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SULFUR AMINO ACID CATABOLISM IN A PIGLET MODEL

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ABSTRACT

A model was developed in growing piglets to study the use of urinary total sulfur excretion as an indicator of sulfur amino acid (SAA) catabolism and the nitrogen (N)/sulfur (S) balance ratio as an indicator of non-protein SAA storage. The recovery of administrated methionine as urinary total S over 48 hours was 106% in well-nourished piglets, but only 69% in protein malnourished piglets. The N/S balance ratio of protein malnourished piglets was lower than that of well-nourished piglets, and this ratio further decreased after methionine administration. We conclude that in a protein malnourished state, relatively more S than N is retained and a significant portion of the S derived from administrated methionine is retained in non-protein pools. These results demonstrate that urinary total S excretion can provide an accurate measure of SAA catabolism; and the N/S balance ratio can provide valuable information about non-protein SAA storage in growing piglets.

RESUME

Nous avons développé un modèle chez le porcelet en croissance afin d'utiliser l'excrétion urinaire de soufre total comme indicateur du catabolisme des acides-aminés soufrés (SAA) et afin d'utiliser le rapport d'équilibre azote (N) / soufre (S) comme indicateur de mise en réserve de SAA non-protéiques. La récupération pendant 48h comme S total urinaire de la méthionine administrée est de 106% chez les porcelets bien nourris, mais est seulement de 69% chez les porcelets malnourris en protéines. Le rapport d'équilibre N/S des porcelets malnourris en protéines était inférieur a celui des porcelets bien nourris, et ce rapport a encore plus diminué après administration de méthionine. Nous concluons que lors de malnutrition en protéines, relativement plus de S que de N est conservé et une part importante du S dérivé de la méthionine administrée est conservé dans les composés non-protéiques. Ces résultats montrent que l'excrétion de soufre total constitue une mesure précise du catabolisme des SAA et que le rapport d'équilibre N/S apporte des informations utiles concernant la mise en réserve des SAA non-protéiques chez le porcelet en croissance.

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Finally, I would like to dedicate this thesis to my Wife Hong Guan and my daughter Qingyi Hou. I love you very much.

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LIST OF ABBREVIATIONS

AP: adequate protein

CDO: cysteine dioxygenase

CSDO: cyteinesulfinate decarboxylase

GCS: γ-glutamylcysteine synthetase

GSH: glutathione

Hcy: homocysteine

LP: Low protein

MET: methionine

N: nitrogen

NRC: National Research Council

PEG: polyethylene glycol

RM: remethylation

S: sulfur

SAA: sulfur amino acid

SAH: S-adenosylhomocysteine

SAM: S-adenosylmethionine

TCEP-HCl: tris-(2-carboxyethyl)phosphine hydrochloride

tHcy: total homocysteine

TM: Transmethylation

TS: Transsulfuration

CONTRIBUTION OF AUTHORS

A manuscript entitled " *Use of urinary sulfur and the nitrogen/sulfur balance ratio as a measure of sulfur amino acid metabolism in a piglet model*" is included as a part of this thesis. The following is the contribution of the authors to the manuscript.

As primary author I was responsible for writing the manuscript and creation the figures and the tables. My contribution to the study in the manuscript including designing the studies, conducting pilot studies, performing the surgeries, carrying out the studies on both well-nourished and protein-depleted piglets, analyzing the samples in the laboratory, gathering and organizing the data, and performing calculations and statistical analyses. Urinary taurine and nitrogen in well-nourished piglets were analyzed by Fahad Saboohi. Farhad Saboohi was also involved in all the laboratory techniques and the methodology for this study.

All the above was conducted under the close guidance and supervision of Dr. Hoffer and Dr. Wykes, they are the originators of the project.

1 INTRODUCTION

Amino acids are allocated in the body to two main pathways; they are either used for protein synthesis or catabolized to urea, ammonia, sulfate, and carbon dioxide (Crim and Munro, 1994). Because the overall partitioning of amino acids between protein synthesis and catabolism is mainly decided in the fed state (Taveroff and Hoffer, 1994; Taveroff et al, 1994; Hoffer et al, 1997; Cayol et al, 1996; Biolo et al, 1992), and 20~50% of essential amino acids are metabolized in first pass by the tissues of the splanchnic bed (Biolo et al, 1992; Hoerr et al, 1993; Matthews et al, 1993), it is very important to find an effective and accurate way to measure amino acid catabolism in the fed state to better understand the factors that regulate whole body protein economy (Taveroff and Hoffer, 1994; Hoffer et al, 1997).

There is no fully satisfactory method for measuring amino acid catabolism in the non-steady state that follows normal food consumption. Historically, urinary nitrogen (N) and urea productions have been used in whole body amino acid catabolism research. Urea is the predominant nitrogenous end product in urine accounting for more than 80% of urinary N (Cheema-Dhadli and Halperin, 1993). However there are concerns with the use of urinary N and urea to assess whole body amino acid catabolism during short-term studies as urinary urea excretion underestimates its true production by 15~30%, due largely to urea hydrolysis (with partial recycling) in the gut (Jahoor and Wolfe, 1987; Hamadeh and Hoffer, 1998; El-Khoury et al, 1994; El-Khoury et al, 1996).

Current research approaches rely on amino acid tracer kinetic models to measure the oxidation of indispensable amino acids in whole body amino acid metabolism research

(Young et al, 1989; Zello et al, 1995; Brunton et al, 1998). However, amino acid oxidation techniques require accurate measurement of expired carbon dioxide production and isotopic enrichment causing this method to be complicated and costly. Both tracer methods require constant feeding regimens to accommodate steady state stochastic modeling, and are both limited in accuracy and precision during the non-steady state that following normal food intake (El-Khoury et al, 1994; Hamadeh and Hoffer, 1998; De Feo P, 1998; Hoffer, 1999).

Due to the shortcomings of both the urea production and tracer amino acid oxidation techniques, it would be valuable to find a simple, inexpensive, non-isotopic method to measure whole body amino acid catabolism in the non-steady state following normal food consumption. Sulfate production has been proposed as a promising alternative (Hamadeh and Hoffer, 2001). Sulfate is produced predominantly from sulfur amino acid (SAA) catabolism (Baker, 1986; Stipanuk, 1999), and unlike urea, sulfate is not subject to losses in the gut, being excreted almost entirely in the urine (Ryan, 1956; Bauer, 1976; Chakmakjian and Bethune, 1966;Hendriks et al, 2001; Hamadeh and Hoffer, 2001). Taurine is the other major sulfur (S) containing end product of SAA; it is also predominantly (95%) excreted in urine (Sturman et al, 1975; Stipanuk, 1999). This suggests that increased S production after a meal could be detected almost entirely in urine.

Because most of the S-containing end products from SAA are excreted in the urine, primarily as inorganic sulfate and taurine, we hypothesis that measures of urinary total S or urinary inorganic sulfate plus taurine would be useful indicators of SAA catabolism and potentially whole body amino acid catabolism.

The SAA's (methionine and cysteine (cystine)) are characterized by their inclusion

of a S atom in addition to a N component. An important feature that distinguishes SAA from total amino acid catabolism is the considerable storage of cysteine in a non-protein reservoir, glutathione (GSH) (Cho et al, 1981; Cho et al, 1984; ; Tateshi et al, 1977). When body GSH pool is constant, SAA intake and the excretion of S-containing end product, mainly as sulfate, will indicate whole body protein balance. But in short-term studies where GSH pool can change, N and S balance may diverge. This divergence may provide information about the body's GSH pool.

This thesis describes studies carried out to explore the usefulness of urinary S and whole body N and S balance ratio as determined in new-born piglets to provide measures of SAA catabolism, whole body protein stores and non-protein SAA stores under different nutritional conditions.

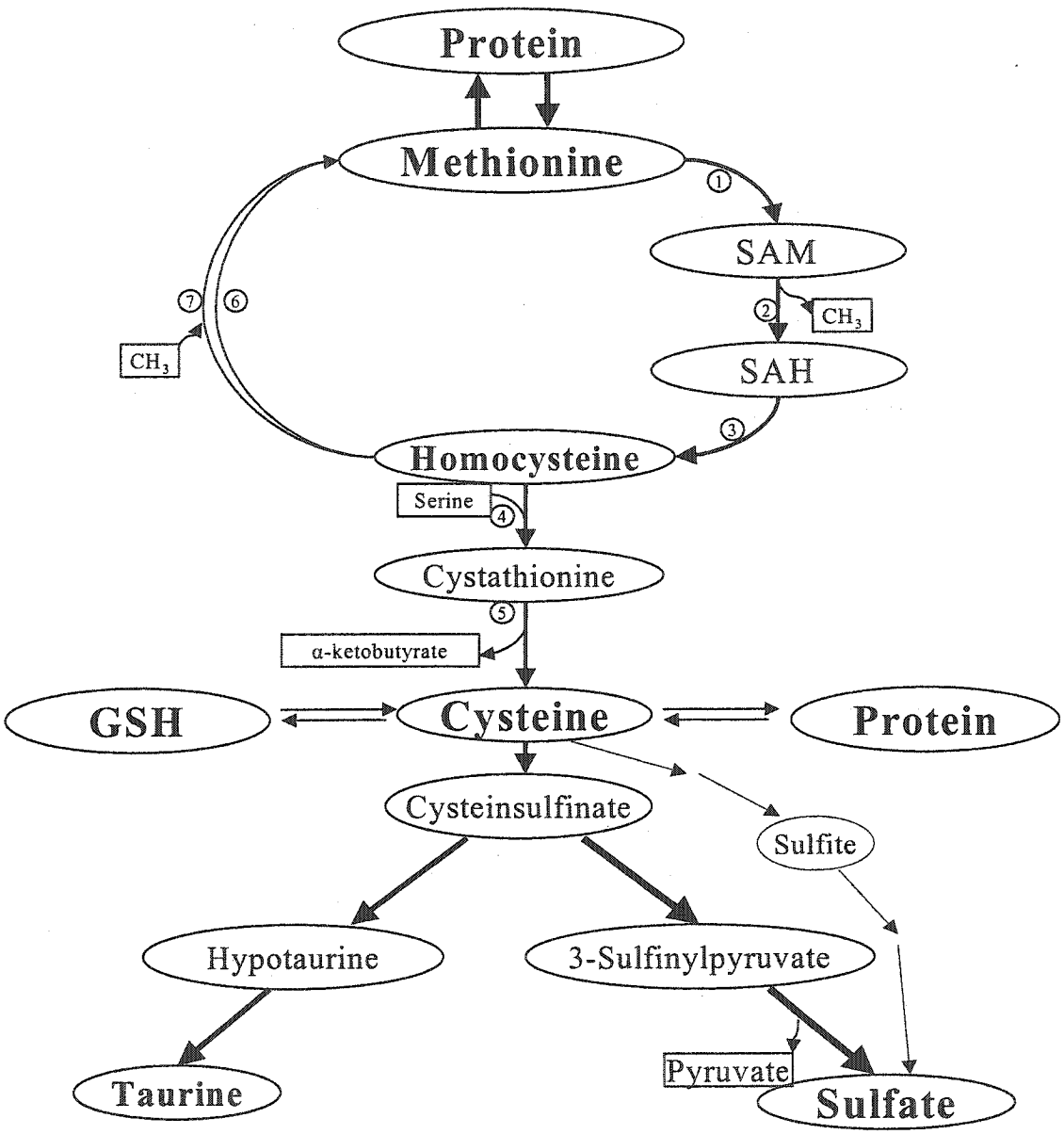

FIGURE 1 Sulfur amino acid metabolism

The circled numbers refer to the numbered reactions. See the text for details.

2 LITERATURE REVIEW

2.1 Sulfur Amino Acid Metabolism (Figure 1)

2.1.1. Metabolism of methionine

Methionine (MET) is a nutritionally essential amino acid for mammals (Griffith, 1987; Stipanuk, 1999). It has three major metabolic functions 1) incorporation into newly synthesized protein, 2) conversion to S-adenosylmethionine (SAM), the dominant biological methyl group donor, 3) conversion to cysteine by the transsulfuration pathway (Griffith, 1987; Stipanuk, 1986). Conversion to cysteine is the only catabolic pathway for MET since transamination is quantitatively insignificant (Stipanuk, 1986; Blom et al, 1989; Tangerman et al, 2000).

MET metabolism involves a series of interconnecting pathways, which can be separated into several distinct steps as below:

1) Formation of SAM (reaction 1): In the first step of MET metabolism, methionine adenosyltransferase (EC 2.5.1.6) catalyzes the formation of SAM by transfer of the adenosyl moiety of ATP to the S atom of MET (Stipanuk, 1986; Stipanuk, 1999; Griffith, 1987). This reaction is irreversible under physiological conditions (Mudd, 1973).

2) Transmethylation (TM) pathway (reaction 2): Catalyzed by a variety of methyl transferases, SAM donates the methyl group to a variety of methyl acceptors. All these reactions produce a common S-containing product, S-adenosylhomocysteine (SAH). These TM reactions are responsible for the biosynthesis of many cellular components, such as creatine and carnitine (Stipanuk, 1986; Griffth, 1987; Stipanuk, 1999). SAH is hydrolyzed by S-adenosylhomocysteine hydrolase (EC 3.3.1.1.), generating homocysteine (Hcy) and

adenosine (Stipanuk, 1986; Griffth, 1987; Stipanuk, 1999) (reaction 3).

4) Transsulfuration (TS) pathway (reactions 4 &5): This is a branch point in MET metabolism. In both humans and rats, about 50% of the Hcy generated from SAH condenses with serine to form cystathionine via cystathionine β-synthase (EC 4.2.1.22.) (reaction 4). Cystathionine is then cleaved to cysteine and α-ketobutyrate in a reaction catalyzed by cystathionase (EC 4.4.1.1) (reaction 5) (Griffith, 1987). Thus the S of Hcy is transferred to serine to form cysteine. The enzymes of the TS pathway are pyridoxine dependent, so inherited defects in the enzymes in this pathway result in hyperhomocysteinemia (Mudd et al, 2001). The TS pathway is impaired in premature infants as a result of absent or low cystathionase activity (Sturman et al, 1970; Zlokin and Anderson, 1982a) and cysteine and GSH metabolism are impaired in premature infants due to cystathioninase deficiency (Vina et al, 1995). This has led to the view that cysteine should be exogenously supplied and therefore is an "essential" amino acid for premature infants.

5) Remethylation (RM) pathway (Reaction 6, 7): The alternative metabolic fate of Hcy is methylation to form MET, completing the MET-Hcy cycle (Finkelstein et al, 1988). In humans, such RM reactions take one of two pathways; one way is the betaine-dependent methylation of Hcy catalyzed by betaine-Hcy methyltransferase (EC 2.1.1.5)(reaction 6) (Finkelstein et al, 1972; Stipanuk, 1999). The alternative pathway is the utilization of 5-methyltetrahydrofolic acid as a methyl donor via MET synthase (EC 2.1.1.13)(reaction 7)(Griffith, 1987; Stipanuk, 1986). Because MET synthase requires cobalamin as a cofactor, deficiencies of folate or cobalamin impair the RM pathway and lead to hyperhomocysteinemia (Mudd et al, 2001).

In the previously described pathways, Hcy is a pivotal regulator of MET metabolism. The RM and TS pathways are competing for available Hcy. Hcy's two metabolic fates serve independent obligatory functions. The average TS rate is determined by dietary MET intake, and methyl group utilization in SAM-dependent biosynthetic reactions determines the TM rate (Mudd and Poole, 1975). High SAM activates cystathionine β-synthase, therefore increases TS to dispose of sufficient MET and under low SAM conditions, TS is decreased and RM is increased (Selhub and Miller, 1992; Selhub, 1999). In humans fed an adequate MET diet, 31~38% of the Hcy formed by TM was remethylated to MET, while 62~69% was catabolized by TS (Storch et al, 1988; Storch et al, 1990). However, during a SAA free diet, the percentage of Hcy remethylated to MET increased to 67%, and TS of Hcy decreased to 33%, as a further consequence the authors also showed that the TS of MET sharply decreased from 12.4 mmol/day (Met-adequate fed state) to 3.5 mmol/day (SAA free fed state). Further, when a cystine-supplemented but MET-free diet was provided, TS of MET further decreased to 2.6 mmol/day, coupled with a 26% decrease in Hcy TS (Storch et al, 1990). These stable isotope results are consistent with data in rats and humans (Finkelstein and Martin, 1984; Finkelstein and Martin, 1986; Mudd and Poole, 1975; Mudd et al, 1980).

2.1.2. Metabolism of cysteine

Plasma cysteine can be derived from 4 sources: (1) from MET via the TS pathway; (2) from protein breakdown; (3) from the diet; (4) from GSH hydrolysis. The oxidation product, cystine, functions similarly to cysteine, and the reaction between cysteine and cystine is freely reversible (Baker, 1986). Cysteine can serve as precursor for synthesis of

proteins and several other essential compounds, such as GSH and Coenzyme A, or be catabolized to inorganic sulfate or taurine. Other than incorporation into protein, the major fates of cysteine are incorporation into GSH or catabolism via cysteinesulfinate dependent pathways or cysteinesulfinate independent pathways (Stipanuk, 1999).

Cysteinesulfinate dependent pathway

The cysteinesulfinate dependent pathway is considered to be the major pathway of cysteine catabolism in mammals, particularly when cysteine availability is high (Stipanuk, 1979; Daniels and Stipanuk, 1982; Griffith, 1987; Bagley and Stipanuk, 1994; Stipanuk, 1999). Cysteine is oxidized to cysteinesulfinate catalyzed by cysteine dioxygenase (CDO) (EC 1.13.11.20), where upon cysteinesulfinate is catabolized either by decarboxylation or transamination. In the decarboxylation pathway, cysteinesulfinate is decarboxylated to hypotaurine catalyzed by cyteinesulfinate decarboxylase (CSDC) (EC 4.1.1.29). Hypotaurine is further nonenzymatically oxidized to taurine. In the transamination pathway, cysteinesulfinate is transaminated to 3-sulfinylpyruvate, and then to pyruvate and sulfinate, sulfite is further oxidized to sulfate by sulfite oxidase (EC 1.8.3.1) (Stipanuk, 1999).

CDO and CSDC are the two key regulatory enzymes in the cysteinesulfinate dependent pathway. CDO activity and CDO protein concentration increased more than 10-fold in cultured rat hepatocytes when the medium was supplemented with methionine and cysteine (Kwon and Stipanuk, 2001), and Higher levels of CDO activity and CDO protein concentration were also observed in the liver of rats fed MET or protein-supplemented diets (Bella et al, 1999). The activity of CSDC decreased and the taurine production is limited when the dietary protein is high (Bagley and Stipanuk, 1994), however when rats fed a low

protein diet supplemented with SAA increased the taurine production in hepatocytes without a change in the activity of CSDC (Bargley and Stipanuk, 1995).

The metabolic partitioning of cysteinesulfinate between transamination and decarboxylation shows considerable species variation (Griffith, 1987), Rats, mice, and dogs have high levels of hepatic CSDC, while cats, monkeys and humans have lower levels of hepatic CSDC, so cats, monkeys and humans can synthesis taurine much less and slower than rats, mice and dogs (Worden and Stipanuk, 1985; Jacobsen et al, 1964; Gaull et al, 1977; Hayes et al, 1980). No information has been published about hepatic CSDC in pigs.

Cysteinesulfinate independent pathway

Cysteinesulfinate independent pathway is also called desulfuration pathway. Many studies have suggested that the production of pyruvate and inorganic sulfate from cyst(e)ine may occur by some pathways that do not involve conversion of cysteine to cysteinesulfinate, (Stipanuk and Beck, 1982; De La Rola and Stipanuk, 1985; Drake and Stipanuk, 1987). One pathway is transamination of cysteine with α-ketoglutarate to form β-mercaptopyruvate, following by desulfuration to release pyruvate and sulfite, which is further oxidated to sulfate (Ishimoto, 1979; Ubuka et al, 1977; Stipanuk, 1986). The other way is cysteine in the form of cystine, is cleaved by γ-cystathionase to thiocysteine, then the S is release as sulfite, which is further oxidated to sulfate (Stipanuk and Beck, 1982).

2.1.3. Taurine Metabolism

Taurine can be derived from cysteine catabolism or from diet and occurs naturally in most mammalian tissue. As an end product of SAA catabolism, taurine also performs important biochemical functions; detoxification, osmoregulation, membrane stabilization,

and neuromodulation (Zelikovic and Chesney, 1990; Chesney et al, 1998). Taurine is different from the amino acids in that a sulfonic group replaces the carboxylic acid and its amine group is located on the beta carbon. For this reason, it is not incorporated into protein, and exists only in the free state (Hayes and Sturman, 1981). Taurine is one of the most abundant free amino acids in the body, and the concentrations within the cell are generally much greater than in plasma (Hayes and Sturman, 1981; Aoki et al, 1973; Vinton et al, 1986). In humans, the metabolism of taurine has been described by two exchangeable pools: a small (0.25g) and rapidly exchanging pool ($t_{1/2}$ ~ 0.1 h) and a large (12.25g) and slowly turnover pool ($t_{1/2}$ ~ 70 h) (Sturman et al, 1975). For humans the whole body taurine pool varies from 12-18 g, and about 75% is located free in skeletal muscle (Hayes, 1985).

Taurine is mainly excreted in the urine, as about 95% of [^{35}S]taurine administrated intravenously to human adults was excreted in urine, roughly 75% as [^{35}S]taurine and 25% as [^{35}S]sulfate (Sturman et al. 1975). The sulfate is formed when taurine is secreted in the bile as taurine-conjugated bile acids and degraded by intestinal microorganisms, then absorbed and transported to the kidney for excretion (Sturman 1973; Sturman et al, 1975; Hepner et al, 1973). Similarly most [^{35}S]taurine administrated orally or intravenously to rats was also excreted as [^{35}S]taurine rather than [^{35}S]sulfate in urine (Hepner et al, 1973). The kidney further regulates the body pool size of taurine through adaptive changes in response to dietary taurine intake (Garcia and Stipanuk, 1992).

2.1.4. Excretion of S-containing compounds

The main source of S-containing compounds in mammalian urine is dietary SAA intake. SAA can be catabolized to sulfate (including inorganic sulfate and ester sulfate),

taurine, and other S-containing compounds, such as mercaptolactace, mercaptoacetate, thiocyanate, thiosulfate (Stipanuk, 1999).

As one predominant end product of SAA catabolism, inorganic sulfate distributes in extracellular water, and it is excreted almost entirely through the renal route (Cocchetto and Levy, 1981; Omvik et al, 1979). In humans, it has been found that 90-100% of the sulfate was recovered as inorganic sulfate in urine over 24 hours following an intravenous administration of a large dose of sodium sulfate (Chakmakjian and Bethune, 1966), and 98% of the sulfate was recovered as inorganic sulfate in urine and extracellular water over 9 h following an intravenous administration of magnesium sulfate (Hamadeh and Hoffer, 2001).

Ester sulfate (also termed "organic sulfate") refers to sulfate esters with phenolic compounds. The predominant source of sulfate esters is unknown but has been attributed to conjugation of sulfate with phenolic compounds formed by bacterial action on aromatic amino acids in the large intestine (Lundquist et al, 1980) or the excretion of steroid hormone metabolites (Finnstrom et al, 1983). It make up 9~15% of total urinary sulfate excretion in humans (Beach et al, 1942; Martensson, 1982; Martensson and Hemansson, 1984).

As the other major S-containing end product of SAA, taurine is excreted primarily in the urine, and because the renal tubules avidly reabsorb SAA, the urinary excretion of SAA (other than taurine) is normally negligible (Paauw and Davis, 1994).

In studies of total urinary S excretion in children and adults, inorganic sulfate accounted for 77~84%, ester sulfate 6~9%, taurine 3%, and cyst(e)ine approximately 0.6%; other S-containing compounds (including MET, Hcy, cystathionine, N-acetylcysteine, mercaptolactace, mecaptoacetate, thiocyanate, thiosulfate) accounted for less than 1.5% of

total S (Martensson, 1982; Martensson and Hemansson, 1984; Lundquist et al, 1980; Stipanuk, 1999). Urinary taurine excreted by one-week old newborn humans is 11% of total S, which is much higher than adults and children (Finnstrom et al, 1983). This increased urinary taurine in newborn infants can attributed to the high taurine content of human milk (Rassin et al, 1978). In rats, the composition of their urinary S containing components is different from human beings. Inorganic sulfate accounted for 69-75% of urinary S, whereas taurine accounted for 17-23%, a much higher percentage than humans (Stipanuk, 1979; Tomozawa et al, 1998; Yukihiro et al, 1998).

2.1.5. Relationship between urinary S-containing end products and SAA catabolism

Because most of the S from MET and cysteine is catabolized into measurable S-containing end products and excreted in the urine (Stipanuk, 1999), quantification of urinary S is potentially a useful indicator of SAA catabolism.

There are few studies that focus on using non-tracer urinary S-containing end products as an indicator of SAA catabolism. Yoshida et al (1989) measured urinary sulfate and taurine for several days following an intragastric injection of L-cysteine in rats fed normal protein diets and found that urinary sulfate and taurine accounted for 95% of the cysteine-S administrated. This group further found that, 24 hours after intraperitoneal injection of cysteine in rats given either low, normal or high protein diets, urinary S (sulfate and taurine) recovery was 59%, 75% and 75% of L-cysteine administrated respectively (Tomozawa et al, 1998; Yukihiro et al, 1998). The low recovery of administrated cysteine in low protein rats is an interesting result, however they did not determine whether the extra

retained cysteine in low protein rats was used for protein synthesis or retained in non-protein S-containing molecules, such as GSH.

2.2 Relationship Between Nutritional Status, SAA Intake, and Tissue GSH Concentration

GSH is a cysteine-containing tripeptide and the most abundant non-protein thiol in mammalian cells (Bray and Taylor, 1993). GSH plays an important role in intracellular protection against toxic compounds, reactive oxygen species, and free radicals (Meister, 1984). Decreased tissue concentrations of GSH have been reported in several disease states and malnutrition, and is associated with an increased risk to oxidative stress and opportunistic infections (Summer and Eisenberg, 1985; Bray and Taylor, 1993; Meister 1991; Jahoor et al, 1995). A clear understanding of the interrelationships between tissue GSH and nutritional status is clinically relevant.

2.2.1. GSH synthesis and homeostasis

GSH synthesis takes place in two steps; firstly, cysteine combines with glutamine to form γ-glutamylcysteine catalyzed by γ-glutamylcysteine synthetase (GCS) (EC 6.3.2.2). This is the rate-limiting step and GCS is the rate-limiting key enzyme in GSH synthesis. Next, glycine combines with γ-glutamylcysteine to form GSH, this reaction is catalyzed by GSH synthetase (Richman and Meister, 1975; Bray and Taylor, 1993). GSH is mainly synthesized in the liver and released into the circulation (Lauterburg et al, 1984; Stipanuk, 1999); then it can be hydrolyzed by GSH γ-transpeptidase located mainly in extrahepatic tissues to yield CysGly, which can be further degraded to release cysteine (Bray and Taylor,

1993). Because GSH is hydrolyzed upon up-take into the cell, each cell must synthesize and regulate its own GSH.

2.2.2 Dietary SAA intake and tissue GSH pool level.

Experiments in rats show that more than half of the SAA taken up by the liver is used for GSH synthesis and when exported into circulation, the kidney removes circulating GSH and releases cysteine back into the plasma (Garcia and Stipanuk, 1992). Tissue GSH concentrations can serve as a cysteine reservoir during the fasting state and SAA-deficient state (Cho et al, 1981; Cho et al, 1984; Tateshi et al, 1977). In humans, the normal turnover of GSH is about 40 mmol/day, nearly two to three times the typical SAA intake, six to seven times the estimated SAA requirement (Fukagawa et al, 1996), and slightly higher than that resulting from protein turnover in the body (~30 mmol/day) (Storch KJ, 1988; Stipanuk, 1999).

GSH metabolism is affected both by general nutritional status and by the SAA content of the diet. There are three regulatory factors involved in the regulation of tissue GSH pool level: 1) SAA intake, 2) function and availability of GSH synthetic enzymes, 3) the uptake and recycling mechanism for GSH (Bray and Taylor, 1993).

SAA intake regulates tissue GSH pool levels. It has been shown in rats that starvation decreases liver GSH to 30~50% of fed-state values (Lauterburg et al, 1984; Rodriguez et al, 1987; Hum et al, 1991). The hepatic GSH level was normalized within 8 hours of re-feeding (Tateishi et al, 1974). Protein-depleted diets decrease hepatic GSH by 20-50% in rats (Deneke et al, 1983; Rodriguez et al, 1987; Bauman et al, 1988a; Hum et al, 1992), and decrease intestinal mucosa GSH by more than 50% in growing piglets (Jahoor et al, 1995)

. When rats were fed a low-protein diet supplemented with either equal-molar cysteine or MET, hepatic GSH concentration increased to the concentration observed in rats fed the normal-protein diet (Bauman et al, 1988a). Similarly, Hunter and Grimble (1997) reported that rats consuming a normal-protein diet, or low-protein diet supplemented with SAA had significantly greater liver and lung GSH concentrations than the rats fed low-protein diets. These results demonstrate that tissue GSH is responsive to dietary protein alternations, and more specifically to the SAA content of the diet.

Tissue GSH concentration is also regulated by the expression of GSH synthetic enzymes (Bray and Taylor, 1993). It has been shown that neither excess dietary protein nor excess SAA increased the liver GSH concentration above the level attained when a protein adequate diet was fed (Boebel and Baker, 1983; Cho et al, 1984; Bauman et al, 1988a; Hum et al, 1992). This is likely due to the fact that the first enzyme in GSH synthesis, GCS, which is regulated by feedback inhibition of GSH (Richman and Meister, 1975). This is further confirmed when rats were fed a diet of excess SAA, the GSH production was markedly lower in hepatocytes and the concentration and activity of GCS, the rate-limiting enzyme in GSH synthesis also decreased in rats fed MET or protein-supplemented diets (Bagley and Stipanuk, 1994). This inhibition may limit the maximum hepatic GSH concentration in vivo and may limit the effectiveness of SAA supplementation to increase GSH concentrations.

It is also reported that GSH synthesizing enzymes are maintained during starvation (Tateishi et al, 1974). There is no significant difference of the activity of GCS when dietary SAA are normal or low (Hunter and Grimble, 1997). However, when rats were previously adapted to a low-protein diet for 2 weeks and then supplemented with SAA, the hepatic GSH

concentration increased more quickly and was sustained at a higher level than a rat that were previously fed normal-protein diet (Bauman et al, 1988b, Taylor et al, 1992). These results suggest that low dietary protein, or low SAA status may increase the response of hepatic GSH concentration to SAA supplementation.

Finally, tissue GSH concentrations are regulated by uptake and efflux mechanisms for GSH. It has been reported that the efflux rate of GSH in mice fed a low-protein diet was significantly lower than the control group fed normal-protein diet (Adachi et al, 1992). Thus this may explain the high concentrations of liver GSH in rats fed a low-protein diet supplemented with SAA (Bauman et al, 1988b, Taylor et al, 1992).

Due to the relationship between SAA and GSH , the possibility exists to use SAA catabolism to assertain valuable information about the the status of the whole body GSH pool.

2.3 N/S Balance Molar Ratio, Urinary N/S Ratio and Non-protein S Stores

2.3.1 Relationship between urinary N and S excretion

Urea and ammonia are the main nitrogenous end products of amino acid metabolism, so their production can be used as a basis for measuring protein turnover and an indicator of whole body amino acid catabolism (Jackson et al, 1983; Waterlow, 1981). Similarly, since sulfate and taurine are the major end product of SAA metabolism, their rate of loss from the body can be used to measure SAA catabolism (Stipanuk, 1999; Hamadeh and Hoffer, 2001). Both of these N and S containing end products are excreted in urine so that total urinary N and S are useful indicators for amino acid catabolism. In humans, 24-h urinary sulfate

excretion is closely correlated with urea-N excretion (r=0.95-0.99, Jourdan et al, 1980; Hamadeh and Hoffer, 2001) and 24-h urinary S excretion is closely correlated with N excretion (r=0.71-0.98; Zlotkin and Anderson, 1982b; Jourdan et al, 1980). Normally there appears to be parallels between N and S excretion because they occur together in dietary protein. In growing animals and children, the large portions of N and S are stored simultaneously in body protein. Therefore, both N and S balance are positive (Eckert, 1948; Beach et al, 1942; Zlotkin and Anderson, 1982b). For all these reasons, S excretion is a potential alternative to N excretion as a measure of whole body amino acid catabolism. However, an important feature that distinguishes SAA catabolism from total amino acid catabolism is the considerable storage of cysteine in a non-protein reservoir, GSH (Cho et al, 1981; Cho et al, 1984; Tateshi et al, 1977; Fukagawa et al, 1996). To the extent that this occurs, urinary S excretion will not simply indicate whole body net proteolysis, but in conjunction with N excretion, it could provide additional insight into the mechanisms governing GSH metabolism (Hamadeh and Hoffer, 2001).

2.3.2 Difference of N/S molar ratio in protein and GSH

Because most of the dietary amino acids retained in the body are used in protein synthesis, almost all the N retained in the body is stored as protein. SAA are also used in protein synthesis and thus their N and S components are subject to the same course (Beach et al, 1942). It has been shown that the N/S molar ratio in animal protein is ~38 (Beach et al, 1943; Block, 1951; Food Policy and Food Science Service, 1970) and in porcine species the N/S molar ratio is also ~38 (Beach et al, 1943; Food Policy and Food Science Service, 1970). However, SAA can also be stored in a non-protein form, GSH, which contains 3 N

and 1 S, so its N/S molar ratio is 3, which is much lower than the N/S molar ratio in protein. This clear difference between the N/S molar ratio in protein and GSH provides the possibility to use the N/S balance ratio or urinary N/S ratio to evaluate whole body tissue GSH status.

2.3.3 Can urinary N/S molar ratio or N/S balance molar ratio evaluate body non-protein S storage?

As early as 1916, studies in dogs showed that feeding periods following fasting showed a marked rise in the urinary N/S ratio (Lewis, 1916). When feeding is continued over a few days there is a gradual decline in the ratio until the normal basal ratio is achieved. This marked rise in the N/S ratio indicates the S retention in the organism is relatively greater than that of N retention. Some other investigators also observed this difference in S and N production between fed and fasting states (Wilson, 1925; Bressani et al, 1965). More recently Cheema-Dhadli and Halperin (1993) described a relative conservation of S greater than N during daytime (~fed state) with relatively greater S losses than N losses overnight (fasting state) in humans. The authors suggested that compounds rich in S versus N, such as GSH or SAA rich protein, were synthesized at an increased rate in the postprandial period. Hamadeh and Hoffer (2001) found that a significant proportion of the S in an oral MET dose or an intravenous mixture of MET and essential amino acids is retained in non-protein bound form. This result is supported by both a sulfate recovery data and a higher N/S balance ratio. Breitkreutz at al (2000) found that HIV positive patients have an abnormally high urinary sulfate/urea ratio compared with HIV negative patients. This suggests that HIV infection may deplete the whole body non-protein S storage, the GSH pool. This result was consistent with other reports that HIV-infection is characterized by a systemic GSH deficiency

(Buhl et al, 1989; Eck et al, 1989; Roederer and Staal, 1993). Together, these studies suggest that urinary N/S ratio may serve as a useful indicator of body non-protein S storage, and provide valuable information about whole body GSH status.

When compared to adults, greater proportions of N and S are stored by the growing organism and simultaneously incorporated into body protein and GSH. Neither excess dietary protein nor excess SAA intake increase tissue GSH concentration above its normal levels (Boebel and Baker, 1983; Cho et al, 1984; Bauman et al, 1988a; Hum et al, 1992). In this situation the retained N and S are used primarily for protein synthesis, and the N/S balance ratio would be similar to the ratio in body protein. However when low SAA diets are fed, tissue GSH pools are depleted, and when SAA are supplemented, a significant portion SAA is used for GSH synthesis to increase tissue GSH level. Therefore, the N/S balance ratio will decrease and become lower than the N/S ratio in body protein thus enabling the possibility to use a simple noninvasive measurement of urinary N/S ratio, or N/S balance ratio to get valuable information about alterations in GSH status.

2.4. Piglet Model

2.4.1 The role of the piglet as a model for amino acid metabolism

The piglet is a suitable model to study nutritional metabolism in the human neonate (Pond and Houpt, 1978; Ball et al, 1996). There are many similarities between piglets and human neonates, including digestive physiology, nutrient requirement patterns, body composition, and function of the kidney (Rowan et al, 1994; Pond and Houpt, 1978; Wykes et al, 1993; Shulman, 1993; Glauser, 1966). Therefore piglets have been widely used in human nutrition research.

Further, the piglet has been shown to be the model of choice to study protein and amino acid metabolism in relation to the human neonate (Garlick et al, 1976; Benevenga, 1986; Wykes et al, 1993; Wykes et al, 1994a; Wykes et al, 1994b). Piglets have a well characterized protein and amino acid metabolism with their estimated indispensable amino acid requirements proportionateley similar to those of the human infants (Ball et al, 1996). Piglets fed a complete elemental diet (described in Wykes et al, 1993) via gastric or venous routes are healthy and grow at rates comparable to sow-fed piglets (Wykes et al, 1993; Bertolo et al, 1999a; Bertolo et al, 1999b).Their body weight increase 6-8% per day in a well nourished state (Wykes et al, 1993; Bertolo et al, 1999a). This rapid growth rate makes it a very sensitive model for the detection of metabolic responses to small differences in amino acid supply in the diet (Wykes et al, 1993).

The piglet model has been used to examine protein kinetic responses to amino acid profile in total parenteral nutrition solutions, malnutrition, and the combined effects of malnutrition and inflammatory stress (Wykes et al, 1993; Ebner et al, 1994; Jahoor et al, 1995; Wykes et al, 1996).

2.4.2. Piglet as a model for SAA metabolism

The importance of SAA metabolism in human nutrition has become increasingly evident in recent years, particularly for infants and children (Famon et al, 1986; Cole et al, 1988; Zlokin and Anderson, 1982a; Vina et al, 1995). Therefore more information is needed on the SAA requirement of infants and children including their metabolic response to inadequate protein intakes. Ethical constraints preclude putting children on nutritionally inadequate diets as well as invasive sampling. Therefore an appropriate animal model is

needed for the study of SAA metabolism in this population. Rats are currently the most common models used in SAA research and many metabolic mechanisms of SAA are based on the rat model (Stipanuk, 1986; Stipanuk, 1999). However, because the rat is born in a metabolically immature state, it is not as good as the piglet to elucidate human infant amino acid metabolism (Reeds and Odle, 1996). Further, the rats small size impedes comparisons to the human infant. Because metabolic activity per unit weight is affected by size and that size affects organ distribution and protein synthesis, the piglet is more like the human than the rat (Benevenga, 1986; Ball et al, 1996).

Most of the SAA research in pigs focuses mainly on SAA requirements. In 1950, Bell first reported that MET is an indispensable amino acid for growth in the pig (Bell et al, 1950). Cysteine is classified as nonessential for pigs, because it can be synthesized from MET; however, it has also been shown that cysteine can provide about 50% of the need for total SAA (NRC 1998), and the sparing capacity of cysteine on the requirement for MET is 40% in both orally fed and intravenously fed piglets (Shoveller et al, 2000). Daabees et al (1984) found that after intrajejunal administration of 2 mmol/kg body weight MET on 8 kg body weight piglets, vena cava plasma MET levels increased and reached peak levels 1 hour after dosing. Values were still elevated 3 hours after dosing, but vena cava plasma taurine and cystine level were not significantly affected by MET loading. These results suggested that piglets do not catabolize MET very quickly after intrajejunal infusion. By contrast, in another study (Newport et al, 1976) fed pigs (4-5kg body weight) low protein diets supplemented with or without MET (50 mmol/kg). The recovery of tracer ^{14}C as $^{14}CO_2$ in

6 hours after a bolus intravenous infusion of a dose of [1-^{14}C]MET was nearly 50% and 15% respectively.

There is still limited information about cysteine catabolism in the piglet. The rat, mouse, and dog have been found to have high levels of CSAD (the rate limiting enzyme in Turine synthesis, Sturman, 1986) as compared to the cat, monkey or man (De La Rose and Stipanuk, 1985), therefore taurine is synthesis more in rat, mouse and dog than cat, monkey or human. However there is still no information about the CSDC activity in the newborn piglet. Like the milk of humans and rhesus monkey with a high taurine concentration (337, and 561 μmol/L, the milk of sows has a even higher taurine concentration (1320 μmol/L) (Stephen et al 1991). This suggestes piglets probably need taurine in their early life like human infants and monkeys. It has been shown that piglets weaned at 7 days of age and fed a milk replacer formula without taurine for 4 weeks had lower plasma and urinary taurine concentration, and liver taurine was decreased, but their growth rate was normal (Stephen and Chavez, 1992). This suggested that exogenous taurine is essential in maintain taurine pool in the newborn piglets, and the piglet may be more like human infants than rats in cysteine catabolism and have limited taurine synthesis ability.

3 RATIONALE

Contemporary methods for measuring amino acid catabolism are determined in the fed-state using tracer-determined amino acid oxidation and urea production. The former is accurate in the steady state, but it is expensive and complicated with both methods marred by problems of accuracy and precision in the non-steady state following food consumption (El-Khoury et al, 1994; Hamadeh and Hoffer, 1998; De Feo P, 1998; Hoffer, 1999). Due to such limitations, it is important to find a simple accurate and non-isotopic method to measure whole body amino acid catabolism.

Sulfate production is a potential alternative to measure whole body amino acid catabolism (Hamadeh and Hoffer, 2001). Because most of the S from the SAA (MET and cysteine) is catabolized into measurable S-containing end products (primarily as inorganic sulfate and taurine), and excreted in the urine (Stipanuk, 1999), quantification of urinary S is potentially a good indicator for SAA catabolism and potentially whole body amino acid catabolism.

SAA's are retained in the body as either protein or non-protein forms. GSH is the major form of non-protein S and it serves as a cysteine reservoir (Cho et al, 1981; Cho et al, 1984; Tateshi et al, 1977; Fukagawa et al, 1996). Protein has a much higher N/S molar ratio than GSH (38 versus 3) (Beach et al, 1943; Block, 1951; Food Policy and Food Science Service, 1970). This difference provides the possibility of using N/S balance ratio in human infants or growing animals to partition retained SAA from protein and non-protein storage, thus providing valuable information about changes in whole body non-protein S storage, presumably the GSH pool.

The piglet is a suitable model to study nutritional metabolism in the human neonate (Pond and Houpt, 1978; Ball et al, 1996). The metabolic similarity of amino acid metabolism in the piglet and human infant and the rapid growth rate of the piglet make it a highly suitable and sensitive animal model to investigate using urinary S excretion as an indicator for whole body SAA catabolism in a non-steady state after a methionine load, and using the N/S balance ratio to partition the retained methionine from protein and non-protein forms in different conditions. This new model may serve as a basis for subsequent clinical studies.

4 OBJECTIVES AND HYPOTHESES

4.1. Objectives

4.1.1 Main Objectives

The objectives of this study were to establish and validate a piglet model to study SAA catabolism and its relationship with N metabolism in different nutritional states.

4.1.2 Specific Objectives

1. To validate the use of sulfate excretion as an indicator of sulfate production.

2. To determine the relationship between urinary S-containing end products and SAA catabolism in well-nourished and protein-depleted piglets.

3. To determine the relationship between N and S balance in well-nourished and protein-depleted piglets.

4.2. Hypotheses

1. An intravenous dose of inorganic sulfate will be fully recovered in the urine, thus validating the use of urinary sulfate excretion as an indicator of sulfate production in this model.
2. An intravenous dose of MET given in surfeit over needs in the well-nourished fed state will be fully catabolized and recoverd as organic and inorganic S in urine.
3. An intravenous dose of MET in the protein-depleted fed state with a low SAA intake will only be partly recovered as urinary S, thus providing a quantitative measure of SAA retention.

4. An intravenous dose of MET will decrease the N/S balance ratio in protein-depleted state. Whereas it will not affect N/S balance ratio in well-nourished state. The decrease of N/S balance ratio in protein-depleted state can provide information about the increase of non-protein S storage after MET infusion.

Urinary Sulfur Excretion and the Nitrogen/Sulfur Balance Ratio Reveal Non-protein Sulfur Amino Acid Retention in Piglets

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5.1. ABSTRACT

We explored the usefulness of urinary sulfur excretion as a measure of sulfur amino acid (SAA) catabolism and the nitrogen/sulfur (N/S) molar balance ratio as an indicator of non-protein SAA storage in growing piglets. After confirming that an intravenous dose of sulfate is fully recovered in urinary sulfate, we measured urinary S recovery following an intravenous dose of methionine in 6 piglets fed an adequate protein diet and 6 piglets fed a low protein diet. As measured over 48 h, recoveries of the administrated methionine as urinary total S, inorganic sulfate and taurine were 106%, 49%, 49% respectively in the adequate protein group, and 69%, 45%, 31% in the low protein group. Total S recovery in low protein group was less than that in the adequate protein group ($p<0.05$) and indicated a net positive balance of the administrated methionine S. Under baseline dietary conditions, the N/S balance ratio in the low protein group was 30, lower ($p<0.05$) than the value of 36 in the adequate protein group. Following methionine administration, this ratio further decreased from 30 to 26 ($p<0.05$). We conclude that in the protein depleted state relatively more S than N is retained from the diet, and under these conditions a significant portion of the S derived from infused methionine is retained in non-protein compounds. Urinary S excretion, a simple non-tracer method, can provide an accurate measure of SAA catabolism. The N/S balance ratio is a potentially useful indicator of changes in non-protein SAA stores in growing piglets.

KEY WORDS: glutathione, methionine, protein restriction, sulfate, taurine, piglets

5.2. INTRODUCTION

Because the extent of partitioning of amino acids between protein synthesis and catabolism is mainly decided in the fed state (Taveroff and Hoffer, 1994; Taveroff et al, 1994; Hoffer et al, 1997; Cayol et al, 1996; Biolo et al, 1992), it is very important to find simple and accurate ways to measure amino acid catabolism in the fed state to better understand the factors that regulate whole body protein economy (Taveroff and Hoffer, 1994; Hoffer et al, 1997). Current methods for measuring fed-state amino acid catabolism are tracer-determined amino acid oxidation and urea production. The former is expensive and laborious, and both methods have problems of accuracy and precision in the non-steady state that follows normal food consumption (El-Khoury et al, 1994; Hamadeh and Hoffer, 1998; De Feo P, 1998; Hoffer, 1999). Sulfate production has been proposed as an alternative (Hamadeh and Hoffer, 2001).

End-product sulfur (S) arises predominantly from the catabolism of the sulfur amino acids (SAA) methionine (MET) and cysteine. Sulfate and taurine are the two main S-containing end products and, once formed, they are excreted almost entirely in the urine (Ryan, 1956; Bauer, 1976; Hendriks et al, 2001; Stipanuk, 1999; Sturman et al, 1975). Measures of urinary S or urinary inorganic sulfate and taurine represent a simple, inexpensive, non-isotopic means to measure SAA catabolism and, in some situations, whole body amino acid catabolism.

An important feature that distinguishes SAA from total amino acid catabolism is the considerable storage of cysteine in a non-protein reservoir, glutathione (GSH) (Cho et a,. 1981; Cho et al, 1984; Tateshi et al, 1977; Fukagawa et al, 1996). This feature also provides the opportunity to use SAA catabolism to infer information about whole body

GSH stores. Because GSH has a much lower ratio of nitrogen (N) /S atoms (GSH contains 3 N and 1 S) than protein, the change of whole body N/S balance ratio in different nutritional states may permit useful information about the body's GSH pool (Cheema-Dhadli and Halperin, 1993; Hamadeh and Hoffer, 2001).

A better understanding of SAA metabolism is important in part because more information is needed on SAA requirements of infants and children including their metabolic response to inadequate protein intakes (Zlokin and Anderson, 1982a; Vina et al, 1995; Famon et al, 1986; Cole et al, 1988). The metabolic similarity of amino acid metabolism in the piglet and the human infant makes it a suitable animal model for this study (Ball et al, 1996; Wykes et al, 1994a) .

The objective of the present study was to validate the method of using urinary S excretion as an accurate measure of whole body SAA catabolism, and to explore the use of the whole body N/S balance ratio as a non-invasive indicator of non-protein SAA storage under different nutritional conditions in a growing piglet model. We first studied the newborn piglet fed adequate protein and surfeit SAA with the aim of producing a state with normally filled GSH stores. We then used this model in a mildly protein malnourished state. Our aim was to deplete the tissue GSH stores, then use urinary S recovery to quantitatively measure SAA retention, and use the N/S molar balance ratio as a noninvasive indicator of non-protein S storage.

5.3. METHODS

Experimental Design

Twelve new-born piglets were placed into an adequate protein group (AP, n = 6) and low protein group (LP, n = 6). The piglets in group AP were continuously fed an elemental diet providing adequate protein and surfeit SAA with sufficient energy for normal growth, and those in group LP a low protein, low SAA diet with sufficient energy. The method of using urinary sulfate or total urinary S as an indicator of sulfate production was validated in the AP piglets by infusing a known amount of sulfate intravenously and measuring its urinary recovery. In both groups a MET infusion study was performed to determine the relationship between urinary S-containing end products and SAA catabolism under conditions of surfeit and deficient SAA intake. Urinary S recovery was used to determine SAA retention, and the N/S molar balance ratio to indicate non-protein S storage.

Animals and surgical procedures

Twelve male piglets (Landrace × Yorkshire) were obtained from Macdonald Farm, McGill University. The 6 piglets in group AP were removed from the sow at 3 days of age for surgery to implant catheters. The 6 piglets in group LP were removed from the sow at 6 days of age for the same procedure. All procedures were approved by the Animal Care Committee of McGill University according to Canadian Council on Animal Care Guideline.

The piglets were pre-medicated with atropine (0.05mg/kg i.m.)(atropine sulfate; MTC Pharmaceuticals, Cambridge, ON), Baytril (2.5 mg/kg i.m) (enrofloxacin/enrofloxacine; Bayer Inc., Etobicoke, ON) and Buprenorphine (7.5 μg/kg i.m.) (buprenorphine hydrochloride, Reckitt & Colman Pharmaceutical, Richmond, VA)

followed by anesthesia induction with 5% isoflurane (MTC Pharmaceuticals, Cambridge, ON). Anesthesia was maintained with 2% isoflurane in 50% oxygen by tightly secured mask. Using a method modified from that of Wykes et al. (1993) the following silastic catheters were aseptically inserted: a venous sampling catheter (1.0 mm id x 2.2 mm od) into the right external jugular; a femoral catheter (0.8 mm id x 2.0 mm od) into the right femoral vein for magnesium sulfate and methionine administration; a feeding catheter (1.57mm id x 3.18mm od) through a hole in the stomach, and a urine sampling catheter (1.02mm id x 2.16mm od) through a hole in the bladder wall. The catheters were filled with saline solution, capped, and secured in the pocket of a mesh jacket worn by the piglet. The piglets were kept in metabolic cages. Each morning, the bladder of each piglet was emptied and the piglets were weighed to the nearest 5 grams on an electronic balance. Urine was collected through the bladder catheter every 24 hours to monitor N balance and S balance.

Diet

The elemental diets were based on the parenteral regimen designed by Wykes et al (1993) and administrated continuously via the gastric catheter. Both diets supplied all nutrients required by neonatal piglets (NRC, 1998) with the exception of the LP diet, which provided 50% of the NRC amino acid requirement. The target infusion rates were 275 $ml.kg^{-1}.d^{-1}$ of undiluted elemental diet and co-infused with lipid (Intralipid 30%, Pharmacia, Mississauga, ON, Canada) at a rate of 34 $ml.kg^{-1}.d^{-1}$. The AP diet supplied 15.8 g amino acid. $kg^{-1}.d^{-1}$ and 1.1 MJ metabolizable energy. $kg^{-1}.d^{-1}$. Amino acids, glucose polymer (Polycose, Ross Laboratories, Columbus, Ohio) and lipid supplied 24%,

38%, and 38% respectively of the total energy. The LP diet supplied the same metabolizable energy as the AP diet with isoenergetic replacement of amino acids by Polycose. The amino acid intake was 6.45 $g.kg^{-1}.d^{-1}$. Amino acid, Polycose and lipid supplied 10%, 52%, and 38% of the total energy.

The dietary amino acid profile (Table 1) was similar to that of milk protein (Wykes et al 1994a, 1994b), with tyrosine supplied as glycyl-tyrosine (Wykes et al, 1994b). For the AP diet, intakes of all the indispensable amino acids exceeded NRC requirements (NRC, 1998), and in particular it provided 120% of the NRC requirement for SAA. (The composition of AP and LP diet was shown in additional Table 6 and 7, and the N and S composition of AP and LP diet was shown in additional Table 8 and 9.)

The diets were administered via the gastric catheter at a constant rate using a COMPAT 199235 enteral feeding pump (Novartis Nutrition, Minneapolis, U.S.A.), with the feeding rates adjusted every morning according to body weight.

To avoid hyperosmolar diarrhea or fluid overload, Polycose was used rather than glucose, and the diets were diluted according to a graded schedule, in which the diet infusion began approximately 3 hours post-surgery at a sub-maximal rate, and gradually increased during day 0 and day 1. The elemental diet solution was diluted 4-fold on the surgery day (day zero), 3-fold for 12 hours then 2.5-fold for 12h on day 1, 2-fold for 12 hours then 1.75-fold for the remainder of days 2 and 3, then 1.5-fold for days 4 to 6. From day 7 to the end of the study, the solution was diluted 1.2-fold. A full rate of diet provision was reached from the beginning of day 3 post-surgery in group AP, and from the beginning of day 4 post-surgery in group LP.

Study Protocol

Sulfate infusion

Two basal 24h urinary sulfate and total S excretions were measured on days 4 and 5 after surgery in the AP piglets. On the beginning of day 6, 1.2 mmol.kg^{-1} of $MgSO_4.7H_2O$ (Sabex, Boucherville, QC, Canada) was infused intravenously over 2 h, and another 24-h urine sample was collected to measure urinary sulfate and total S excretion.

Methionine infusion

In the AP piglets, 24h urine collections were made on days 8 and 9 post-surgery to measure baseline urinary excretions. At the beginning of day 10, MET (1.2 mmol.kg^{-1}) was infused intravenously as 0.6mmol. kg^{-1}.h^{-1} MET over 2 h from a 0.2 mol/L stock solution. This stock solution was made by dissolving 7.46 g MET (Ajinimoto U.S.A. Inc.) in sterile 0.9% sodium chloride to 250 ml final solution, followed by sterilization by passage through a 0.22µm CORNING 430769 Filter System (CORNING Costar, Corning, NY, U.S.A.). Following the MET infusion, 24-h urine collections were made to measure urinary S excretion over the following two days. Blood samples were also obtained just prior to and 24 and 48 h after the MET infusion. The piglets were then killed by an intravenous injection of sodium pentobarbital (Euthanyl 750 mg).

In the LP piglets, a MET infusion study was performed when they were the same age as the AP piglets (13 days) using the same infusion and measurement procedure.

Analytical Methods

Total S, inorganic sulfate, and ester sulfate

The methods are based on the turbidimetry of barium sulfate in the presence of a small amount of preformed barium sulfate and the stabilizing agent polyethylene glycol (PEG), as described by Lundquist et al (1980), with some modifications.

Total S

Total S was determined after wet oxidation of the sample with nitric acid and perchloric acid in the presence of catalytic vanadate (Smith, 1953). A 3ml urine sample was transferred to a 150 mm x 25 mm Kimax digestion tube (VWR International) and dried under plain airflow at 120 ° C. Five ml of digestion mixture was added to the digestion tube at room temperature. This digestion mixture was prepared by dissolving 0.2125 g ammonium metavanadate (Sigma Chemical, St Louis, MO) in 130 ml 70% nitric acid, and dissolving 0.94 g potassium dichromate (Fisher Scientific, New Jersey) in 33.5 ml water. They were then mixed with 150 ml 70% perchloric acid. All water was distilled and de-ionized. The sample was heated at 140 ° C for 2 hours leaving the end of the tube uncovered to allow evaporation of all nitric acid. It is necessary that the digestion tube is sufficiently long to prevent sample loss through bumping but sufficiently short that all the nitric acid evaporates without reflux near the top of the tube. A funnel was then placed on the mouth of the tube and the temperature was raised to 220° C for 3 hours to oxidize all S containing molecules to inorganic sulfate. Complete oxidation is indicated by a change in sample color from green to orange as chromic ion (green) is oxidized to dichromate (orange). After cooling, 20 ml of acid diluent was added, and

well mixed to dissolve precipitates. The acid diluent was made by mixing 31.5 ml of glacial acetic acid with 12.5 ml of 37% hydrochloric acid and 12.5 ml of 85% phosphoric acid, with the volume made up to 500 ml with de-ionized water. The contents of the digestion tube were transferred to a long 100-ml tube. The digestion tube was washed with de-ionized water and the washings added to the 100-ml tube and the final volume made up to the 50 ml mark. For the sulfate analysis, 1 ml of the diluted digestate was mixed with 3 ml of de-ionized water and 1 ml of freshly maded Barium-PEG-sulfate reagent. The barium-PEG-sulfate reagent was made in two steps. First, 7.5 g of PEG (Sigma Chemical, St Louis, MO) and 0.4885 g barium chloride (Fisher Scientific, New Jersey) were added to de-ionized water to make a 50 ml solution. Then 40 ml of this solution were transferred to a flask where 80 µl of 50 mM sodium sulfate were added with efficient stirring. After the diluted digestate was mixed with the barium-PEG-sulfate solution, it was left at room temperature for precisely 23 minutes, then mixed and transferred to a 2 ml cuvette and read precisely 25 min after the digestate and barium-PEG-sulfate were mixed, using a Perkin-Elma Lambda 3A UV/VIS spectrophotometer set to record absorbance at 600 nm. The sulfate content of the digestate was determined from a standard curve and the concentration of total S per liter of urine then calculated, using the appropriate dilution factor.

Inorganic sulfate

A 0.1 ml urine sample was diluted to 3 ml with de-ionized water. To this was added 1 ml of 0.5 M HCl and 1 ml of Barium-PEG-sulfate, followed by thorough mixing. The sample was then treated as described above.

Ester sulfate:

Inorganic sulfate was eliminated from the urine by barium chloride precipitation. Three ml of a solution of 0.977 g of $BaCl_2.2H_2O$ and 4.1 ml 37% HCl in 100 ml were mixed with 1.5 ml of urine. The mixture was centrifuged at 1400 g for 10 min at room temperature, and 1.5 ml of the supernatant applied to 1-ml columns of cation exchange resin (AG 50W-X8, 100-200 mesh hydrogen form, Bio-Rad Laboratories, Richmond, CA). Water (2.5 ml) was added, and the eluate was collected in 15 ml Kimax screw-cap glass tubes. The sealed tubes were heated at 100 °C for 30 min in a water bath to hydrolyze sulfate esters. After cooling to room temperature, 2 ml of the resulting solution was mixed with 1ml of Barium-PEG-sulfate reagent and mixed well. The other 2 ml was mixed with 1ml of de-ionized water and served as a control, since the hydrolysate of urine samples may develop a faint blue color. The sulfate measurement was made as described above. The difference between the absorbance of the control and de-ionized water was substracted from the absorbance obtained with the complete assay.

Methionine, taurine and homocysteine

Plasma MET and taurine and urinary taurine were derivatized with phenylisothiothiocyanate and measured by HPLC using a Waters HPLC system and PicoTag C18 reverse phase column with an ultraviolet detector set at 254 nm as described previously (Robitaille and Hoffer, 1988). The MET peak eluted at 10.5 min, and the taurine peak eluted at 5.5 min. Plasma total homocysteine (tHcy) was measured by fluorescence HPLC following reduction of all disulfides using tris-(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl; Pierce Chemical Co., Rockford, IL,

USA; 10%in water), and conversion to a fluorescent derivative with monobromobimane (Calbiochem,San Diego, CA) using a method modified from that of Hum et al (1991).

Total N, urea and creatinine

Total urinary N was measured by high-temperature combustion followed by chemiluminescent detection of nitric oxide using a ANTEK model 7000B total N analyzer (ANTEK Instruments L.P. Houston, TX). Serum and urine urea and creatinine were analysed using a Hitachi 917 automated analyzer (Laval, QC, Canada).

Calculations

N balance

N balance (N_{bal}) was calculated as:

N_{bal} (mmol.kg^{-1}.d^{-1}) = N_{in} (mmol.kg^{-1}.d^{-1}) - N_{out} (mmol.kg^{-1}.d^{-1}).

N retention (N_{ret}) was calculated as:

N_{ret} (%) = N_{bal} (mmol.kg^{-1}.d^{-1}) / N_{in} (mmol.kg^{-1}.d^{-1}) × 100.

N_{out} was equal to urinary N only; stool outputs were negligible.

S balance

S balance ($_{bal}$) was calculated as:

S_{bal} (mmol.kg^{-1}.d^{-1}) = S_{in} (mmol.kg^{-1}.d^{-1}) - S_{out} (mmol.kg^{-1}.d^{-1}).

SAA retention (SAA_{ret}) was calculated as:

SAA_{ret} (%) = S_{bal} (mmol.kg^{-1}.d^{-1}) / SAA_{in} (mmol.kg^{-1}.d^{-1}) × 100.

S_{out} was equal to urinary total S only; stool outputs were negligible.

N/S molar balance ratio

The N/S molar balance ratio was calculated as: N_{bal} ($mmol.kg^{-1}.d^{-1}$) / S_{bal} ($mmol.kg^{-1}.d^{-1}$).

Urinary S recovery after sulfate or methionine administration

The recovery of infused magnesium sulfate or MET as urinary sulfate or S was calculated from the increase in urinary sulfate or S excretion above a stable baseline excretion that was due to dietary and endogenous SAA catabolism. Urinary sulfate or S production due to the infused substrate was equal to total sulfate or S production minus baseline sulfate or S production over the same period of time. This assumes that prior to the infusion, baseline excretion remained constant over the subsequent 2 days. The absolute dose of MET infused was determined by the body weight on the morning of the infusion. Since excretions were expressed per kg body weight, it was necessary to take account of the slightly greater body weight on the second 24 h collection period. This was done by multiplying the excretion due to the administrated MET per kg for the second 24 h period by body weight day 2 / body weight day 1.

Statistical Analysis

Two-way repeated measures ANOVA (Sigmastat for Windows version 2.03, SPSS Inc.) was used to determine significant differences in N balance, S balance, N/S balance ratio, N retention, SAA retention, the 2 factors being diet (AP versus LP) and different study states (before and after MET infusion). Two-way repeated measures

ANOVA was used to determined significant differences in body weight, plasma SAA concentrations, and S recoveries of in MET infusion study, the 2 factors being diet (AP versus LP) and time. Within the same group, plasma SAA concentrations and urinary S excretion over time were analyzed by one-way repeated ANOVA. When ANOVA results showed significance, Newman-Keuls test was used post hoc to determine the source of difference. Paired t-test was used to determine significance in urinary S and sulfate recoveries with 100%. Differences between data were considered significant at $P<0.05$. All results are presented as means ± SEM.

5.4. RESULTS

Growth.

The piglets in both groups were healthy and active throughout the study; their growth curves are shown in Figure 2. After a full intake of elemental diet were achieved, the growth rates of the AP piglets (79.4 ± 2.8 $g.kg^{-1}.d^{-1}$) was similar to that previously reported for sow-raised piglets (79 $g.kg^{-1}.d^{-1}$) (Wykes et al, 1993). The growth of the LP piglets was not significantly slower (70.5 ± 5.4 $g.kg^{-1}.d^{-1}$; p=0.11).

Daily urinary S excretion.

Fig. 3 shows daily urinary excretion of total S, inorganic sulfate, taurine, and ester sulfate. For the AP piglets, average daily excretion of total S, inorganic sulfate, taurine, and ester sulfate during the baseline periods (days 7,8,11,12) were 2.51 ± 0.08, 1.69 ± 0.06, 0.67 ± 0.03, and 0.036 ± 0.002 $mmol.kg^{-1}.d^{-1}$ respectively, and unchanging over these days. Inorganic sulfate, taurine, ester sulfate and other S components accounted for

67.5 ± 1.5%, 27.0 ± 1.2%, 1.5 ± 0.1% and 4.0 ± 1.2% respectively of the total. In the LP group, average daily urinary total S excretion during the baseline period (days 11,12) was lower than this (1.40 ± 0.02 mmol.kg $^{-1}$.d $^{-1}$; p<0.05), with inorganic sulfate, taurine, ester sulfate, and other S components accounting for 78.9 ± 1.8%, 11.7 ± 1.2%, 3.0 ± 0.2%, and 6.4 ± 1.5%, respectively of the total and constant over days 11-12 (Figure 3). Urinary inorganic sulfate and taurine excretion was also lower (p<0.05) than those of the AP piglets. Urinary ester sulfate excretion was not affected by diet or sulfate or MET administration.

Sulfate and methionine infusion studies

When 1.2 mmol.kg^{-1} of magnesium sulfate was administrated to the AP piglets, urinary total S and inorganic sulfate excretion increased from baseline levels of 2.50 ± 0.13 and 1.86 ± 0.19 mmol.kg^{-1}.d^{-1} to 3.65 ± 0.13 and 3.04 ± 0.17 mmol.kg^{-1}.d^{-1} over the subsequent 24h, returning to the baseline level in one day (Figure 3). Taurine excretion was not affected. If it is assumed that endogenous urinary total S and inorganic sulfate excretion remained constant over the 24h that followed the magnesium sulfate infusion, the urinary excretions of total S and inorganic sulfate above baseline excretions accounted for 95.9 ± 3.1 %and 98.6 ± 2.7 % respectively of the total sulfate infused (Table 2).

When 1.2 mmol.kg^{-1} of MET was administrated to the AP piglets, urinary total S, inorganic sulfate and taurine excretion increased above the baseline level (p<0.001) over the first 24 h following the infusion. Total S and taurine excretion remained above the baseline level (p<0.05) over the second 24 h, but inorganic sulfate excretion had returned

to its baseline level. When the same dose of MET was administrated to the LP piglets, urinary total S, inorganic sulfate and taurine excretion increased ($p<0.001$) over the following 24 and 48 h.

Table 3 shows urinary S recovery following the MET infusion. The calculation assumes that basal urinary S excretion over the two 24-h baseline periods remained constant over the 48 h immediately following the infusion. In the AP group, 70% of the MET dose was catabolized and excreted as total S within 24 h, 44% as inorganic sulfate and 18% as taurine. When measured over 48 h, the cumulative MET S recovery as urinary total S was 106% (not significantly different from 100%), with 49% as inorganic sulfate and 49% as taurine. Plasma MET, taurine and tHcy concentrations were not significantly affected by the MET infusion (Table 4). In the LP group, urinary total S recovery of the infused MET was 32% after 24 h and 69% after 48 h (Table 3), both lower than in the AP group ($p<0.001$). MET recovery as urinary inorganic sulfate was 24% in the first 24 h, lower than for the AP group ($p<0.001$). After 48h, the recovery as urinary taurine was 31%, which was also lower than for the AP group ($p<0.05$). Baseline plasma MET, taurine and tHcy concentrations were lower in the LP group ($p<0.05$), and unlike the AP group 24 h after the MET infusion, their plasma MET was 4.1-fold above the baseline level ($p<0.05$), and plasma tHcy 2.5-fold above the baseline level ($p<0.05$), both returning to normal by 48 h. Twenty-four and 48 hours after the MET infusion, plasma taurine concentrations were 1.3-fold and 1.4-fold above the baseline level ($p<0.05$; Table 4.). Before the MET administration, the creatinine clearance in LP piglets

was lower than AP piglets ($p<0.05$). Serum urea concentrations in LP piglets were lower than AP piglets ($p<0.05$).

In both groups, N balance was 3% less positive in the 48 h following MET administration ($p<0.05$; Table 5). In the AP group, 48 h S balance and the N/S balance ratio following MET administration were not significantly different from the baseline, but in the LP group, the 48 h S balance was more positive following the MET infusion, increasing from a baseline value of 3.81 mmol/kg/48h to 4.12 mmol/kg/48h ($p<0.001$). The 48h baseline N/S balance ratio in LP group was 30, lower than the ratio of 36 in the AP group ($p<0.001$). Following the MET infusion the 48 h N/S balance ratio in these piglets decreased even further to 26 ($p=0.001$; Table 5).

5.5. DISCUSSION

This study has established a model in the growing piglet for studying SAA metabolism in which the use of urinary S excretion is used as an indicator of whole body SAA catabolism. To determine whether urinary sulfate serves as a valid indicator of sulfate production in this model, we administrated inorganic sulfate intravenously in a physiologically relevant dose corresponding to the amount of S in MET used in subsequent studies. The urinary sulfate recovery measurement was carried out after verifying that daily endogenous total S and sulfate excretions were constant over the measurement period. Cumulative urinary total S and sulfate recovery was 96 % and 99 % over 24 h following the magnesium sulfate infusion. This is in agreement with the 90-100% recovery in urine as inorganic sulfate over 24h following intravenous

administration of a large dose of sodium sulfate (Chakmakjian and Bethune, 1966) and 98% recovery as inorganic sulfate in urine and extracellular water over 9 h following an intravenous administration of magnesium sulfate in adult human (Hamadeh and Hoffer, 2001). This result confirms that sulfate produced in the body is quantitatively excreted within 24 h in the urine of growing piglets.

Having determined that urinary sulfate can provide accurate information about sulfate production, we investigated what fraction of the S in an intravenous dose of MET can be accounted for as increased urinary excretion of S containing components 24 h and 48 h following its administration in well-nourished (AP) and protein-depleted (LP) piglets. Because all the piglets were fed continuously through a stomach catheter at a rate adjusted every morning according to their body weight, each piglet received the same amount of diet per kilogram body weight every day. It was therefore expected that SAA catabolism and S excretion would be constant, as was indeed observed (Figure 3).

In the well-nourished state with surfeit SAA intake, the increases in urinary total S, inorganic sulfate, and taurine excretion following intravenous MET accounted for 70%, 44%, 18% (at 24 h), and 106%, 49%, 49% (at 48 h) of the S in the administrated methionine. This suggests that within 48 h all of the administrated surfeit MET was catabolized and excreted in the urine as S containing end products, half as inorganic sulfate (which was almost entirely excreted within the first 24 h), and the other half as taurine (which was largely excreted in the second 24 h). Plasma MET, taurine and tHcy concentrations were not different from their baseline levels 24 h and 48h after MET infusion (Table 4). Taken together, these results suggest that 24 h after its administration,

the infused MET was already completely metabolized, with 44% catabolized to inorganic sulfate and excreted in urine and 18% converted to taurine and excreted in urine. Over the second 24 h following the MET infusion, 29% of the dose was recovered as urinary taurine, and only 5 % as inorganic sulfate. Taurine is actively transported into cells (mainly in muscle), where it has a large and slowly exchanged body pool (Jacobsen and Smith, 1968; Sturman, 1973; Sturman et al, 1975). We presume that most of the recovered taurine was formed in the first 24 h after the MET infusion, but some of it was retained in the muscle taurine pool and released over the subsequent 24 h.

This is the first report in piglets that within 48h of surfeit MET infusion all of the dose is fully catabolized and excreted in urine as S-containing end products. This result is in agreement with the 95% recovery in urine as inorganic sulfate and taurine over a few days following administration of 5 mmol/kg of L-cysteine in well-nourished rats (Yoshida et al, 1989). The observation that all of the administrated MET was recovered as urinary S over 48h in a well–nourished state with surfeit SAA intake validates the concept of using urinary S excretion as a useful indicator of SAA catabolism in the growing piglet.

Following validation of this method in the well-nourished state, we used the same method to determine the fate of administrated MET during mild protein depletion. When the same dose of MET was administrated to the LP piglets, urinary total S recovery was 32% over 24 h and 69% over 48 h, both significantly lower than in the well-nourished state. Thus 24 h and 48 h after the MET infusion, 68% and 31% of the S in the infused MET was still retained in the body. As indicated in table 4, and unlike in the AP group, plasma MET, tHcy, and taurine concentrations were substantially higher than baseline

level at 24h. Circulating MET can account for only 12% of the infused MET dose if it is assumed that intracellular and extracellular MET concentrations are approximately equal (Bergstrom et al, 1990) and total body water is 75% of body weight (Wykes et al, 1993). After accounting for this, 56% of the infused MET was retained in other S-containing forms 24 h after its administration. Forty-eight hours after the MET infusion, plasma MET and tHcy had returned to close to baseline. Although 12% of the infused MET was catabolized in the second 24 h, and some part of it may have been catabolized to taurine and retained in the muscle taurine pool, 31% of the infused MET remained unaccounted for 48 h after its administration. After MET administration, there was an unexplained small but significant reduction in the N balance (ruling out retention of the MET in new body protein synthesis), whereas there was a significant increase in S balance. This suggests that the retained MET must have been retained as non-protein S, such as GSH or taurine.

An important feature that distinguishes SAA catabolism from total amino acid catabolism is the considerable storage of cysteine in GSH (Cho et al, 1981; Tateishi et al, 1977; Tateishi et al, 1981; Cheema-Dhadli and Halperin, 1993; Bauman et al, 1988a; Bauman et al, 1988b; Hunter and Grimble, 1997). Because of this unique metabolic feature of SAA, S balance will not only indicate whole body net proteolysis but can also provide information about whole body GSH. Apart from protein, GSH is the main non-protein SAA storage pool in the body. The normal turnover of GSH in humans is nearly two to three times typical SAA intakes (Stipanuk, 1999). The N/S molar ratio in animal protein is ~38 (Wilson, 1925; Beach et al, 1943; Food Policy and Food Science Service,

1970) whereas the N/S molar ratio in GSH is 3. Because of this feature, a simple whole body N/S balance ratio can provide information as to partitioning of conserved SAA between protein and GSH storage.

In this study, the basal N/S balance ratio in AP group piglets was 36, and it remained 35 48 h after MET administration. This is close to the N/S molar ratio of 38 reported for pork protein (Beach et al, 1943; Food Policy and Food Science Service, 1970), suggesting that in the well-nourished state, both before and after MET administration, the dietary SAA retained in the body were largely in the protein pool and there was no extra accumulation of non-protein S. This would be consistent with reports that excess SAA does not increase liver GSH concentration above the level when a diet adequate in protein is fed (Boebel and Baker 1983; Cho et al, 1984; Bauman et al, 1988a; Hum et al, 1992).

The growth rate of the AP piglets was 79.4 $g.kg^{-1}.d^{-1}$, similar to that reported for sow-fed piglet (Wykes et al, 1993). We compared the body weight gain with N balance data on the assumption that 1 g N is equivalent to 6.25 g protein and 33 g lean tissue (Lukaski et al, 1981; Forbes, 1999). Using this calculation, 79% of the body weight gain was lean tissue and 14.9% was protein, similar to the 14.5% protein gain reported in parenterally fed piglets (Wykes et al, 1993). In the LP piglets only 36% of body weight gain was lean tissue, so that 6.9% of the weight gain was protein. Because newborn piglets and newborn human infants have similar protein and lean tissue body composition (Shulman, 1993), this result suggests that the growing piglet is potentially a very sensitive model to study the effects of different parenteral and enteral feeding regimens on body

composition, can provide a useful comparison for clinical human infant body composition studies.

Prior to the MET infusion, the basal N and S balances in the LP group were 58% and 50% less positive than in the AP group. Their N and SAA retention rates were 8% and 16% higher, indicating greater efficiency of utilization of dietary amino acids, and their N/S balance ratio of 30 was significantly lower. The reason for the constantly lower N/S balance ratio in piglets fed a protein-deficient diet is not entirely clear. We suggest that while the protein-deficient piglets gained muscle protein at a markedly reduced rate, their gain of splanchnic tissue rich in GSH was less compromised. Such an occurrence would reduce the N/S balance ratio as observed. This possibility is supported by the observation that following MET administration the 48h N/S balance ratio of the LP piglets decreased even further from 30 to 26. Protein-deficient diets are known to decrease hepatic GSH by 20-50% in rats (Deneke et al, 1983; Rodriguez et al, 1987; Bauman et al, 1988a; Hum et al, 1992), and to decrease intestinal mucosa GSH by more than 50% in growing piglets (Jahoor et al, 1995). The further decrease of N/S balance ratio after MET administration suggests in this protein-depleted state, the piglets retained a part of the administrated MET to increase their tissue GSH pool level. Tissue GSH concentrations were not measured in this study, but the reduction in the N/S balance ratio we observed is consistent with reports in rats that tissue GSH concentrations increase when MET or cysteine are provided to protein depleted animals (Bauman et al, 1988a; Bauman et al, 1988b; Hunter and Grimble, 1997). Future studies which include tissue GSH concentration will be carried out to test these predictions.

In human neonates and adults ester sulfate accounts for 17% and 6-10% of urinary total S (Lundquist et al, 1980; Finnstrom et al, 1983; Martensson, 1982; Martensson and Hermansson, 1984), whereas it accounted for only 1.5~3% of urinary total S excretion in the piglets in this study, and it did not increase after methionine administration. Ester sulfate (also termed "organic sulfate") refers to sulfate esters with phenolic compounds. Sulfated compounds such as tyrosine sulfate and other sulfate metabolites occur in small concentrations in normal tissues (Bodanszky et al, 1977; Bauch et al, 1988). The predominant source of urinary sulfate esters is unknown, but has been attributed to the excretion of steroid hormone metabolites (Finnstrom et al, 1983) or sulfate conjugation of aromatic amino acids in the intestinal tract (Lundquist et al 1980). Because urinary ester sulfate was constant and accounted only a very small part of total S excretion in piglets, it appears to be unimportant in SAA catabolism in these animals.

Taurine is normally added to elemental diets as a semi-indispensable amino acid for the piglet (Bertolo et al, 1999b). However we observed in this study that urinary taurine excretion was 125% of dietary taurine in the AP piglets. It suggests that when dietary MET and cysteine intake are adequate, newborn piglets do not require dietary taurine.

An interesting observation was that before the MET administration, plasma MET and tHcy levels in LP group were both lower than those in AP group ($p<0.05$), and 24h after MET administration, plasma MET and tHcy levels were still above their baseline level in the LP group, but in the AP group plasma MET and tHcy concentrations remained unchanged 24 h after the MET infusion. This suggests that protein-depleted piglets down-regulate the enzyme activity in the transsulfuration pathway.

In adult humans, higher protein or MET intakes either have no effect or decrease fasting tHcy (Stolzenberg-Solomon et al, 1999; Shimakawa et al 1997; Mann et al, 1999; Ward et al, 2000). This is presumed to occur because a high amino acid intake increase the glomerular filtration rate (Brandle et al, 1996; Skov et al, 1999), and this lower plasma tHcy (Wollesen et al, 1999). In this study, the reduced protein intake did decrease the glomerular filtration rate as shown by lower creatinine clearance than AP group ($p<0.05$), but even so, unlike the adult humans, the plasma tHcy was significantly reduced, and 24h following the MET load, there was an increase in plasma tHcy. It suggests that in piglets, the decrease in dietary MET intake lowers plasma tHcy concentration more than a reduction in glomerular filtration rate increases it.

In summary, we have established a model in the growing piglet for SAA metabolism research. We validated the method of using urinary S excretion as an accurate indicator of whole body SAA catabolism. Using this method we measured SAA retention following a MET administration, and we showed that in the protein-depleted state, a significant fraction of a dose of intravenous MET S appears to be stored in the body in non-protein forms, presumably as GSH and taurine. We suggest that this non-tracer, non-invasive method can potentially provide valuable information about the whole body SAA metabolism and non-protein S storage in animals and humans.

TABLE 1 Comparison of the amino acid intake of AP, LP elemental diet with NRC requirement of piglets

	Elemental Diet & NRC (g. $kg^{-1}.d^{-1}$)			Elemental Diet & NRC (mmol. $kg^{-1}.d^{-1}$)		
Amino Acid	AP	LP	NRC*	AP	LP	NRC*
Arginine	0.92	0.19	0.38	5.31	1.09	2.16
Glycine	0.56	0.3		7.47	4.00	
Alanine	1.47	0.75		16.50	8.43	
Serine	0.87	0.45		8.25	4.29	
Glutamate	1.60	0.8		10.90	5.44	
Proline	1.26	0.64		10.98	5.57	
Methionine	**0.36**	**0.125**	**0.25**	**2.42**	**0.84**	**1.68**
Cysteine	**0.31**	**0.15**	**0.30** †	**2.57**	**1.24**	**2.48** †
Phenylalanine	0.61	0.29	0.58	3.69	1.76	3.48
Tyrosine	0.39	0.15	0.30 †	2.15	0.83	1.66 †
Threonine	0.87	0.32	0.63	7.43	2.69	5.34
Tryptophan	0.39	0.09	0.18	1.93	0.44	0.88
Histidine	0.47	0.15	0.30	3.05	0.97	1.94
Aspartate	0.99	0.5		7.43	3.76	
Isoleucine	0.79	0.27	0.53	6.05	2.02	4.01
Leucine	1.77	0.48	0.95	13.48	3.63	7.25
Valine	0.87	0.33	0.65	7.43	2.78	5.56
Lysine	1.26	0.48	0.95	8.65	3.25	6.51
Taurine	**0.07**	**0.04**		**0.55**	**0.28**	
Total	15.84	6.48	14.4-15.0	126.24	53.30	

*Based on the National Research Council requirement for 3-5 kg piglets, assuming average weight of 4 kg (NRC 1998).

† Cysteine and tyrosine requirements were calculated by subtracting methionine and phenylalanine from total sulfur and aromatic amino acid requirements, respectively.

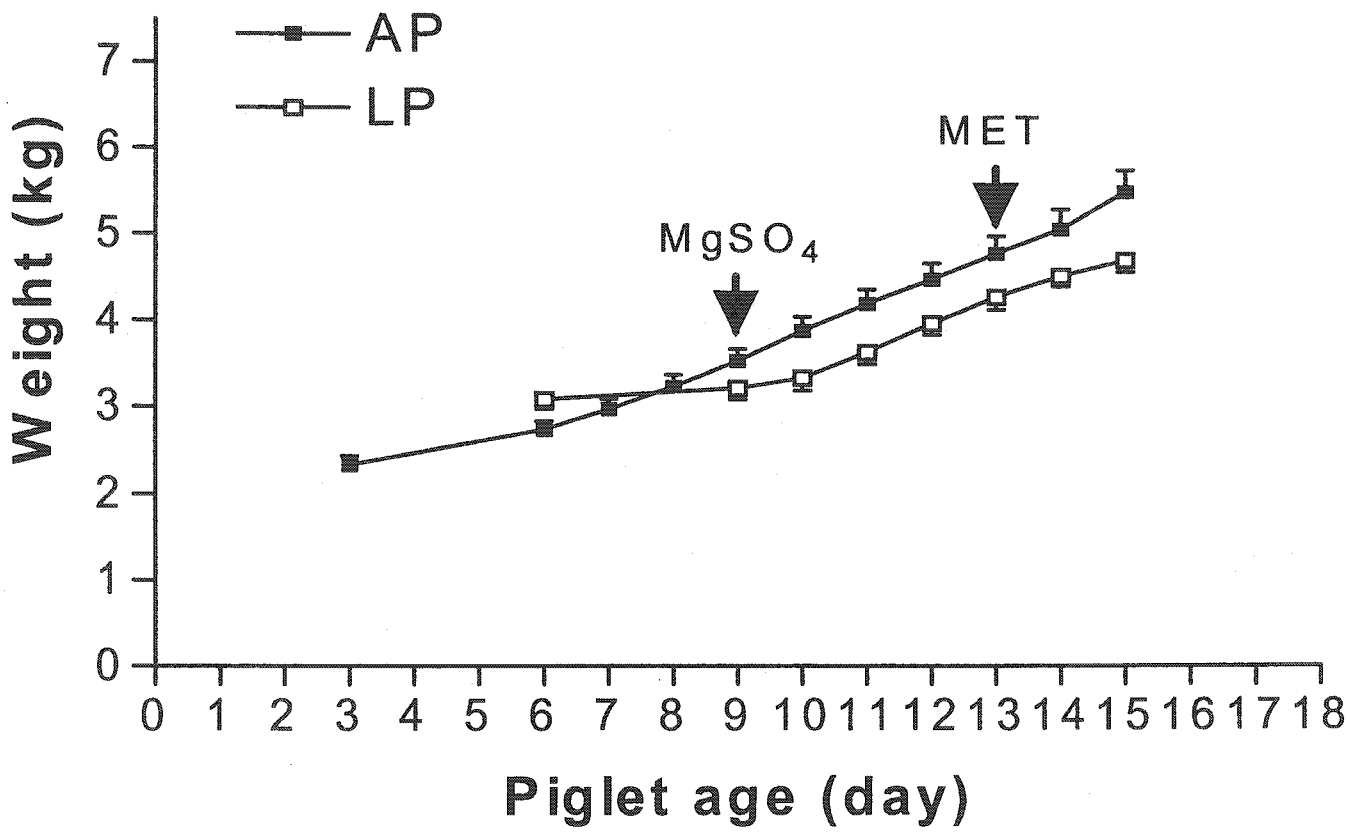

FIGURE 2. Weight (mean ± SEM) of 6 piglets fed adequate protein (AP) and 6 piglets fed low protein (LP) diet. $MgSO_4$ indicates $MgSO_4$ infusion in the AP piglets; MET indicates methionine infusion in both groups.

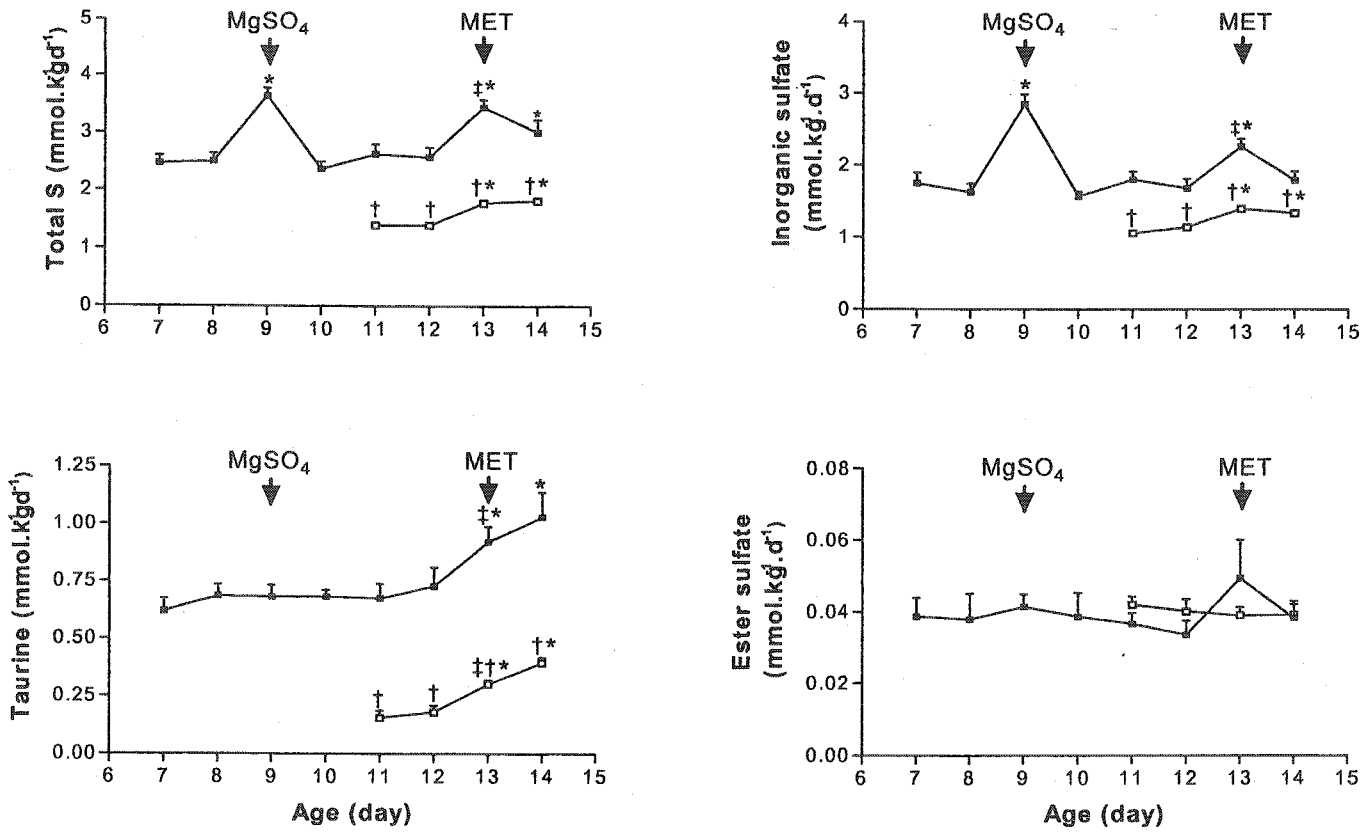

FIGURE 3. Urinary excretion of total S, inorganic sulfate, taurine, and ester sulfate in AP piglets (■) and LP piglets (□). Values are means ± SEM. * significantly different from baseline (days 7,8,11,12) ($p < 0.05$); † significantly different from AP ($p < 0.05$); ‡ significantly different from the next day following MET infusion (day 14).

TABLE 2. Urinary total S and sulfate excretion over 24 h after administration of magnesium sulfate

Sulfate infusion	1.20
Basal excretion	
Total S	2.50 ± 0.13
Sulfate	1.86 ± 0.19
Excretion following sulfate infusion	
Total S	3.65 ± 0.13
Sulfate	3.04 ± 0.17
Excretion above basal	
Total S	1.15 ± 0.04
Sulfate	1.18 ± 0.03
Recovery, (% of infused dose)	
Total S	95.85 ± 3.09 *
Sulfate	98.56 ± 2.73 *

Values are mean ± SEM (in $mmol.kg^{-1}$)

* not significantly different from 100%

TABLE 3. Urinary sulfate, taurine, and total S excretion over 24 h and 48 h after administration of methionine in piglets fed adequate protein (AP) or low protein (LP) diet

	AP		LP	
	24 h	48 h	24 h	48 h
Basal excretion				
Sulfate	1735 ± 120	3470 ± 24	1099 ± 17	2199 ± 33
Taurine	702 ± 71	1405 ± 14	167 ± 29	333 ± 58
Total S	2608 ± 153	5216 ± 306	1398 ± 41	2797 ± 82
Excretion following methionine infusion				
Sulfate	2259 ± 110	4051 ± 208	1394 ± 18	2730 ± 33
Taurine	920 ± 68	1953 ± 169	300 ± 17	696 ± 38
Total S	3450 ± 130	6462 ± 311	1782 ± 37	3609 ± 55
Excretion above basal				
Sulfate	524 ± 43	585 ± 48	295 ± 15	541 ± 35
Taurine	218 ± 46	567 ± 75	134 ± 16	373 ± 37
Total S	842 ± 88	1269 ± 32	383 ± 30	831 ± 57
Recovery (% of infused dose)				
Sulfate	43.6 ± 3.6	48.7 ± 4.0	23.9 ± 0.9*	45.4 ± 3.3†
Taurine	18.0 ± 3.8	48.8 ± 6.6†	11.1 ± 1.4	31.0 ± 3.1*†
Total S	70.1 ± 7.3	105.5 ± 2.9†	32.0 ± 2.5*	69.2 ± 4.8*†

1200 μmol.kg^{-1} methionine was infused on the first day

Values are mean ± SEM (in μmol. kg^{-1}), n = 6

Basal excretion refers to excretion over the 2 days prior to methionine infusion

* Significantly different from AP group, $p < 0.05$

† Significantly different from 24 h, $p < 0.05$

TABLE 4. Circulating metabolite concentrations and creatinine clearance before and after methionine administration in piglets fed adequate protein (AP) or low protein (LP) diet

		0 h	24 h	48 h
AP				
Plasma	Methionine ($\mu mol.L^{-1}$)	119 ± 14	112 ± 19	112 ± 13
Plasma	Taurine ($\mu mol.L^{-1}$)	137 ± 19	139 ± 22	140 ± 19
Plasma	Homocysteine ($\mu mol.L^{-1}$)	29 ± 3	37 ± 6	39 ± 8
Serum	Urea ($mmol.L^{-1}$)	2.22 ± 0.28	2.58 ± 0.41	3.08 ± 0.50 *
	Creatinine clearance ($ml.min^{-1}.kg^{-1}$)	5.7 ± 0.6	4.4 ± 0.3 *	4.2 ± 0.4 *
LP				
Plasma	Methionine ($\mu mol.L^{-1}$)	61 ± 7 †	251 ± 36 *	84 ± 6
Plasma	Taurine ($\mu mol.L^{-1}$)	47 ± 3 †	63 ± 3 *	68 ± 7 *
Plasma	Homocysteine ($\mu mol.L^{-1}$)	9 ± 1 †	23 ± 5 *	12 ± 1
Serum	Urea ($mmol.L^{-1}$)	0.30 ± 0.04 †	0.35 ± 0.07 †	0.50 ± 0.16 †
	Creatinine clearance ($ml.min^{-1}.kg^{-1}$)	3.6 ± 0.2 †	3.5 ± 0.2	4.4 ± 0.2

Value are mean ± SEM.

† Significantly different from AP group ($p < 0.05$)

* Significantly different from 0 h ($p < 0.05$)

TABLE 5. S and N balance and N/S molar balance ratio 48 h before and after methionine infusion in piglets fed adequate protein (AP) or low protein (LP) diet

Group	AP		LP	
Study Period	Prior-MET	After-MET	Prior-MET	After-MET
N metabolism (mmol/kg)				
N intake	315.2 ± 1.0	317.5 ± 0.6	121.3 ± 0.3	121.2 ± 0.7
N output	44.2 ± 4.7	53.5 ± 6.1	8.7 ± 0.8	12.3 ± 1.0
N balance	271.0 ± 5.0	264.4 ± 6.0 *	112.7 ± 1.0 †	108.9 ± 1.2 †*
N retention (%)	86.0 ± 1.5	83.2 ± 1.9 *	92.9 ± 0.6 †	89.9 ± 0.8 †*
S metabolism (mmol/kg)				
S intake	12.87 ± 0.06	14.13 ± 0.03	6.60 ± 0.02	7.73 ± 0.05
SAA intake	10.96 ± 0.05	12.22 ± 0.02	4.69 ± 0.01	5.82 ± 0.05
S output	5.22 ± 0.31	6.46 ± 0.31	2.80 ± 0.08	3.61 ± 0.06
S balance	7.65 ± 0.28	7.67 ± 0.32	3.81 ± 0.09 †	4.12 ± 0.06 †*
SAA retention (%)	69.8 ± 2.7	62.7 ± 2.6 *	81.1 ± 1.8 †	70.8 ± 0.8 †*
N/S balance ratio	35.6 ± 1.1	34.7 ± 1.3	29.7 ± 0.5 †	26.4 ± 0.1 †*

Values are mean ± SEM, n = 6

* Significantly different from Prior-MET, $p<0.05$

† Significantly different from AP group, $p < 0.05$

6. GENERAL CONCLUSION

6.1 Summary of Results

A model was established in growing piglets to study SAA metabolism. We validated the method of using urinary total S excretion as an indicator of whole body SAA catabolism. We also validated the method of using the whole body N/S balance ratio as an indicator of changes in whole body non-protein S storage. Using this non-tracer, non-invasive method we quantitatively measured SAA retention following MET administration, and showed that in a protein depleted state, relatively more S than N is retained, indicating that a significant portion of the S derived from administrated MET is retained in non-protein compounds, presumably GSH and taurine.

6.2 Significance

This model can be used to investigate SAA metabolism in growing piglets under different nutritional conditions and can serve as a basis for future clinical studies in humans. Using this non-invasive method of measuring urinary S recovery and whole body N/S balance ratio following MET administration, we demonstrated that a significant portion of the S derived from MET is retained in non-protein pools, presumably GSH and taurine. This finding is consistent with more invasive measurements of tissue GSH concentration. Potentially this non-invasive approach may provide useful information on whole body GSH status in clinical studies of human infants.

6.3 Future Research

Tissue GSH concentrations were not measured in this study, but the reduction in the N/S balance ratio after MET administration in protein depleted piglets is consistent with reports in rats that tissue GSH concentrations increase when MET or cysteine are provided to protein depleted animals (Bauman et al, 1988a; Bauman et al, 1988b; Hunter and Grimble, 1997). Future studies which include measurement of tissue GSH concentration will be carried out to test these predictions.

In this study, we showed that by using urinary S excretion we can measure the catabolism of MET. As an essential amino acid, the catabolism of MET can potentially serve as an indicator of whole body amino acid catabolism. It will be interesting to use this simple and non-invasive method in studies of amino acid requirements for human infants and children.

Appendix A

Pilot Study Results

Several pilot studies were conducted to develop the final protocol for this piglet model. A discussion of these results would be helpful to understand the rationale for the study design in this thesis, and as a guide for other investigators who want to use this model.

1. Why was an elemental diet used in this study?

Initially I fed piglets continuously by stomach catheter with a commercial milk replacer formula ("Wet-Nurser", Jefo Nutrition Inc, St-Hyacinthe, QC). The target intake was protein 16g.kg^{-1}.d^{-1}, energy 260 kcal.kg^{-1}.d^{-1} (1.1 MJ. kg^{-1}.d^{-1}). Following a 2-h baseline urine collection, an intravenous dose of MET (1.3 mmol.kg^{-1}) was administrated over 2 h, and urine and blood samples were collected every hour for 8 h. To my surprise the 8 h urinary inorganic sulfate recovery was 0%, and there was no increase in the serum sulfate concentration. I obtained the same results after trying different MET doses (2.0 and 2.6 mmol/kg) in both fed and fasting states in another piglet. Speculating that piglets have a limited capacity to catabolize MET in this situation, I analyzed the amino acid concentrations in their serum and urine and found that even after 10 hours of fasting, the serum MET concentration was 550 μmol/L, 10 times higher than the reference MET level in piglets (Wykes et al 1994a; Bertolo et al 2000). In the fed state prior to the MET infusion, serum MET was 2017 μmol/L, which was 40 times higher than normal. The cystine concentration was only 20 μmol/L, which was similar to the reference cystine level (Bertolo et al 2000). Two hours after a MET infusion (1.3 mmol.kg^{-1}), the serum MET concentration increased from 2017 to 3324 μmol/L with no increase in plasma cystine concentration. Three hours after a high dose of MET infusion (2.6 mmol/kg),

urinary MET concentration was 737 μmol/L, which was much higher than 82 μmol/L, the urinary MET concentration 3 hours after a low dose (2.0 mmol/kg) MET infusion in the fasting state.

These pilot study results suggested:

1) The MET content of the milk replacer formula was much higher than the requirement of piglets and even the capacity for degradation.

   We were able to ascertain that crystalline MET was an ingredient of the diet, although the company would not disclose the amounts. Because we decided to switch to an elemental diet, we did not analyze the actual MET content ourselves.

2) Piglets have a limited capacity to catabolize MET to cysteine and cystine.

3) When plasma MET is high, urinary MET excretion is increased presumably because the maximal tubular reabsorption is exceeded.

Based on these results, we made following adjustments in study design:

1) An elemental diet with crystalline amino acids was used to better control dietary SAA supply.

2) A low dose of MET (1.2 mmol/kg) rather than a high dose (2.6 mmol/kg) was used in MET load experiments to prevent MET loss in the urine.

3) The more complex measurement of total S measurement was added to that of inorganic sulfate to make sure all S containing components in urine were accounted for.

2. Improvement in the design of the elemental diet

In the next pilot study, we designed an elemental diet mainly based on the total parenteral nutrition regimen previously designed by Wykes (Wykes et al, 1993) and the elemental diet was administrated following the method described by Bertolo et al (1999). Several problems had to be resolved:

1) The piglets developed diarrhea when we decreased the dilution to 1.5-fold and gave a full strength feeding (480 ml/kg/d) three days after surgery as described by Bertolo (1999).
2) Diarrhea persisted when we increased the final dilution to 1.75.
3) When the dilution was increased to 2-fold (584 ml/kg/d), diarrhea was avoided, but after 5 days some piglets showed symptoms characteristic of fluid overload. They breathed heavily and had edema present, and a fatal end-point was reached in one piglet.

These pilot study results suggested that this elemental diet had a high osmolarity that caused diarrhea if not diluted, but that excessive dilution would cause fluid over load.

Based on these results, we made the following adjustments to the elemental diet:

1) Glucose polymer (Polycose) rather than glucose was used as the carbohydrate energy source in the diet to reduce osmolarity.
2) A dilution schedule was developed to prevent diarrhea and fluid overload.

The diet infusion began approximately 3 hours post-surgery at sub-maximal rates and gradually increased during day 0 and day1. The elemental diet solution was diluted

4-fold on the surgery day (day zero), then 3-fold for 12 h and 2.5-fold for 12h on day 1, 2-fold for 12 h, 1.75-fold for 12 h on day 2 and kept at this dilution for day 3. It was diluted 1.5-fold for day 4, 5, and 6, and from day 7 to the end of the study was 1.2-fold diluted. A full rate of diet provision was reached from the beginning of day 3 in AP piglets, and day 4 in LP piglets.

3 . Design of the magnesium sulfate infusion study and MET infusion study

1) Using this improved elemental diet, 90% of the intravenous dose of magnesium sulfate was recovered in urine as inorganic sulfate over 8 hours. Therefore we established a 24 h urinary collection following the magnesium sulfate infusion for recovery of the entire infused sulfate.
2) We continuously infused MET supplemented (60 $\mu mol.kg^{-1}.h^{-1}$, iv) in the fed state and collected urine every hour. After 20 h, 59% of the infused S was recovered as inorganic sulfate and 73% was recovered as total S in urine. These results showed that piglets needed more than 20 hours to catabolize the infused MET, and that in addition to inorganic sulfate, MET was catabolized to other forms of S containing compounds, possibly taurine and ester sulfate.

Based on these pilot results, we made the following adjustment to the final design of MET infusion study:

1) After the MET infusion, urine was collected 48h.
2) In order to make the baseline urine collection of endogenous excretion more comparable to the period following MET infusion, the baseline was divided into two 24 h periods to demonstrate constant basal excretion rate. This would justify

subtracting basal excretion from total to calculate S recovery following MET infusion.

3) Because piglets grow very fast (8% increase in body weight per day), dietary intake had to be adjusted every morning according to net body weight even after MET administration to make sure daily basal S excretion per kg body weight was constant..

4) Urinary taurine and ester sulfate were analyzed in the MET infusion study.

Appendix B

Additional Tables

TABLE 6. Composition of adequate protein (AP) elemental diet

(Except for 30% Intralipid)

Amino Acid	$g.L^{-1}$	Nutrient/Product	$g.L^{-1}$
Arginine [1]	3.36	Polycose [4]	94.39
Glycine [1]	1.45	K_2HPO_4 [2]	1.57
Alanine [1]	5.34	KH_2PO_4 [2]	1.08
Serine [1]	3.15	K acetate [2]	1.46
Glutamate [1]	5.83	NaCl [2]	2.15
Proline [1]	4.59	Calcium Gluconate [2]	6.36
Methionine [1]	1.31		
Cysteine [1]	1.13	$MgSO_4.7H_2O$ [2]	0.78
Phenylalanine [1]	2.21	$ZnSO_4.H_2O$ [2]	0.09
Glycyl-Tyrosine [3]	1.86	$MnSO_4$ [2] solution (1.65g in 100 ml)	0.30 ml
Threonine [1]	3.16		
Tryptophan [1]	1.43	* Iron Dextran [5] (50 mg/ml diluted to 10 mg/ml)	1.00 ml
Histidine [1]	1.72	* Micro + 6 Mineral Mixture [6]	5.00 ml
Aspartate [1]	3.59		
Isoleucine [1]	2.88	* Multi 12 Pediatric Vitamin Mixture [6]	5.00 ml
Leucine [1]	6.42		
Valine [1]	3.16		
Lysine-HCl [1]	5.75		
Taurine [2]	0.25		
Total	**58.59**		

[1] Ajinimoto U.S.A; [2] SIGMA St Louis MO; [3] Chem-Impix International, Wood Dale, IL; [4] Ross Laboratories, Columbus, Ohio [5] Luipold Pharmaceuticals, Shirley, NY; [6] Sabex, Boucherville, QC; * add just before feeding.

TABLE 7. Composition of low protein (LP) elemental diets

(Except for 30% Intralipid)

Amino Acid	$g.L^{-1}$	Nutrient/Product	$g.L^{-1}$
Arginine [1]	0.69	Polycose [4]	131.34
Glycine [1]	0.86	K_2HPO_4 [2]	1.57
Alanine [1]	2.73	KH_2PO_4 [2]	1.08
Serine [1]	1.64	K acetate [2]	1.46
Glutamate [1]	2.91	NaCl [2]	2.15
Proline [1]	2.33	Calcium Gluconate [2]	6.36
Methionine [1]	0.46		
Cysteine [1]	0.55	$MgSO_4.7H_2O$ [2]	0.78
Phenylalanine [1]	1.06	$ZnSO_4.H_2O$ [2]	0.09
Glycyl-Tyrosine [3]	0.72	$MnSO_4$ [2] solution (1.65g in 100 ml)	0.30 ml
Threonine [1]	1.15		
Tryptophan [1]	0.33	* Iron Dextran [5] (50 mg/ml diluted to 10 mg/ml)	1.00 ml
Histidine [1]	0.55	* Micro + 6 Mineral Mixture [6]	5.00 ml
Aspartate [1]	1.82		
Isoleucine [1]	0.96	* Multi 12 Pediatric Vitamin Mixture [6]	5.00 ml
Leucine [1]	1.73		
Valine [1]	1.18		
Lysine-HCl [1]	1.73		
Taurine [2]	0.13		
Total	**23.49**		

[1] Ajinimoto U.S.A; [2] SIGMA St Louis MO; [3] Chem-Impix International, Wood Dale, IL; [4] Ross Laboratories, Columbus, Ohio [5] Luipold Pharmaceuticals, Shirley, NY; [6] Sabex, Boucherville, QC; * add just before feeding

TABLE 8. N and S composition of adequate protein (AP) elemental diet

Amino Acid	$g.L^{-1}$	$(mmol.L^{-1})$	N $(mmol.L^{-1})$	S $(mmol.L^{-1})$	$g.kg^{-1}.d^{-1}$	$mmol.kg^{-1}.d^{-1}$	N $(mmol.kg^{-1}.d^{-1})$	S $(mmol.kg^{-1}.d^{-1})$
Arginine	3.36	19.31	77.24		0.92	5.31	21.24	
Glycine	1.45	19.33	19.33		0.40	5.32	5.32	
Alanine	5.34	60.00	60.00		1.47	16.50	16.50	
Serine	3.15	30.00	30.00		0.87	8.25	8.25	
Glutamate	5.83	39.66	39.66		1.60	10.91	10.91	
Proline	4.59	39.91	39.91		1.26	10.98	10.98	
Methionine	1.31	8.79	8.79	8.79	0.36	2.42	2.42	2.42
Cysteine	1.13	9.32	9.32	9.32	0.31	2.56	2.56	2.56
Phenylalanine	2.21	13.39	13.39		0.61	3.68	3.68	
Glycyl-Tyrosine	1.86	7.82	15.63		0.51	2.15	4.30	
Threonine	3.16	27.01	27.01		0.87	7.43	7.43	
Tryptophan	1.43	7.01	14.02		0.39	1.93	3.86	
Histidine	1.72	11.10	33.29		0.47	3.05	9.15	
Aspartate	3.59	26.99	26.99		0.99	7.42	7.42	
Isoleucine	2.88	21.98	21.98		0.79	6.05	6.05	
Leucine	6.42	49.01	49.01		1.77	13.48	13.48	
Valine	3.16	27.01	27.01		0.87	7.43	7.43	
Lysine-HCl	5.75	31.42	62.84		1.58	8.64	17.28	
Taurine	0.25	2.00	2.00	2.00	0.07	0.55	0.55	0.55
$MgSO_4.7H_2O$	*0.78*	*3.17*	*0.00*	*3.17*	*0.21*	*0.87*	*0.00*	*0.87*
Total			**577.43**	**23.28**			**158.79**	**6.40**

TABLE 9. N and S composition of low protein (LP) elemental diet

Amino Acid	$g.L^{-1}$	$(mmol.L^{-1})$	N $(mmol.L^{-1})$	S $(mmol.L^{-1})$	$g.kg^{-1}.d^{-1}$	$mmol.kg^{-1}.d^{-1}$	N $(mmol.kg^{-1}.d^{-1})$	S $(mmol.kg^{-1}.d^{-1})$
Arginine	0.69	3.97	15.89		0.19	1.09	4.37	
Glycine	0.86	11.52	11.52		0.24	3.17	3.17	
Alanine	2.73	30.64	30.64		0.75	8.43	8.43	
Serine	1.64	15.58	15.58		0.45	4.28	4.28	
Glutamate	2.91	19.79	19.79		0.80	5.44	5.44	
Proline	2.33	20.23	20.23		0.64	5.56	5.56	
Methionine	0.46	3.05	3.05	3.05	0.13	0.84	0.84	0.84
Cysteine	0.55	4.50	4.50	4.50	0.15	1.24	1.24	1.24
Phenylalanine	1.06	6.39	6.39		0.29	1.76	1.76	
Glycyl-Tyrosine	0.72	3.01	6.02		0.20	0.83	1.66	
Threonine	1.15	9.79	9.79		0.31	2.69	2.69	
Tryptophan	0.33	1.60	3.21		0.09	0.44	0.88	
Histidine	0.55	3.52	10.55		0.15	0.97	2.90	
Aspartate	1.82	13.67	13.67		0.50	3.76	3.76	
Isoleucine	0.96	7.36	7.36		0.27	2.02	2.02	
Leucine	1.73	13.18	13.18		0.47	3.63	3.63	
Valine	1.18	10.10	10.10		0.33	2.78	2.78	
Lysine-HCl	1.73	9.44	18.87		0.47	2.60	5.19	
Taurine	0.13	1.02	1.02	1.02	0.03	0.28	0.28	0.28
$MgSO_4.7H_2O$	*0.78*	*3.17*	*0.00*	*3.17*	*0.21*	*0.87*	*0.00*	*0.87*
Total			**221.37**	**11.74**			**60.88**	**3.23**

TABLE 10. Daily S and N balance and N/S molar balance ratio before and after methionine infusion

Group	Adequate-Protein			Low-Protein		
Study Period	Prior-MET	Post-MET		Prior-MET	Post-MET	
	Mean-24h	1st 24h	2nd 24h	Mean-24h	1st 24h	2nd 24h
N metabolism(mmol.kg $^{-1}$)						
N intake	157.6 ± 0.8	159.3 ± 0.6	158.2 ± 0.2	60.7 ± 0.1	60.4 ± 0.8	60.8 ± 0.1
N output	22.1 ± 1.7	24.9 ± 2.4	28.6 ± 3.9	4.3 ± 0.3	6.3 ± 0.4	6.0 ± 0.5
N balance	135.5 ± 1.9	134.4 ± 2.2	129.6 ± 3.9*	56.3 ± 0.3 †	54.1 ± 1.2 †*	54.8 ± 0.5 †*
N retention (%)	86.0 ± 1.1	84.4 ± 1.5 *	81.9 ± 2.5 *	92.9 ± 0.5 †	92.9 ± 0.5 †	90.2 ± 0.8 †*
S metabolism (mmol.kg^{-1})						
S intake	6.43 ± 0.04	7.66 ± 0.02	6.47 ± 0.01	3.30 ± 0.01	4.42 ± 0.05	3.31 ± 0.01
SAA intake	5.48 ± 0.03	6.71 ± 0.02	5.52 ± 0.01	2.35 ± 0.01	3.49 ± 0.03	2.35 ± 0.004
S output	2.61 ± 0.11	3.45 ± 0.13	3.01 ± 0.22	1.40 ± 0.03	1.78 ± 0.04	1.83 ± 0.02
S balance	3.82 ± 0.11	4.21 ± 0.15*	3.46 ± 0.22*	1.90 ± 0.03 †	2.64 ± 0.05 †*	1.48 ± 0.02 †*
SAA retention (%)	69.7 ± 2.2	62.7 ± 2.2*	62.7 ± 4.5*	81.1 ± 1.4 †	75.6 ± 1.4 †	63.0 ± 0.9*
N/S balance ratio	35.7 ± 0.9	32.1 ± 1.1*	38.1 ± 1.9*	29.7 ± 0.4 †	20.5 ± 0.2 †*	37.0 ± 0.2*

Values are mean ± SEM, n = 6

*Significantly different from Prior-MET, $p<0.05$

† Significantly different from AP group, $p < 0.05$

Appendix C

Additional Figures

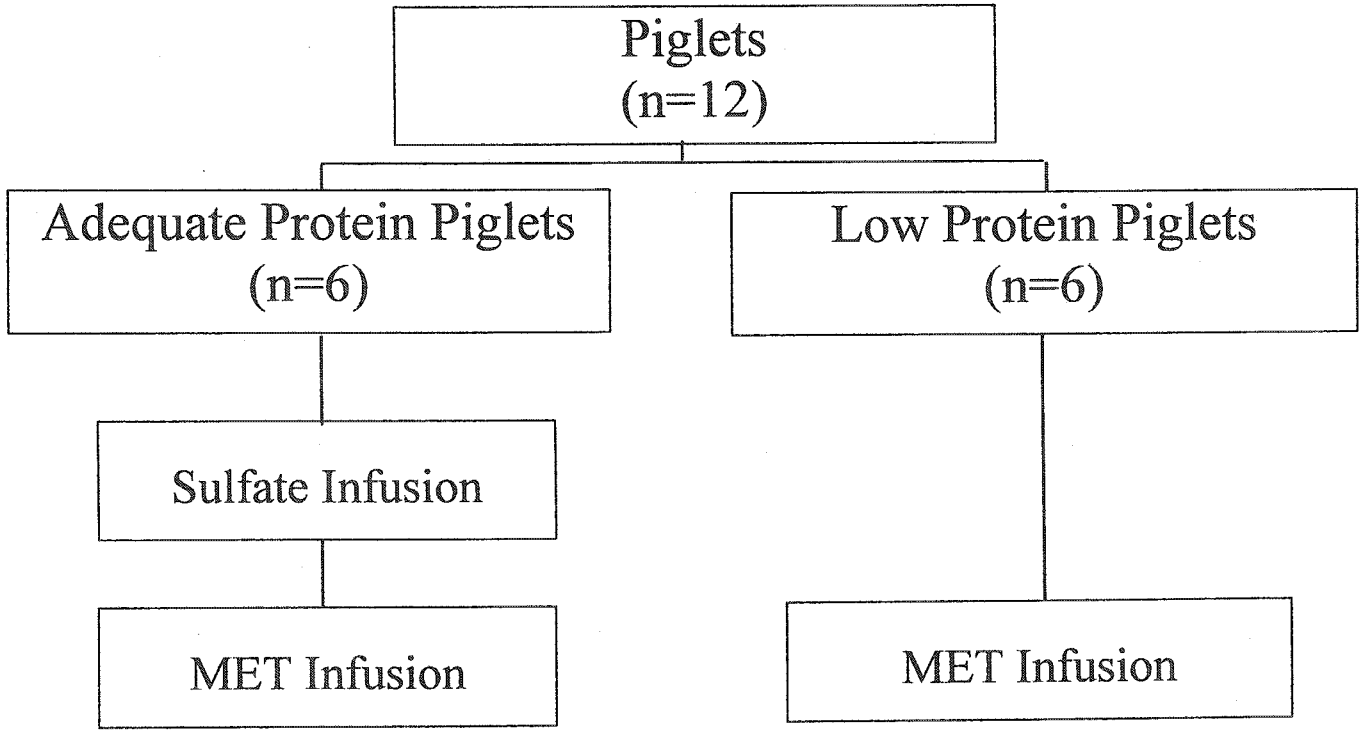

FIGURE 4. Study design

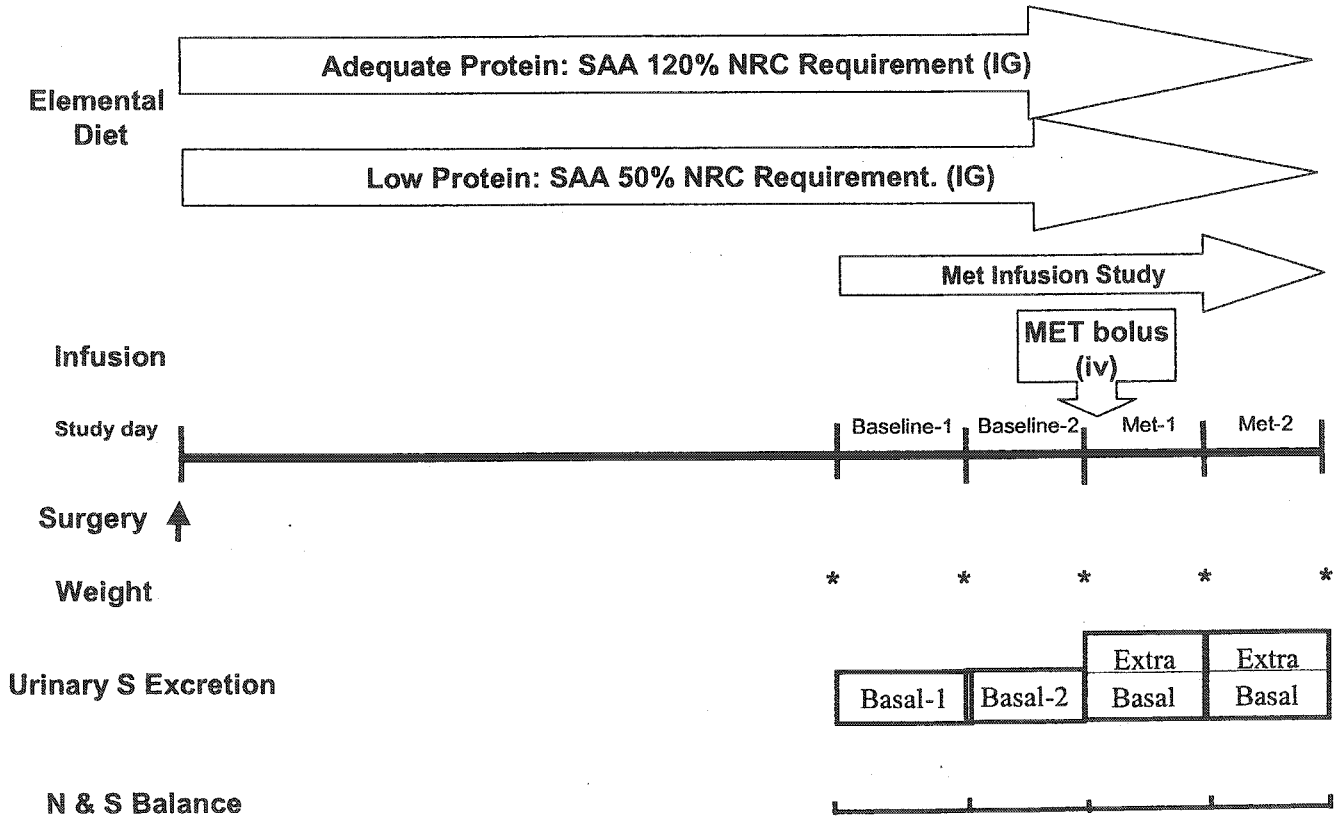

FIGURE 5. Experimental protocol of methionine infusion study in adequate protein and low protein piglets. In both groups, methionine infusion was performed when piglets were 13 days of age. In adequate protein group, sulfate infusion was performed 4 days prior to the methionine infusion.

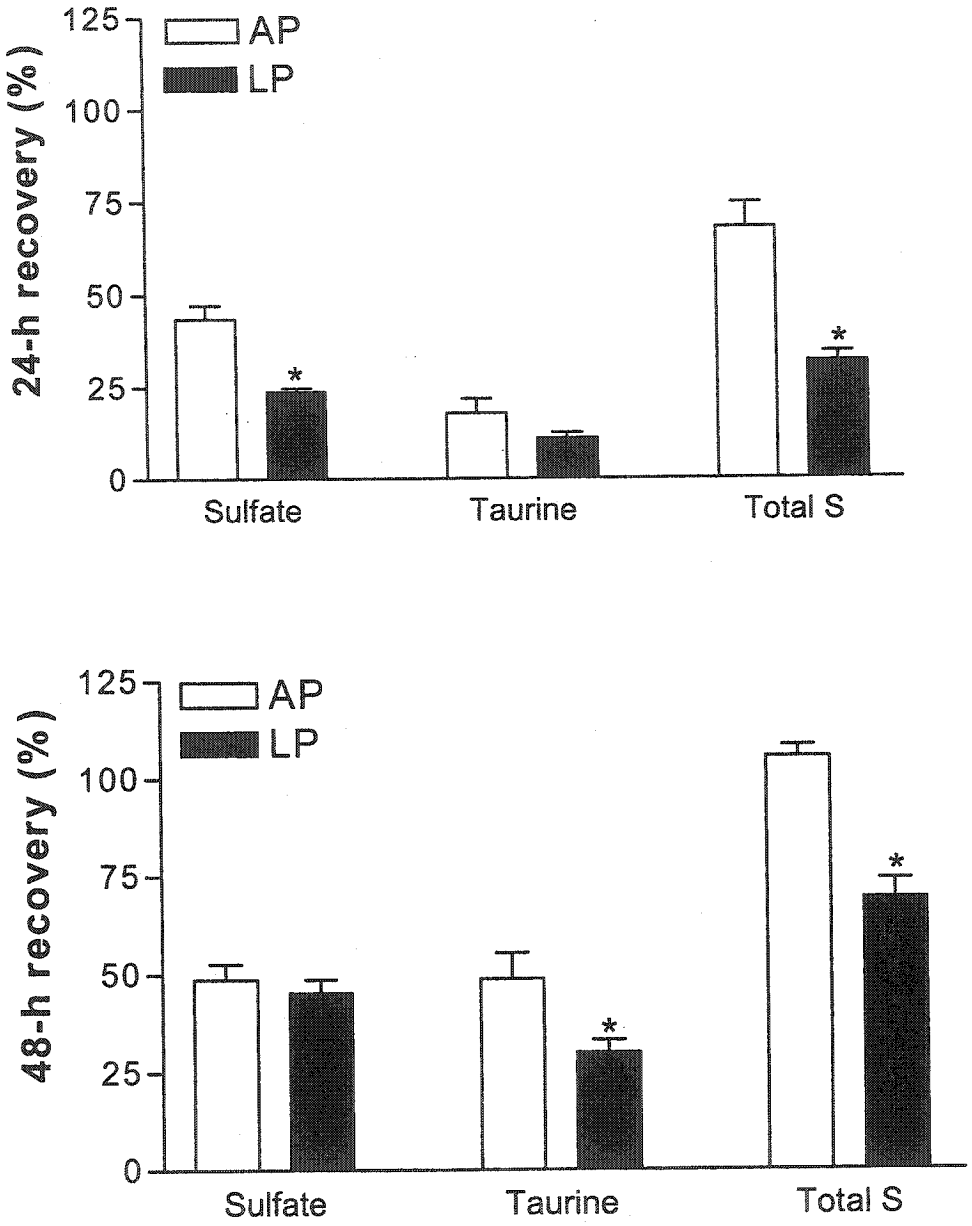

FIGURE 6. Urinary S recoveries 24 and 48 h after methionine infusion in adequate protein piglets (AP) and low protein piglets (LP). * Significantly different from AP group ($p<0.05$)

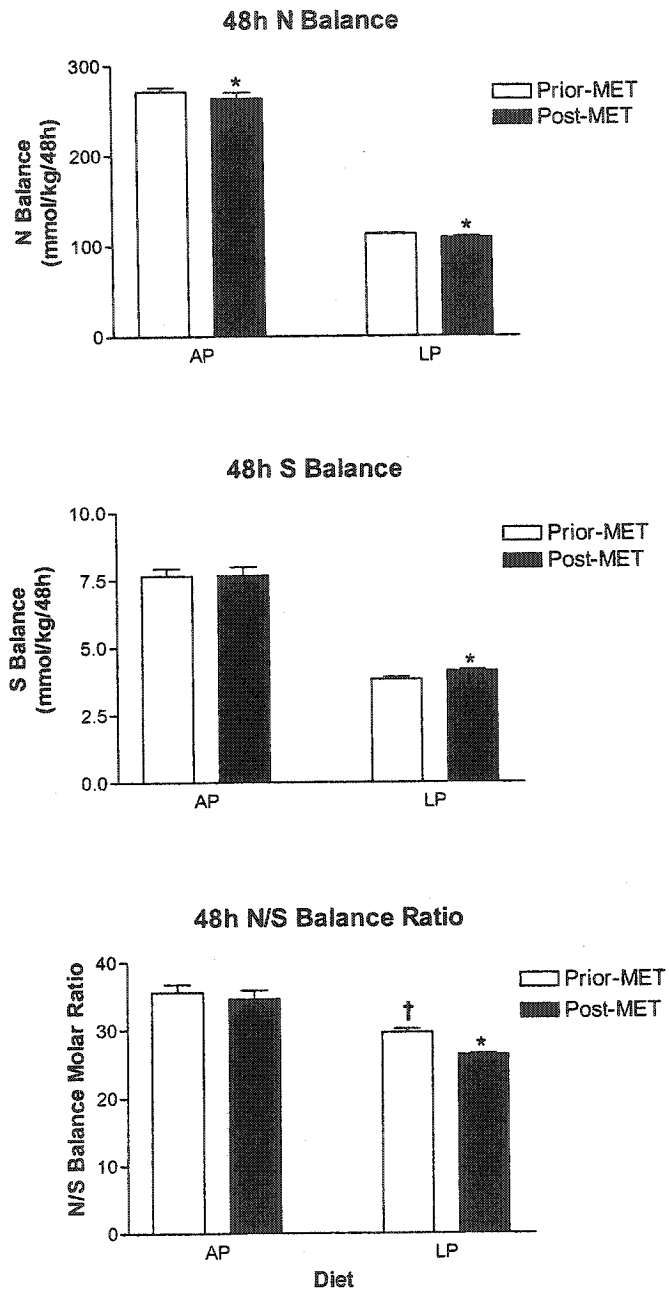

FIGURE 7. N, S balance and N/S balance ratio 48 h before and after methionine infusion in adequate protein piglets (AP) and low protein piglets (LP).

*** Significantly different from Prior-MET; † significantly different from AP.**

Appendix D

Animal Use Protocol

closed. Aug 22

McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at www.mcgill.ca/fgsr/rgo/animal/

Protocol #: 4053
Investigator #: 865
Approval End Date: Dec 31, 2002
Facility Committee: AGR

☐ Pilot ☐ New Application ☒ Renewal of Protocol # 4053

Title (must match the title of the funding source application): Sulfur Amino Acid Metabolism in Man (Piglet Model Studies)

1. Investigator Data:

Principal Investigator: Drs. Linda Wykes, John Hoffer **Office #**

Department: Dietetics and Human Nutrition/ / Lady Davis Research Institute, **Fax#**

Address: Macdonald Campus of McGill Univ., 21,111 Lakeshore Road, Ste. Anne de Bellevue **Email:**

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Dr. Linda Wykes **Work #:** **Emergency #:**

Name: Dr. Chunsheng Hou **Work #:** **Emergency #:**

ACTION	✓	DATE
P.I.	✓	Dec 18'01
FACC	✓	"
RGO	✓	"
VET	✓	"
DB	✓	"
Completed		

3. Funding Source:

External ☒
Source (s): CIHR
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: Sept 1999-Sept 2003

Internal ☐
Source (s):
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period:

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y): or ongoing ☒

Expected Date of Completion of Animal Use (d/m/y): or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator: **Date:** 26/11/01

Approval Signatures:

Chair, Facility Animal Care Committee:		**Date:** Dec 19/01.
University Veterinarian:		**Date:** Dec 19/2001
Chair, Ethics Subcommittee(as per UACC policy):		**Date:** 2/14/02
Approved Period for Animal Use	**Beginning:** Jan. 1, 2002	**Ending:** Dec 31, 2002

☐ This protocol has been approved with the modifications noted in Section 13.

DEC 19 2001

4. Research Personnel and Qualifications: List the names of all individuals who will be in contact with animals in this study (including the Principal Investigator) and their employment classification (investigator, technician, research assistant, undergraduate/graduate student, fellow). Indicate any training received (e.g workshops, lectures, etc.). The PI certifies that all personnel listed here have suitable training and/or experience, or will be provided with the specific training which qualifies them to perform the procedures described in the protocol. Each person listed in this section must sign to indicate that s/he has read this protocol. (Space will expand as needed.)

Name	Classification	Training Information	Signature
Linda Wykes PhD	Investigator	12 years experience with piglet model	
John Hoffer MD	Investigator	Physician	
Chunsheng Hou MD	Graduate student	Surgeon, 3 years animal research and teaching experience in China, 1 year experience with piglet model.	
Mark Warren	Graduate student	3 years experience with piglet model	
Deborah Martin	Technician	12 years experience former ARC and vet practice emergency technician.	
Winnie Cheung	Undergraduate Student	In training	

* Enter the first name, press 'enter', then the 2nd name... complete the first column, then the 2nd, then the 3rd
** If an undergraduate student is involved , the role of the student and the supervision received must be described.

5. Summary (In language that will be understood by members of the general public)

a) RATIONALE: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge.

More information is needed on protein and amino acid requirement of infants and children as well as their metabolize responses to inadequate protein and amino acid intake. However ethical constraints preclude putting children on nutritionally inadequate diets as well as invasive sampling. We aim to use young growing piglet as a model to study the relationship between two components of protein (nitrogen and sulphur) and to develop a non-invasive protocol that can be adapted for clinical studies. We wish to determine the relationship between sulphur balance and nitrogen balance in the fed state with normal or low sulphur amino acid (SAA) intake, and use N/S balance to predict changes in tissue protein gain and nonprotein sulphur store using piglet as a model. Metabolism of the sulphur amino acids is important for 2 reasons. First, most of the body's sulfur (S) is in the form of protein-bound S-amino acids. Since almost all the S is rapidly turning over so we hypothesize that net gain or loss of S from the body is a valid measure of the gain or loss of body proteins. Under usual conditions almost all S lost from the body is excreted in the urine as sulphur mainly as the inorganic sulfate anion, we are currently investigating the usefulness of inorganic, total (inorganic + organic) sulphate, and total sulphur excretion to determine how rapid tissue gain or loss of S-amino acids is influenced by nutrient intake and metabolic stimuli. Secondly, one of the body's defenses against oxidant or reactive intermediate-induced tissue damage is glutathione (GSH), a tripeptide consisting of the amino acids glycine, glutamic acid, and the sulfhydryl amino acid, cysteine. We hypothesize that an important component of the fraction of the gain or loss of body S can be attributed to the net GSH synthesis or catabolism. We propose to validate the use of N/S balance ratio and stable isotope measurement of GSH synthesis to pridict nonprotein sulphur store to estimate whole body GSH stores of humans or animals without resort to tissue biopsy.

b) SPECIFIC OBJECTIVES OF THE STUDY: Summarize in point form the primary objectives of this study.

1) To develop a noninvasive N/S balance model in the fed state with normal sulphur amino acid intake by testing response to supplemental sulphate or methionine.
2) To determine N/S balance and GSH synthesis in the fed state during normal and low sulphur amino acid intake.

c) PROGRESS REPORT: If this is a renewal of an ongoing project, BRIEFLY summarize what was accomplished during the prior approval period and indicate if and how the current goals differ from those in the original application.

1) In pilot study, commercial milk replacer diet was found to be unsuitable for SAA studies because of its very high methionine content. The new feeding regimen was developed to ensure adequate nutrient intake with low fluid intake (to minimize risk of fluid overload) and low osmolarity (to minimize risk of diarrhea).
2) Sampling protocols and analytical methods were developed to measure sulphur recovery from a sulphate or methionine supplement.
3) Study1 is well under way with 2 animals completed, 4 more scheduled.
4) In our new design we will use N/S balance ratio and stable measurement of GSH synthesis, a noninvasive method to learn nonprotein sulphur store other than use radio isotope 35 S, a invasive method.

d) SUMMARY OF PROCEDURES FOR ANIMAL USE REPORT TO THE CCAC: Using KEY WORDS ONLY, list the procedures used (e.g. anaesthesia, breeding colony, injection IP, gavage, drug administration, major survival surgery, euthanasia by exsanguination, behavioural studies). Refer to Appendix 1of the Guidelines for a more complete list of suggested key words.

Anaesthesia, major surgery, survival surgery, injection, intramuscular injections, special diet, cannulation, iv blood sampling, drug administration, enthanasia.

6. Animals To Be Used

a) Purpose of Animal Use (Check one):

1. ☐ **Studies of a fundamental nature/basic research**
2. ☒ **Studies for medical purposes relating to human/animal diseases/disorders**
3. ☐ **Regulatory testing**
4. ☐ **Development of products/appliances for human/veterinary medicine**

b) Will the project involve breeding animals? NO☒ YES☐
Will the project involve the generation of genetically altered animals? NO☒ YES☐
Will field studies be conducted? NO☒ YES☐

c) Description of Animals

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	pig					
Supplier/Source	Macdonald farm					
Strain						
Sex	M/F					
Age/Wt	2-4 day/ 1.5-2.5kg					
# To be purchased	16					
# Produced by in-house breeding						
# Other (e.g.field studies)						
#needed at one time	4					
# per cage	1					
TOTAL# /YEAR	16					

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals.

. Justification of Animal Usage

a) Please justify the number of animals requested for each species described in the table 6c above, BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT. Include information on experimental and control groups, # per group, and failure rates. Also justify in terms of statistical requirements, product yield, etc. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. The numbers of animals are for one year only, not the length of funding. Use the table below when applicable (space will expand as needed).

Two studies of 8 piglets each, each piglet will be studied twice, study 1: sulphate and methionine infusion, study 2: low and enough sulphur amino acid intake. Group sizes (n=8) were calculated to provide an 80% probability of detecting a difference of 30% with a within group coefficient or variation of 20%.

Test Agents or Procedures e.g. 2 Drugs	# of Animals and Species Per Group e.g. 6 rats	Dosage and/or Route of Administration e.g. .03, .05 mg/kg – IM, IP (4 variables)	# of endpoints e.g. 1, 7, 10 days (3 variables)	Other variables (i.e. sex, weight, genotypes,etc.) e.g. Male, Female groups (2 variables)	Total number of animals per year e.g. 2 x 6 x 4 x 3 x 2 = 288
Normal SAA MgSO4 Methionine	8	 1.2 mmol/kg, IV 1.2 mmol/kg, IV			8
Low SAA Methionine	8	 2.58 mmol/kg/d IG			8

*** For the above table, enter the first agent/procedure, press 'enter', then the 2nd agent... complete the first column, then the 2nd, then the 3rd...**

b) Please justify the need for live animals versus alternate methods (e.g. tissue culture, computer simulation).

Live animals are needed for metabolic "in vivo" kinetic studies.

c) Describe the characteristics of the animal species selected that justifies its use in the proposed study (consider characteristics such as body size, species, strain, data from previous studies or unique anatomic/physiological features)

The piglet is the preferred model for amino acid metabolism studies because of its well-characterised protein and amino acid metabolism, similar pattern of amino acid requirements and amino acid body composition, metabolic similarity to the human (particularly the digestive system), sensitivity to the amino acid profile in the diet and diet restriction, and size, which accommodates the sampling schedule (Pencharz et al.,1996; Ball et al., 1996).

This study is similar to currently approved protocols 3476 and 3817 (Wykes). All surgical procedures have been performed previously (Wykes et al. J Nutr 1993).

8. Animal Husbandry and Care

a) **Special cages** NO ☐ YES ☒ **Specify:** Stainless steel rabbit cages (77cm long x 65cm wide x 40cm high), adapted for piglets with plastic-coated small-opening flooring, modified for access piglet from above and for metabolic collection.

Special diet NO ☐ YES ☒ **Specify:** Elemental diet based on Wykes et al. J Nutrition 1993; 123:1248-1259 with polycose substituted for glucose to decrease osmolarity. It will supply NRC nutrient requirement for neonatal piglet.

Special handling NO ☐ YES ☒ **Specify:** Piglets will be fitted with a jacket to house catheters in a pocket and to allow the piglets freedom of movement while enteral feeding is administered through a tether/swivel apparatus. The tether will be attached to an anchor button on the piglet's jacket, and the swivel will be bolted to the top of the cage. The piglet will not be restrained. Piglets will be weighed and have both femoral and jugular catheters flushed daily with 2 ml of saline (0.9%) and they will have access to a ball and squeaky toy,

b) Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)?

NO ☒ YES ☐ **Specify:**

c)

Multiple institution facility housing: NO ☒ YES ☐

Indicate all facilities where animals will be housed:	**Building:**	Macdonald-Steward building	**Room No:**	MS B-50
Indicate area(s) where animal use procedures will be conducted:	**Building:**	LARU	**Room No:**	Surgery

If animal housing and animal use are in different locations, briefly describe procedures for transporting animals:
Pigs will be transported in dog carrying cages in a car, the car will be warmed, and the cages will be covered with blanket. Two persons will observe the piglets during transporting period.

9. Standard Operating Procedures (SOPs)

Complete this section if you plan to use any of the UACC SOPs listed below. IT IS UACC POLICY THAT THESE SOPS BE USED WHEN APPLICABLE. Any proposed variation of the SOPs must be described and justified. The Standard Operating Procedures can be found at the UACC website at www.mcgill.ca/fgsr/rgo/animal/ . The completed and signed SOP form must be attached to the protocol.

Check all SOPs that will be used:

Blood Collection (UACC#1)	☐	**Production of Monoclonal Antibodies (UACC#7)**	☐
Anaesthesia (rodents) (UACC#2)	☐	**Production of Polyclonal Antibodies(UACC#8)**	☐
Analgesia (rodents/larger species) (UACC#3)	☐	**Collection of Amphibian Oocytes (UACC#9)**	☐
Breeding (transgenics/knockouts) (UACC#4)	☐	**Rodent Surgery (UACC#10)**	☐
Transgenic Generation (UACC#5)	☐	**Neonatal Rodent Anaesthesia and Euthanasia (UACC#11)**	☐
Knockout/in Generation (UACC#6)	☐		☐

10. Description of Procedures

a) **FOR EACH EXPERIMENTAL GROUP, DESCRIBE ALL PROCEDURES AND TECHNIQUES IN THE ORDER IN WHICH THEY WILL BE PERFORMED - surgical procedures, immunizations, behavioural tests, immobilization and restraint, food/water deprivation, requirements for post-operative care, sample collection, substance administration, special monitoring, etc. IF A PROCEDURE IS COVERED BY AN SOP, WRITE "AS PER SOP", NO FURTHER DETAIL IS REQUIRED. Appendix 2 of the Guidelines provides a sample list of points that should be addressed in this section.**

Piglets: Three-day old piglets will be obtained from Macdonald Farm, McGill University, and will undergo surgery then transfer to the animal facility, where they will be housed in individual metabolic cages with visual and aural contact. They will have a ball and squeaky toy for entertainment. Room temperature will be maintained at 27oC with a 12-hour light/dark cycle.

Surgery Catheters will be implanted aseptically into a jugular vein, femoral vein, stomach and urinary bladder. Piglets will be premedicated with atropine (0.05mg/kg i.m.), 15 minutes before induction of anaesthesia with 5% isoflurane, Enrolfloxacin/enrolfloxacine (Baytril: Bayer) (2.5 mg/kg) i.m. will be given as a preoperative antibiotic and Buprenorphine (0.0075 mg/kg i.m.) as a preemptive analgesic. Anaesthesia will be maintained with 2% isoflurane in 50% oxygen by tightly secured mask. "Inhalation agents are well tolerated and mask induction can be carried out on small pigs with ease (Becker, 1986). Intubation of the trachea is difficult for anatomical reasons (Lumb and Jones, 1984; Flecknell, 1987; Green, 1982)" (CCAC Guide Vol. 1(2nd Ed.) 1993, Chapter XI, G,i). Piglets will be kept warm during surgery on a heated operating table. The neck, groin and abdominal area will be shaved and scrubbed clean sequentially with Hibitane, and iodine, before sterile draping. Silastic catheters (0.8 mm id x 2.0 mm od x 1m) will be inserted into the left external jugular and femoral veins, anchored in place with grommets fitting snugly over the catheter wall, and sewn to stable underlying tissue. The stomach and bladder catheters will be placed through a hole in the stomach and bladder wall respectively, and held in place using a purse-string suture. All catheters will be tunnelled subcutaneously to exit laterally on the side of the chest. The incisions will be closed with a double row of sutures, and covered with antibiotic cream. The catheters will be filled with saline solution, capped, and secured in the pocket of a mesh jacket worn by the piglet. Each surgery will be performed by two of the named research personnel (Wykes, Hou, Warren, Martin).

Postoperative Care: Following surgery, piglets will be kept warm and continuously observed with special attention paid to monitoring the health (temperature, color of mucus membranes, and urine production) and behavior (activity, interest in the environment, general demeanor) of the piglets. Buprenorphine (0.0075 mg/kg i.m.) will be administered every 12

hours for the first 24 hours after surgery and then repeated as necessary.

Diet: Piglets will receive an elemental and complete diet (Meet the require of NRC requirement for the neonatal piglet, 1998 except with adequate (120% of NRC98), or deficient(58% of NRC98) levels of sulphur amino acid (SAA)) continuously feeding via gastric catheter after surgery. The targeted intakes will be: amino acid: 15g/kg/d (100% of NRC98) and energy: 260 kcal/kg/d (120% of NRC98).

Study Procedure
Study 1
1) Piglets will receive the adequate SAA diet by gastric catheter for 11 days.
2) On days 4,5,7,8,9, 24h urine collections (collected through the metabolic funnel) will be used for baseline N/S balance.
3) On day 6, a supplement of MgSO4 (1.2 mmol/kg) will be given iv through the femoral catheter with S excretion measured in urine collected every 4h through the bladder catheter for the next 24h.
4) Similarly urine will be collected every 4 hour through bladder catheter on day 10 following a supplement of methionine (1.2 mmol/kg), and the 24h urine of day 11 will be collected through metabolic funnel.
5) Blood will be sampled through the jugular catheter (3 ml every 4h for the first 12h of day 6, 10, and another 3 ml for the next 12h, total volume will be 24 ml, (about 10% of blood volume of a 3 kg piglet) to measure plasma sulphate and amino acids
6) The piglets will be killed with an intravenous injection of sodium pentobarbital (Euthanyl 750 mg) after 11 days study.

Study 2
1) Piglets will be studied for 11 days. They will receive the deficient SAA diet through the gastric catheter for 7 days. On day 8 they will be switched to the adequate diet.
2) On days 4,5,6,7, 24h urine collections (collected through the metabolic funnel) will be used to measure N/S balance in low SAA fed state.
3) Similarly on days 8,9,10,11, 24h urine collections (collected through the metabolic funnel) will be used to measure N/S balance in normal SAA fed state
4) On day 7, a stable isotope infusion will be performed, [1,2-13C2]Glycine will be infused through the jugular catheter for 12 hours, and 2.0 ml of blood will be sampled through femoral catheter at hours 0,3,4,5,6,9,10,11,12, , the total blood sampled will be 18 ml (about 8% of blood volume of a 3kg piglets). The piglets will be swiched to adequate SAA diet just in the middle of this study to study the effect of repletion on red blood cell GSH synthesis.
5) Similaly, on day 11. [1,2-13C2]Glycine will be infused for 6 hours, and 2 ml of blood will be sampled at hours 0,3,4,5,6 (total blood sampled will be 10 ml, about 4% of blood volume), and red blood cell GSH synthesis in the stable adequate SAA fed state will be measure. At the end of the 6 hour infusion, piglets will be killed with an intravenous injection of sodium pentobarbital (Euthanyl 750 mg). Tissues (liver, lung, kidney, and muscle) will be sampled post mortem.

b) Field Studies – Provide all relevant details. Procedures to be conducted (e.g. surgery, blood collection, tagging etc.) should be described above.

Method of capture/restraint, duration of captivity, potential injury/mortality, monitoring frequency:

Transportation and /or housing of animals in the field:

Special handling required:

Capture of non-target species, potential injury/mortality:

Will captured animals be released at or near the capture site YES ☐ NO ☒
If not, specify if they will be relocated to other locations and/or populations.

Describe any potential ecological disruption this study may cause:

It is the responsibility of the investigator to obtain all necessary permits for work with wildlife. Copies of these permits must be forwarded to the Research Ethics Officer (Animal Studies) when they are obtained.

c) Pre-Anaesthetic/Anaesthetic/Analgesic Agents: List all drugs that will be used to minimize pain, distress or discomfort. Table will expand as needed. (*complete 1st column pressing 'enter' after each species, then 2nd column…)

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency
Piglet	Atropine	0.08 mg/kg	0.2-0.3 ml	IM	1
	Isoflurane	2-5%			
	Buprenorphine	0.0075 mg/kg	0.15-0.2 ml	IM	2

d) Administration of non-anaesthetic substances: List all non-anaesthetic agents under study in the experimental component of the protocol, including but not limited to drugs, infectious agents, viruses (table will expand as needed). (*complete 1st column pressing 'enter' after each species, then 2nd column…)

Species	Agent	Dosage (mg/kg)	Total volume (ml) per administration	Route	Frequency
Piglet	Enrolfloxacin (Baytril)	2.5 mg/kg	0.2-0.3 ml	IM	1

e) Endpoints : 1) Experimental – for each experimental group indicate survival time .
2) Clinical - describe the conditions, complications, and criteria (e.g. >20% wt.loss, tumour size, vocalizing, lack of grooming) that would lead to euthanasia of an animal before the expected completion of the experiment (specify per species and project if multiple projects involved).

1. Experimental: 11 days
2. Clinical end point:
   1) Diarrhea (Criteria: not recover after 2 days antibiotic therapy and elemental diet dilution)
   2) Technical catheter problems that can't be resolved.
   3) Incidence of sepsis (fever, demeanor changes, major incision infection).

Specify person(s) who will be responsible for animal monitoring and post-operative care
Name: Chunsheng Hou **Phone#:** 249-8897 (daytime)
457-9567 (night)

f) Method of Euthanasia – According to CCAC guidelines, justification must be provided for use of any physical method of euthanasia without prior use of anaesthesia (justify here):

Specify Species	
Pig	☒ anaesthetic overdose, list agent/dose/route: Sodium pentobarbital (Euthanyl 750 mg) IV.
	☐ exsanguination with anaesthesia, list agent/dose/route:
	☐ decapitation without anaesthesia ☐ decapitation with anaesthesia,list agent/dose/route:
	☐ cervical dislocation
	☐ CO_2 chamber
	☐ other (specify)
	☐ not applicable (explain)

11. Category of Invasiveness: B ☐ C ☐ D ☒ E ☐

Categories of Invasiveness (from the CCAC *Categories of Invasiveness in Animal Experiments*). Please refer to this document for a more detailed description of categories.

Category A: Studies or experiments on most invertebrates or no entire living material.

Category B: Studies or experiments causing little or no discomfort or stress. *These might include holding animals captive, injection, percutaneous blood sampling, accepted euthanasia for tissue harvest, acute non-survival experiments in which the animals are completely anaesthetized.*

Category C: Studies or experiments involving minor stress or pain of short duration. *These might include cannulation or catheterizations of blood vessels or body cavities under anaesthesia, minor surgery under anaesthesia, such as biopsy; short periods of restraint, overnight food and/or water deprivation which exceed periods of abstinence in nature; behavioural experiments on conscious animals that involve short-term stressful restraint.*

Category D: Studies or experiments that involve moderate to severe distress or discomfort. *These might include major surgery under anaesthesia with subsequent recovery, prolonged (several hours or more) periods of physical restraint; induction of behavioural stresses, immunization with complete Freund's adjuvant, application of noxious stimuli, procedures that produce pain, production of transgenics (in accordance with University policy).*

Category E: Procedures that involve inflicting severe pain, near, at or above the pain threshold of unanaesthetized, conscious animals. *Not confined to but may include exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs or chemicals at levels that (may) markedly impair physiological systems and which cause death, severe pain or extreme distress or physical trauma on unanaesthetized animals.* According to University policy, E level studies are not permitted.

12. Potential Hazards to Personnel and Animals It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation Safety permits before this protocol is submitted for review. A copy of these certificates must be attached, if applicable.

No hazardous materials will be used in this study: ☒

a) Indicate which of the following will be used in animals:
☐Toxic chemicals ☐Radioisotopes ☐Carcinogens ☐Infectious agents ☐Transplantable tumours

b) Complete the following table for each agent to be used (use additional page as required).

Agent			
Dosage			
Route of administration			
Frequency of administration			
Duration of administration			
Number of animals involved			
Survival time after administration			

c) After administration the animals will be housed in: ☐ the animal care facility
☐ laboratory under supervision of laboratory personnel

Please note that cages must be appropriately labeled at all times.

Describe potential health risk (s) to humans or animals:

e) Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:

13. Reviewer's Modifications (to be completed by ACC only): The Animal Care Committee has made the following modification(s) to this protocol during the review process. Please make these changes to your copy. You must comply with the recommended changes as a condition of approval.

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