physiological or nutritional states and age of the subjects (Young et al., 1991; Hiramatsu et al., 1994; Fukagawa et al., 1996; Raguso et al., 1997,2000; Jahoor et al., 2006) and 26 μmol/kg/h in piglets (Riedijk et al., 2007). However, TS determination using the isotopic model with deuterium labeled serine in humans was not achieved in the studies by Gregory group because of that production of [^{13}C]cysteine from infused [3-^{13}C]serine was not detected, which is in agreement with the current study (Davis et al., 2004, 2005, 2006).

The indirect method using double-labeled methionine measures the rate of methionine oxidation to CO_2, which might bring in overestimation from production of $^{13}CO_2$ derived from sequestration of S-adenosylmethionine for polyamine synthesis. So it is the consideration of overestimating TS from using the indirect model that drove us to explore the direct modle for TS measrement. In this experiment the failure of detecting cysteine (m+3) metabolically derived from serine (m+3) might be, for one thing, due to great intracecullar dilution of infused serine as mentioned above. Transsulphuration takes place mainly in the liver. The tracers were infused in the blood first and then introduced to liver via the circulatory system. In this experiment, serine (m+3) plateau

enrichment in plasma was reached during the first hour of infusion and maintained until the end of infusion. At the same time, plasma serine (m+3) plateau enrichment levels observed in this study were already fairly high (12-16%) in comparison with other kinetic studies. However, only very little amount of serine (m+3) (APE: 0.2-0.4%) was left in the liver due to the very large intracellular dilution as reflected from the slope value in the regression equation between enrichments in plasma and liver. For another thing, despite of the great intracellular dilution of serine (m+3), glycine (m+1) and methionine (m+1) enrichments were detected instead. This challenged us to consider why serine (m+3) was utilized for one-carbon metabolism other than cysteine synthesis. This could be due to the fact that one-carbon metabolism is widely compartmentalized in both cytosolic and mitochondrial pools (Gregory et al., 2000). Transsulphuration, instead, is mainly compartmentalized in mitochondrial. Therefore, the infused serine (m+3) can serve as a substrate for formation of glycine and methionine directly. However, to go to transsulphuration pathway, the infused serine has to shuttle through cytosol to mitochondria. In addition, the feeding state during infusion might be also a factor that prevented us from detecting cysteine (m+3). As a result of vitamin B_6 deficiency, feed intake was much lower than that in regular commercial pigs at the same age. Because pigs were pair-fed during infusion as well, feed intakes for both control and B_6 deficient pigs were low, which provided only 1.5 times of maintenance metabolizable energy. Protein intake was low accordingly. This condition favors remethylation rather than transsulphuration (Stipanuk and Benevenga, 1977; Finkelstein et al., 1986; Storch et al., 1990) due to low production of S-adenosylmethionine, an activator of transsulphuration pathway (Finkelstein, 1990). Because TS was not able to be determined, the impact of

vitamin B_6 deficiency on the rate of endogenous synthesis of cysteine *in vivo* in pigs cannot be determined in this study.

In this study the deuterium labeled hydrogen from infused serine can be transferred to produce labeled other compounds. When L-[2,3,3-d_3] labeled serine and unlabeled THF are catalyzed by SHMT, the deuterium atom at the second carbon of serine is left in the carbon skeleton to produce [$^2H_1$]glycine. [$^2H_2$]labeled one carbon unit is transferred to THF to give rise to 5,10-CD_2-THF. The latter is then used to produce [$^2H_2$]methionine. In the meanwhile, deuterium labeled serine can also go to mitochondrion to undergo a series of metabolism to give rise to [$^2H_1$]formate. The produced [$^2H_1$]formate can be shuttled back to cytosol to pass its singly labeled one carbon unit to methionine (Gregory et al., 2000). Therefore, the triple labeled serine can transfer its labeling to have single deuterium labeled [$^2H_1$]glycine, single deuterium labeled [$^2H_1$]methionine and double deuterium labeled [$^2H_2$]methionine through a series of one-carbon unit metabolism.

As illustrated in Figure 1, PLP dependent SHMT catalyzes reversible transfer of one-carbon units between THF and 5,10-CH_2-THF. In this experiment, glycine (m+1) enrichment converted from serine (m+3) was higher in pigs deficient of vitamin B_6 than that in control due to the interconvertable enzyme reaction. This implies that the turn over of glycine via SHMT might be reduced by vitamin B_6 deficiency based on reciprocal relationship between flux and enrichment. Therefore, vitamin B_6 deficiency might have a down-regulation of one carbon unit supply. To our knowledge, this is the first report showing the impact of vitamin B_6 status on one-carbon unit metabolism in a pig model. Since one-carbon units are required for remethylation, the reduced vitamin B_6 status

might finally result in lower methionine formation through remethylation pathway. However, we did not detect statistical difference in methionine (m+1) enrichment, a parameter reflecting a portion of remethylation ($P>0.05$).

In summary, in addition to re-confirmation of the perturbation role of vitamin B_6 deficiency in the static measures of sulphur amino acid metabolism including enzymatic activies and thiol concentrations, vitamin B_6 deficiency resulted in decreased cysteine and serine turn over rates and 1-C metabolism as shown in the increased enrichments. i.e. the kinetics of sulphur amino acids and 1-C metabolisms were compromised as a result of vitamin B_6 deficiency in pigs.

CHAPTER EIGHT

GENERAL DISCUSSION

The objective of this study was to investigate the impact of vitamin B_6 deficiency on transsulphuration in terms of both static measurement of biochemical indices in sulphur amino acid metabolism and kinetic measurement of transsulphuration in a pig model. The role that vitamin B_6 deficiency plays in sulphur amino acid and 1-C metabolism has been well documented (Smolin and Benevenga, 1982,1984; Smolin et al., 1983; Martinez et al., 2000; Scheer et al., 2005; Davis et al., 2005,2006; Lima et al., 2006). However, the reported results lack consistency. In particular, most of reports mentioned above did not employ a pair-feeding protocol, which creates an experimental environment for a net comparison between control and B_6 deficiency. So the observed biochemical indices might be confounded by the intake of folate, vitamin B_{12}, or methionine induced as a result of loss of appetite from B_6 deficiency treatment. This might be one of the reasons to explain the contradictory observations. Furthermore, species-specific information on changes in enzymatic activities by vitamin B_6 status in a pig model was not available until the current study. While it is important to understand the qualitative effects of vitamin B_6 deficiency with respect to the perturbation of sulphur amino acid metabolism, it is crucial that we have quantitative knowledge on the degree to which transsulphuration is affected by vitamin B_6 deficiency. To date, limited information regarding kinetic measurements in vitamin B_6 deficiency exists. As such, the current study provides a static picture of biochemical indices including metabolites and

enzymes in sulphur amino acid metabolism in piglets subjected to dietary vitamin B_6 depletion and graded levels of vitamin B_6 repletion. Additionally, the isotope dilution study using L-[3-^{13}C] cysteine and L-[2,3,3-d_3] serine provided critical information as per the net impact of vitamin B_6 deficiency on transsulphuration in pigs.

8.1 Response of growth to depletion/repletion

In this study, consistent reductions in growth and feed efficiency by vitamin B_6 deficiency were observed in both Chapters 4 and 5. Unlike most traditional animal experiments where growth was a result of a response to feed intake, feed intake between control and B_6 deficiency groups in this study was the same due to the implementation of a pair-feeding design for the purpose of avoiding confounding effects of nutrient intake on biochemical indices. It follows that the metabolic effects of vitamin B_6 deficiency rather than feed intake caused the decline in growth performance based on the central role of vitamin B_6 in intermediary metabolism including amino acids, one-carbon units, lipids, glucose and neurotransmitter biosynthesis (Mackey et al., 2006). Feed intake in vitamin B_6 deficient pigs did drop with the progression of vitamin B_6 depletion. Consequently, feed intake in control pigs was not enough to maintain normal growth curve as a result of pair-feeding protocol. However, a study in which piglets were fed diets based on commercial ingredients, supplementation of graded levels of pyridoxine hydrochloride at 0, 10, 50, and 100 mg/kg, respectively showed no difference in growth performance (Matte et al., 2005). This implies that commercial feed ingredients contain enough vitamin B_6 to meet the requirement for the potential of growth. As such, the semi-purified diet used in this study served as an excellent tool to obtain a pig model deficient of vitamin B_6. The observation in Chapter 5 that average daily gain at a

repletion level of 0.75 mg/kg pyridoxine·HCL on week 1 repletion was lower than the highest repletion level of 3 mg/kg ($P<0.05$) while there were no differences between the medium-high (1.5-3 mg/kg) repletion levels ($P>0.05$) provides evidence that vitamin B_6 intake at the repletion level of 0.75 mg/kg was still limiting growth, even though feed intake was not different.

8.2 Sensitive indicators to vitamin B_6 status

Plasma PLP concentration is the most commonly used direct measure of vitamin B_6 status. To our knowledge, the current study provides the first temporal characterization of changes in B_6 status in response to depletion and subsequent repletion, as measured by plasma PLP, in pigs. Decrease in plasma PLP concentration by vitamin B_6 deficiency in pigs is consistent with the reports in other models (Martinez et al., 2000; Davis et al., 2005; Scheer et al., 2005). The strength of the current study lies in temporal characterization of not only plasma PLP but also additional biochemical measures including plasma homocysteine, cysteine, and other amino acid concentrations, in response to B_6 deficiency in the pig.

Accumulation of homocysteine and reduction in cysteine by vitamin B_6 deficiency were observed in Chapters 4 and 5, consistent with the literature (Smolin and Benevenga, 1982,1984;Smolin et al., 1983; Martinez et al., 2000). However, severe hyperhomocysteinemia (greater than 300 μM) in B_6 depleted animals was observed starting from week 3 in Chapter 4 and week 4 in Chapter 5, respectively, which was not consistent with Chapter 7 where homocysteine was only 31 μM. This could be due to that the abrupt shift in thiol concentrations was missed in Chapter 7.

Homocysteine and cysteine are well documented to be sensitive to vitamin B_6 deficiency (Smolin and Benevenga, 1982,1984; Smolin et al., 1983; Martinez et al., 2000). However, little is known concerning the response of homocysteine and cysteine to graded levels of vitamin B_6 repletion. The pattern of changes for both total homocysteine and cysteine mirrored the temporal and dose-dependent responses observed for plasma PLP. Nonlinear regression analysis showed that the data set between plasma PLP and homocysteine fits an inverse first order equation HCY=-37.2963+1387.2212/PLP ($P<0.0001$; $R^2=0.7163$). This means that when vitamin B_6 status reverses back to normal, hyperhomocysteinmia will disappear consequently.

8.3 Difference in enzyme activity response to depletion/repletion

The data for enzyme activities of CBS, CGL and SHMT in this study is a validation for the co-factor role of vitamin B_6 as evidenced the decreased and increased activities upon depletion and repletion, respectively. These observations are also consistent with the limited literature in rats (Smolin and Benevenga, 1984;Lima et al., 2006). However, we found that in comparison with CBS activity, CGL activity was less sensitive to vitamin B_6 depletion but more responsive to B_6 repletion. This is because CGL has lower affinity than CBS and SHMT for PLP, which means that it takes longer time for CGL to loose PLP and to combine PLP.

8.4 Kinetic measures assist in defining the role of B_6 deficiency in the regulation of transsulphuration

The novelty of this study lies in the kinetic measurements of serine and cysteine using isotope dilution technique, which allowed us to have a better understanding of serine and cysteine availability *in vivo* at the whole-body level under nutritional

regulation of vitamin B_6. The present experiment, to our knowledge, is the first study to provide *in vivo* cysteine kinetics using isotope dilution technique in a pig model deficient of vitamin B_6. Cysteine flux was decreased by vitamin B_6 depletion in this study, which is not in agreement with the measurement in humans (Davis et al., 2006). The very low slope value in the regression equation between plasma and liver serine (m+3) enrichments indicates the existence of very large intracellular dilution of infused serine tracer, which further disclosures part of reasons why we were not able to determine transsulphuration rate using the direct stable isotope model. Furthermore, together with reduced serine flux, the increased labeling transfer from serine to glycine implies that 1-C units supply might be down regulated by vitamin B_6 deficiency. The detection of one-carbon unit transfer other than cysteine production from infused serine could be due to the cellular compartmentation of different metabolisms.

CHAPTER NINE

CONCLUSIONS AND FUTURE RESEARCH

CONCLUSIONS

1) **Vitamin B_6 deficiency reduced weight gain and feed efficiency through its metabolic role**

Due to the application of pair-feeding protocol, the observed reduction of weight gain by dietary vitamin B_6 depletion confirms the adverse metabolic role of low vitamin B_6 status in the *de novo* synthesis of protein and lipid.

2) **Plasma PLP concentrations were responsive to dietary implementation of vitamin B_6 depletion and repletion**

Plasma PLP concentrations in pigs consuming vitamin B_6 deficient diets were lower than controls throughout 6-week depletion period reaching a nadir of 14% of the control animals' value by trial's end. Dose-dependent response of plasma PLP concentration to vitamin B_6 repletion was observed throughout 2-week repletion. As the repletion dose increased, repletion rate increased and the days reaching 90% of plateau PLP decreased.

3) **Vitamin B_6 deficiency perturbed sulphur amino acid metabolism**

Both apo and total hepatic CBS, CGL activities were reduced at the end of week 4 and week 6. Plasma homocysteine increased and cysteine, by contrast, decreased as a result of dietary vitamin B_6 depletion. Decrease in cysteine flux was resulted from vitamin B_6 deficiency as well.

4) **The responses of plasma PLP and homocysteine concentrations to graded levels of vitamin B_6 repletion shed light on increasing vitamin B_6 requirement by 50% in 10-20 kg pigs**

Plasma PLP concentrations increased as repletion dose increased. At day 14 repletion, plasma PLP concentrations at the two higher repletion levels (2.25mg/kg and 3.00 mg /kg) resumed to pre-depletion levels, which were statistically same. At the same time, plasma PLP concentrations from the two lower repletion levels (0.75 mg/kg and 1.50 mg/kg) were still lower than those in higher repletion levels. Plasma homocysteine concentrations demonstrated similar pattern to PLP. Based on the response of these two parameters to the repletion levels, 2.25 mg/kg was the optimum repletion dose, which was 150% of current NRC (1998) requirement for vitamin B_6 in term of pyridoxine hydrochloride.

5) Vitamin B_6 deficiency lowered one carbon unit supply in the whole body

Decrease in serine flux by vitamin B_6 deficiency was observed together with reduced enzymatic activities of SHMT in one carbon metabolism. The increased glycine (m+1) enrichment delivered the message that glycine flux through SHMT reaction was reduced as well.

6) Large intracellular dilution of serine was found in a pig model

Using stable isotope L-[2,3,3-d_3]serine, a slope value of 2% was derived from the regression equation between plasma serine (m+3) enrichment and liver serine (m+3) enrichment in pigs. This indicates that 98% of the infused tracer was lost in the liver with only 2% left intracellularly.

FUTURE RESEARCH

1) **Kinetic measurements of transsulphuration using dual labelled methionine in a pig model deficient of vitamin B_6**

From the current study we discovered that there was a huge intracellular dilution of infused serine (m+3), which prevented us from detecting the label of cysteine (m+3) metabolically derived from serine (m+3). Transsulphuration rate was consequently, not available. However, kinetic measures of transsulphuration in vitamin B_6 deficient pigs play significant role in exploring the nutritional regulation of transsulphuration via vitamin B_6 deficiency. In the meanwhile, the model using dual labeled methionine has been extensively reported to be able to determine transsulphuration rate though this model is supposed to bring in the overestimation of transsulphuration rate. In case of the difficulty in dermining transsulphuration rate using the direct model in pigs, the dual labeled indirect model would be a necessary tool to quantify transsulphuration rate in pigs deficient of vitamin B_6.

2) **One-carbon unit metabolism in a pig model deficient of vitamin B_6**

Vitamin B_6 serves as a cofactor for SHMT, which functions in 1-C metabolism to shuttle one carbon units between glycine and serine. The results from this study show that upon vitamin B_6 deficiency, SHMT activity decreased, plasma serine concentration increased, whole body serine flux decreased and glycine enrichment increased. While 1-C metabolism is not the primary focus in this study, the prelimary results shed light on the changes in 1-C metabolism by vitamin B_6 deficiency. 1-C metabolism has been confirmed to be compartmented in both mitochondrial and cytosol. In addition, the

separate serine flux through the folate cycle and transsulphuration, respectively, together with glycine flux would be definitely an asset in understanding 1-C metabolism in pigs deficient of vitamin B_6. All of the above perspectives call for particular studies to investigate 1-C metabolism in vitamin B_6 deficient pigs.

3) **Impact of vitamin B_6 deficiency on protein kinetics**

Vitamin B_6 serves as a cofactor for over 100 enzymes, especially those involved in amino acid metabolism. In the cuurent study, we observed decreased weight gain in vitamin B_6 deficient pigs, which is attributed to the negative effect of vitamin B_6 deficiency on protein metabolism. Kinetic studies employing isotopically labelled leucine in a pig model deficient of vitamin B_6 would provide important knowledge for us to understand changes in whole body protein metabolism when vitamin B_6 status is low. This will be definitely important to human clinics and animal production.

4) **Examination of key enzymes in the transsulphuration pathway at molecular level in pigs subjected to vitamin B_6 deficiency**

Enzyme activity is a critical parameter to judge the impact of vitamin B_6 deficiency on transsulphuration. The activities of enzymes in the transsulphuration pathway have been examined to be lower in the vitamin B_6 deficient pigs in this study. In addition to the biochemical measurement of enzyme activity, the dermination of enzyme protein levels at molecular level would further provide a systematic picture of the impact of vitamin B_6 deficiency on transsulphuration. One thing that needs to be mentioned is that there is difficulty in the availability of antibodies to CBS and CGL in pigs as most of the marketed products are developed for rats and humans at the time of conducting this study.

5) Regulation of transsulphuration by glutathione

Glutathione is a major antioxidant in tissue. It is reported that redox regulation of the transsulphuration pathway occurs at the level of CBS (Banerjee and Zou, 2005) and that flux through CBS was increased under oxidizing conditions (e.g., H_2O_2 or t-butylhydroperoxide). So the impact of glutathione on metabolites and enzymes as well as kinetics in the transsulphuration pathway in vitamin B_6 deficient pigs will provide critical knowledge to human clinical and appied animal nutrition studies.

CHAPTER TEN

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APPENDIX I

Effect of vitamin B_6 deficiency on plasma amino acid concentrations in piglets subjected to 6 weeks dietary vitamin B_6 depletion

Amino acid	Week	$+B_6$	$-B_6$	P value
Serine (μmol/L)	0	169.10±19.58[b]	160.95±15.99[b]	0.7495
	3	280.88±17.51[a]	273.78±15.99[a]	0.7670
	6	118.57±17.51[b]	178.73±15.99[b]	**0.0175**
Glycine (μmol/L)	0	1946.92±300.40[a]	1364.95±265.73	0.1675
	3	2529.67±300.40[a]	1506.43±385.61	0.0602
	6	1319.41±335.86[b]	723.69±64.68	0.1075
Methionine (μmol/L)	0	117.21±12.76[b]	115.61±25.40	0.9565
	3	128.85±12.76[b]	261.67±59.06	0.0869
	6	70.73±12.76[a]	173.58±71.42	0.2461

Note: 1) Data are presented as least square mean ± standard error. 2) P values are derived from difference of main effect of diet. P values < 0.05 are considered significant. 3) The same shoulder letters within a column for each amino acid mean non-significant (P>0.05).

APPENDIX II

Estimates of plasma PLP concentration and repletion rate by Richards equation

	0.75 mg/kg	1.5 mg/kg	2.25 mg/kg	3 mg/kg
PLP_f	14.1 ± 3.01	20.3 ± 1.61	29.2 ± 3.55	25.8 ± 1.19
k	0.21 ± 0.044	0.41 ± 0.056	0.36 ± 0.048	0.72 ± 0.090
PLP_{90f}	14.1	10.4	12.7	6.2

Note:

PLP_f is PLP concentration at final repletion time;

k is repletion rate;

PLP_{90f} is repletion time required for reaching 90% of plateau PLP concention.

APPENDIX III

Impact of graded level of vitamin B_6 repletion on plasma PLP concentration in vitamin B_6 depleted pigs