SULPHUR AMINO ACID REQUIREMENT AND METABOLISM IN THE TOTAL PARENTERAL NUTRITION (TPN) FED HUMAN NEONATE

By

Glenda Courtney-Martin

A thesis submitted in conformity with the requirements for the degree of PhD

Graduate Department of Nutritional Sciences

University of Toronto

© Copyright by Glenda Courtney-Martin (2009)
SULPHUR AMINO ACID REQUIREMENT AND METABOLISM IN THE TPN FED HUMAN NEONATE

Doctor of Philosophy, 2009

Glenda Courtney-Martin

Graduate Department of Nutritional Sciences

University of Toronto

ABSTRACT

Except for tyrosine, the amino acid requirement of parenterally fed (PN) human neonates has not been derived. Methionine and cysteine are indispensable and dispensable sulphur amino acids respectively. Cysteine is synthesized from methionine. Cysteine is unstable in solution, and is left out or added in very small amounts to amino acid solutions. Methionine is added to compensate for the lack of cysteine, assuming that the neonate will convert methionine to cysteine to meet the body’s metabolic demand.

Methionine is hepatotoxic and there is evidence that the neonate has limited ability for its conversion to cysteine. To determine the requirement of the neonate for methionine, PN-fed, stable, post-surgical neonates received graded intakes of methionine. The mean methionine requirement was estimated to be 49 mg.kg⁻¹.day⁻¹, which is 48 to 90% of the methionine content of current commercial amino acid solutions.

Because cysteine is the rate limiting substrate for glutathione (GSH) synthesis and current methods of determining amino acid requirement measure requirement for protein synthesis, SAA requirements for maintenance of GSH status was deleniated in healthy adult males and in PN-fed human neonates. GSH kinetics was measured in healthy men
receiving the mean methionine requirement and graded intakes of cysteine. GSH synthesis did not change with the addition of cysteine. Additionally, PN-fed post-surgical neonates received a methionine-adequate cysteine-free PN followed by cysteine supplemented PN for two 3-day periods and GSH kinetics measured on days 3 and 6. There was no change in GSH synthesis in response to cysteine supplementation.

It is concluded that the PN-fed human neonate is capable of synthesizing enough cysteine from methionine not only for protein synthesis but for GSH synthesis. For both healthy men and stable post-surgical neonates, the requirement for GSH synthesis is met at the sulphur amino acid requirement derived using the indicator amino acid technique.
ACKNOWLEDGEMENTS

To my husband, Okan, whose unwavering love and support was beyond heroic. This accomplishment is as much yours as it is mine. And to my daughter Kimberly, for sure you keep the wind beneath my wings but you have suffered much for this. I am sorry. I love you much. Mom.

To Dr. P, Thanks for being the best teacher and mentor I could hope for. I am truly blessed to have had the opportunity to be your student. May God return to you “pressed down, shaken together and running over” all that you have so generously given. God’s blessings always.

To Dr. Ball, Thanks for your sound, always intellectually stimulating and brilliant guidance.

To Mahroukh, Thanks for helping me achieve one of the primary objectives for this PhD: to become versed in the methods and machinery that we use in the lab. Thanks for your enthusiasm with method developing. Much appreciated.

To Rajavel Elango, Arshad Humayun, Karen Chapman, Christopher Tomlinson, Joann Herridge and Jean Hsu. The camaraderie, support, and variations in humour helped to make the time fly.
To my friend Catharine Rocchi, your support from the beginning to end is most sincerely appreciated. Thanks for your faithfulness.

To my office mates, Deb Harrison, Marnie Mckenzie, and Julia Maxwell. Thanks so much for all your support and encouragement.

Heartfelt thanks to all my subjects, NICU nurses and staff especially Nicole daSilva, Pharmacy staff, especially Mark Bedford and the nurses in the CIU, especially Maria Mione and Roberta Gardiner.
TABLE OF CONTENTS

ABSTRACT  ii
ACKNOWLEDGEMENTS  iv
TABLE OF CONTENTS  vi
LIST OF TABLES  x
LIST OF FIGURES  xi
PUBLICATIONS AND PRESENTATIONS ARISING FROM THESIS  xiii
ABBREVIATIONS USED IN THESIS  xiv

1 INTRODUCTION  1

2 LITERATURE REVIEW  7

2.1 Sulphur Amino Acid Metabolism  7
  2.1.1 Sulphur Amino Acid Metabolism Using Nitrogen Balance  12
  2.1.2 Sulphur Amino Acid Metabolism Using Stable Isotope Tracers  13
  2.1.3 Sulphur Amino Acid Metabolism Animal Studies  16

2.2 Sulphur Amino Acid Requirement of the Adult Human  18
  2.2.1 Nitrogen Balance  18
  2.2.2 Sulphur Amino Acid Requirements by Stable Isotope Tracer Kinetic Method  20

2.3 Sulphur Amino Acid Requirement of the Human Infant  26
  2.3.1 Nitrogen Balance  26
  2.3.2 Indicator Amino Acid Oxidation (IAAO)  28

2.4 Sulphur Amino Acid Metabolism: Effect of Route of Feeding  30

2.5 Is Cysteine a Conditionally Essential Amino Acid in Human Neonates?  34
  2.5.1 In Vitro Studies:  34
  2.5.2 Plasma Amino Acids  38
  2.5.3 Nitrogen Balance  39
  2.5.4 Stable Isotope Tracer Studies  41

2.6 Glutathione  43
  2.6.1 Introduction to Glutathione Metabolism  43
  2.6.2 Glutathione: Turnover and Metabolism  48
  2.6.4 Glutathione Kinetic Measurement:  51
  2.6.5 Glutathione Metabolism and Synthesis Rates:  55

2.7 Cysteine Metabolism  64
  2.7.1 In Vitro Studies  64
  2.7.2 in Vivo Studies  67

3 THESIS SCOPE AND MOTIVATION  72

3.1 Rationale  72

3.2 Hypothesis and Objectives  74
6.4 Results .......................................................... 157
6.5 Discussion ...................................................... 164

7. DOES THE ADDITION OF CYSTEINE TO THE TOTAL SULPHUR AMINO
ACID REQUIREMENT (METHIONINE ONLY) INCREASE ERYTHROCYTES
GLUTATHIONE SYNTHESIS IN THE TPN FED HUMAN NEONATE: A PILOT
STUDY. .............................................................. 169
7.1 Abstract .......................................................... 169
7.2 Introduction ...................................................... 170
7.3 Subjects and Methods ........................................... 173
  7.3.1 Subjects ......................................................... 173
  7.3.2 Experimental design ........................................ 175
  7.3.3 Study Diets .................................................... 179
  7.3.4 Tracer Protocol ............................................... 183
  7.3.5 Sample Collection .......................................... 183
  7.3.6 Sample Analysis ........................................... 184
  7.3.7 Calculations .................................................. 186
  7.3.8 Statistical Analysis ........................................ 187
7.4 Results .......................................................... 188
  7.4.1 Clinical characteristics and nutrient intake .............. 188
  7.4.2 Intracellular glycine enrichment ........................... 188
  7.4.3 Glutathione kinetics ........................................ 191
7.5 Discussion ...................................................... 197

8 GENERAL DISCUSSION, CONCLUSION AND FUTURE DIRECTIONS ____ 201
  8.1 General Discussion and Conclusions ................................ 201
  8.2 Future Directions .............................................. 208

9 REFERENCES ...................................................... 210

10 APPENDICES ...................................................... 227
  10.1: Mean plasma cysteine concentration of adult males who participated in study
number 3 (chapter 6) in response to increasing cysteine intake. ...................... 227
  10.2: Mean plasma homocysteine intake of adult males in response to increasing cysteine
intake ........................................................................ 228
  10.3 CONSENT FORMS ............................................... 229
  10.4 STUDY DAY CALCULATIONS .................................. 240
  10.5 Amino Acid Composition of PN Solution ....................... 242
  10.6 Study day list of events ....................................... 243
  10.7 Order form for TPN Research Pharmacy: Total SAA requirement of the TPN-fed
post-surgical human neonate. .................................................. 245
  10.8 Study day calculations: Methionine-adequate cysteine-free diet does not limit
glutathione synthesis in young healthy adult males. .................................. 247
  10.9 Study day calculations: Methionine-adequate cysteine-free diet does not limit
glutathione synthesis in young healthy adult males. .................................. 255
11. Composition of crystalline L-amino acid mixtures used to determine “Does the addition of cysteine to the total sulphur amino acid requirement (methionine only) increase erythrocyte glutathione synthesis in the TPN fed human neonate. __________ 263

11.1 calculation form for study “Does the addition of cysteine to the total sulphur amino acid requirement (methionine only) increase erythrocyte glutathione synthesis in the TPN-fed human neonate. Study days 1 & 2____________________________________ 265

11.2 calculation form for study “Does the addition of cysteine to the total sulphur amino acid requirement (methionine only) increase erythrocyte glutathione synthesis in the TPN-fed human neonate. Study day 3.________________________________________ 267

11.3 calculation form for study “Does the addition of cysteine to the total sulphur amino acid requirement (methionine only) increase erythrocyte glutathione synthesis in the TPN-fed human neonate. Study days 4 & 5.________________________________________ 269

11.4 calculation form for study “Does the addition of cysteine to the total sulphur amino acid requirement (methionine only) increase erythrocyte glutathione synthesis in the TPN-fed human neonate. Study day 6.________________________________________ 271

11.5 Comparison of breakpoint _____________________________________________ 273

11.5.1 Breakpoint determination _____________________________________________ 273
LIST OF TABLES

Table 4.1 HPLC stability analysis of TPN bulk solution................................................. 89
Table 4.2 HPLC stability analysis of individual amino acids........................................... 91
Table 5.1 Subject characteristics of TPN fed neonates.................................................... 102
Table 5.2 Amino acid composition of PN solution administered to neonates.............. 106
Table 5.3 Nutrient intake of neonates who participated in methionine requirement study 
................................................................................................................................. 111
Table 6.1 Subject characteristics of adult men ............................................................... 141
Table 6.2 Composition of protein free powder and flavoured crystals......................... 147
Table 6.3 Amino acid composition............................................................................... 149
Table 6.4 Individual FSR at varying cysteine intake levels............................................ 162

Table 7.1 Subject characteristics of TPN-fed neonates studied on TPN with and without 
cysteine ....................................................................................................................... 174
Table 7.2 Amino acid composition of amino acid solutions administered to neonates 
studied on TPN with and without cysteine ............................................................. 177
Table 7.3 Individual nutrient intakes of neonates studied on TPN with and without 
cysteine ....................................................................................................................... 182
Table 8.1 Comparison between methionine concentrations in currently available 
commercial solutions and proposed concentration based on requirement estimate 204
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Structure of the sulphur amino acids methionine and cysteine</td>
<td>8</td>
</tr>
<tr>
<td>2.2</td>
<td>Pathways of methionine metabolism</td>
<td>11</td>
</tr>
<tr>
<td>2.3</td>
<td>Glutathione metabolism</td>
<td>45</td>
</tr>
<tr>
<td>2.4</td>
<td>Structure of reduced glutathione (GSH)</td>
<td>46</td>
</tr>
<tr>
<td>2.5</td>
<td>Structure of glutathione disulfide (GSSH) (Oxidized form of GSH)</td>
<td>46</td>
</tr>
<tr>
<td>2.6</td>
<td>Pathways to cysteine metabolism</td>
<td>65</td>
</tr>
<tr>
<td>4.1</td>
<td>HPLC chromatogram</td>
<td>92</td>
</tr>
<tr>
<td>5.1</td>
<td>Parenteral methionine requirement. The effect of methionine intake on phenylalanine oxidation in the TPN fed human neonate</td>
<td>122</td>
</tr>
<tr>
<td>5.2</td>
<td>Parenteral methionine requirement. The effect of increasing methionine intake on $F^{13}CO_2$ in the TPN fed human neonate</td>
<td>123</td>
</tr>
<tr>
<td>5.3</td>
<td>Plasma methionine concentration in response to graded intakes of methionine</td>
<td>126</td>
</tr>
<tr>
<td>5.4</td>
<td>Plasma homocysteine concentration in response to graded intakes of methionine</td>
<td>127</td>
</tr>
<tr>
<td>5.5</td>
<td>Plasma cystathionine concentration in response to graded intakes of methionine</td>
<td>129</td>
</tr>
<tr>
<td>6.1</td>
<td>Mean tracer-to-tracee molar ratio (mol % above baseline) of erythrocyte free glycine</td>
<td>159</td>
</tr>
<tr>
<td>6.2</td>
<td>Glutathione kinetec in response to graded intakes of cysteine</td>
<td>161</td>
</tr>
<tr>
<td>6.3</td>
<td>Urinary sulphate excretion in response to graded cysteine intakes</td>
<td>163</td>
</tr>
<tr>
<td>7.1</td>
<td>Net tracer/tracee molar ratio of erythrocyte free glycine</td>
<td>190</td>
</tr>
<tr>
<td>7.2</td>
<td>Glutathione kinetice in neonates fed TPN with and without cysteine</td>
<td>193</td>
</tr>
<tr>
<td>7.3</td>
<td>Glutathione FSR of neonates fed TPN with and without cysteine</td>
<td>194</td>
</tr>
<tr>
<td>7.4</td>
<td>Individual glutathione synthesis rates of neonates fed TPN with and without cysteine</td>
<td>195</td>
</tr>
</tbody>
</table>
Figure 7. 5 Plasma cysteine concentration of neonates fed TPN with and without added cysteine .............................................................. 196

Figure 10. 1 Mean plasma cysteine concentration of adult males in response to graded cysteine intakes ........................................................................ 227

Figure 10. 2 Mean plasma homocysteine concentration of adult males in response to graded cysteine intakes ............................................................... 228
PUBLICATIONS AND PRESENTATIONS ARISING FROM THESIS

Publications:


Glenda Courtney-Martin, Mahroukh Rafii, Linda J. Wykes, Ronald O. Ball, and Paul B. Pencharz. **Methionine-adequate cysteine-free diet does not limit erythrocyte glutathione synthesis in young healthy adult males.** J Nutr 138: 2172-2178, 2008 (Chapter 6)

**Presentation and abstract:**

ABBREVIATIONS USED IN THESIS

AA   Amino acid/s
AdoMet S-Adenosylmethionine
ALT  Alanine aminotransferase
ANOVA Analysis of variance
APE  Atom percent excess
ASR  Absolute synthesis rate
AST  Aspartate aminotransferase
BSO  Buthionine-SR-sulfoximine
CDO  Cysteine dioxygenase
CI   Continuos infusion
CSDC Cysteinesulfinate decarboxylase
DAAO Direct amino acid oxidation
FSR  Fractional synthesis rate
GCMS Gas chromatography mass spectrometry
GCS $\gamma$-glutamylcysteine synthetase
GGT  Gamma glutamyl transferase
GSH  Glutathione
GSSH Glutathione disulfide
HPLC High performance liquid chromatography
IAAO Indicator amino acid oxidation
ID   Intraduodenal
IV   Intravenous
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCMS/MS</td>
<td>Liquid chromatography mass spectrometry/mass spectrometry</td>
</tr>
<tr>
<td>N</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl cysteine</td>
</tr>
<tr>
<td>PN</td>
<td>Parenteral nutrition</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended dietary allowance</td>
</tr>
<tr>
<td>RM</td>
<td>Remethylation</td>
</tr>
<tr>
<td>SAA</td>
<td>Sulphur amino acids</td>
</tr>
<tr>
<td>TM</td>
<td>Transmethylation</td>
</tr>
<tr>
<td>TPN</td>
<td>Total parenteral nutrition</td>
</tr>
<tr>
<td>TS</td>
<td>Transsulphuration</td>
</tr>
<tr>
<td>TSAA</td>
<td>Total sulphur amino acids</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

Total parenteral nutrition (TPN) as the words imply, is the provision of an individual’s entire nutrient requirement via the intravenous route. The state of the art depicts that the first requirement for TPN is the provision of a source of $\alpha$-amino nitrogen in the form of both dispensable and indispensable amino acids (Heird and Winters 1975). In this current day, the $\alpha$-amino nitrogen source is provided as a mixture of crystalline amino acids. The second requirement is the provision of sufficient non-protein calories to meet the total energy requirement. These non-protein calories are provided in the form of dextrose, and lipids, with the lipids also serving as a source of essential fatty acids. Vitamins and minerals are provided in the form of additives specially formulated for use in intravenous nutrition. Finally electrolytes are also provided, usually in an amount consistent with maintenance requirement (Heird and Driscoll 1975).

The first documented report on the attempted use of complete intravenous nutrition was in 1944 by Helfrick and Abelson in an infant with Hirschsprung’s disease (Helfrick 1944). However, the successful implementation of TPN as depicted by appropriated growth and development took another twenty years to occur. Building on the work of Wretlind and co-workers (Shenkin 1978), in 1968 Dudrick and his co-workers published on the successful growth and development of beagle puppies (Dudrick, Wilmore et al. 1968) as well as a human infant fed solely by TPN (Wilmore and Dudrick 1968).

This successful implementation of parenteral nutrition (PN) forty years ago provided a major therapeutic break-through that has improved survival rates among
children and adults with gastrointestinal diseases and feeding intolerance. However, the neonate who is unable to tolerate enteral feeding, particularly the surgical neonate presents a special challenge to the clinician. Depending on the extent of the gastrointestinal failure and the length of time to recovery, a neonate could be managed on TPN for an extended length of time. Under such circumstances in particular, but in any neonate requiring TPN, it becomes obvious that despite its contribution to the management of these patients PN is not benign. It is associated with several complications including metabolic imbalances, sepsis, thromboembolism and total parenteral nutrition (TPN) associated cholestasis. All of these are potentially life threatening, but it is cholestasis, and its associated liver failure that is the most common cause of death in that population (Fisher 1989).

TPN cholestasis is a progressive disease characterized by a sequential pattern of histological changes in the liver of individuals on TPN (Moss, Das et al. 1993). In the infant, a series of abnormal changes occur beginning with biliary stasis as early as 5 days after the initiation of TPN. This is followed by portal inflammation, bile duct proliferation and finally fibrosis (Moss, Das et al. 1993). Resolution of TPN cholestatic changes is expected once TPN therapy is withdrawn (Spivak and Grand 1983). However, depending on the extent of liver damage, death can occur as a result of liver failure.

In the clinical setting, TPN-associated liver disease is monitored and sometimes diagnosed using measurements of serum transaminases namely; aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma glutamyl transferase (GGT), as well as markers of cholestasis namely, alkaline phosphatase and conjugated bilirubin concentrations. Using a rat model of TPN cholestasis Demiracan et
al. (Demircan, Ergun et al. 1999) correlated histological degree of cholestasis with routine laboratory values. They found no correlation between liver histology and any of the routine tests. In an effort to find a more representative test, they correlated liver histology with serum bile salts. The results showed a direct correlation between bile salts and degree of cholestatic changes in the liver. Bile salts, being a relatively expensive test, is not routinely used in the clinical setting.

Although the sequela of liver damage in TPN cholestasis has now been characterized from liver biopsies (Moss, Das et al. 1993), the pathophysiology of TPN cholestasis remains unclear. Nevertheless, many risk factors have been identified such as immaturity of liver function, and low gestational age (Beath, Davies et al. 1996), early exposure to TPN (Beath, Davies et al. 1996), sepsis, (Beath, Davies et al. 1996), intestinal stasis and bacterial overgrowth (Kubota, Yonekura et al. 2000), prolonged starvation, intestinal disease, surgical resection and the duration of TPN (Belli, Albrecht et al. 2003). None of these factors have proved to be causal (Loff, Waag et al. 1998), hence the role of specific components of the TPN solution: amino acids (Belli, Fournier et al. 1987; Brown, Thunberg et al. 1989; Moss, Haynes et al. 1999), dextrose (Belli, Albrecht et al. 2003), lipids (La Scala, Le Coultre et al. 1993), minerals, and the deficiency of certain micronutrients (Whalen, Shamberger et al. 1990) have been studied. Of the three macronutrients used for TPN, amino acids have attracted the most attention. Direct toxicity of the amino acids has been shown in *vitro* (Belli, Fournier et al. 1987; Moss, Haynes et al. 1999). In rats receiving two different amino acid solutions (Vamin vs. Travasol) for 5 days, Belli *et al.* (Belli, Fournier et al. 1987) showed that animals infused with Travasol had a decreased bile flow when compared to animals infused with
Vamin. They attributed this to the difference in the amino acid pattern of the two solutions. The increased cholestatic effect of Travasol was attributed to its higher concentration of the methyl donor amino acid (methionine).

A number of studies have shown that methionine levels are elevated during TPN (Bell, Filer et al. 1983; Moss, Das et al. 1993). Moss et al. (Moss, Das et al. 1993) showed that the TPN solution was directly hepatotoxic and that toxicity was mediated by one or more sulphur-containing amino acids. In that study, the serum concentration of methionine was markedly elevated in animals on TPN compared to controls. Young infants fed TPN (Travasol) showed higher plasma methionine and lower plasma serine concentrations than orally fed infants (Bell, Filer et al. 1983).

In a follow-up study designed to test whether infusion of methionine into normally fed rabbits injure the liver in a manner similar to TPN, Moss et al (Moss, Haynes et al. 1999) infused methionine via a central venous catheter into rabbits fed rabbit chow ad libitum. Two other groups served as controls: a TPN-fed group and a standard rabbit chow-fed group. After 14 days on the diets, the administration of intravenous methionine to normally fed animals injured the liver in a similar manner to that seen in the liver of animals receiving TPN. This led the authors to propose that the methionine levels in current TPN solutions may be too high for the neonate and could precipitate liver damage associated with TPN. In addition to high methionine levels, the TPN-fed neonate has been found to have a low plasma cysteine concentrations (Malloy, Rassin et al. 1984; Miller, Jahoor et al. 1995). Low plasma cysteine has been related to the decrease in transsulfuration due to the immaturity of the enzymes responsible for transsulfuration in the neonatal liver (Gaull, Sturman et al. 1972; Zlotkin and Anderson
Low glutathione concentration and synthesis rates have also been reported in the neonate (Vina, Vento et al. 1995). High methionine together with low cysteine and consequent deficiency of glutathione could act synergistically to potentate liver damage and contribute to TPN cholestasis.

Except for tyrosine (Roberts, Ball et al. 2001) the amino acid requirements of the human neonate during TPN feeding have not been experimentally derived. Our group has developed a neonatal piglet model (Wykes, Ball et al. 1993) for the study of amino acid requirement and metabolism in TPN feeding. Using this model, we have determined that the requirement of several amino acids is lower with TPN feeding than with enteral feeding. The requirement for methionine, in particular, was found to be 30% lower in TPN feeding compared to enteral feeding. If this is also true for the human neonate, it may be possible to formulate a TPN solution that does not damage the liver if an appropriate and lower intake of methionine is identified. Given the extensive morbidity of TPN-related liver disease and the corresponding increased cost to the health care system, it is a high priority to develop such a TPN solution.

The purpose of this thesis is to determine the total sulphur amino acid requirement (methionine only) for the TPN-fed human neonate and to study the metabolism of glutathione in response to cysteine intake, first in adult males and then in the TPN-fed neonate. The thesis is organized into several chapters: chapter two presents a thorough literature background on sulphur amino acid metabolism, requirement, and glutathione metabolism. Chapter three outlines and discusses the objectives of the thesis. Chapter four outlines the methods and processes involved in the development of TPN solutions de novo along with the validation process for the appropriate use of such solutions. The
three other studies presented in this thesis will be presented in chapters five to seven.

Chapter eight is the conclusion chapter for the thesis with suggestions for future research.
The sulphur amino acids are methionine and cysteine (Figure 2.1, P8). They are so named because of the presence of a sulphur atom in their molecule. These two amino acids and one of their metabolites; glutathione, are the focus of this thesis. This thesis attempts to bridge a number of important gaps in the literature; firstly it will provide novel an unprecedented information on the total sulphur amino requirement of the TPN-fed human neonate. In addition, using sophisticated stable isotope techniques, it will provide further clarification on an ongoing debate in the literature as to whether cysteine is a conditionally essential amino acid in the TPN-fed human neonate. The development and validation of the piglet model as a surrogate for the study of amino acid requirements and metabolism in the human neonate provided the base from which we were able to launch into this work with confidence. We chose to begin our series of experiments in the human neonate with the sulphur amino acids because of the evidence which suggests that methionine is the most toxic of all the amino acids with implications in TPN-associated liver disease. The interest in TPN-associated liver disease and its relationship with methionine stems out of my career as a clinical dietitian.

2.1 Sulphur Amino Acid Metabolism

Methionine is a dietary indispensable amino acid (AA) required for normal growth and development of humans (Rose 1938; Holt and Snyderman 1961; Snyderman, Boyer et al. 1964; Holt 1968; Fomon, Ziegler et al. 1986), other mammals
Figure 2.1 Structure of the sulphur amino acids methionine and cysteine
(Finkelstein, Martin et al. 1988) as well as avian species. Methionine is metabolized via three major metabolic pathways; transmethylation, remethylation and transsulfuration (Figure 2.2). It is a substrate for protein synthesis and it serves as the major methyl group donor in vivo (Stipanuk 1986; Griffith 1987); serving as a source of the methyl group for DNA and RNA intermediates. Methionine is a methyl acceptor for 5-methyltetrahydrofolate homocysteine methyl transferase (methionine synthase), the only reaction which allows for the recycling of this form of folate. As well, it serves as a methyl acceptor for the catabolism of betaine and choline and is required for synthesis of cysteine.

Although methionine is a metabolic precursor for cysteine (DU Vigneaud 1944), only the sulphur atom from methionine is transferred to cysteine, the carbon skeleton is donated by serine (DU Vigneaud 1944). Cysteine is not a precursor for methionine because of the irreversibility of the cystathionine-β-synthase reaction (Rose 1938) (Figure 2.2). “Consequently, any substitution by cysteine for dietary methionine requirement can only be via inhibition of the sulphur amino acid pathway that leads to synthesis of the transsulphuration metabolites, including cysteine itself” (Ball, Courtney-Martin et al. 2006).

Womak and Rose (Womack 1941), and Rose and Wixon, (Rose and Wixom 1955) were the first to demonstrate a sparing effect of cysteine on the methionine requirements; first in rats, then in humans. More recently, the sparing effect of cysteine on the methionine requirements in rats have been convincingly substantiated by the elegant work of Finkelstein et al (Finkelstein, Martin et al. 1986; Finkelstein, Martin et al. 1988). In food and companion animals (e.g.,
101
RM
NADPH
5-10 Methylene THF
Serine
THF
5-Methyl-THF
NAD
NADPH
13
B12
14
12
11
Proteins
Methionine
ATP
3
S-Adenosyl - Methionine
Polyamines
Creatine
2
CH3 + Acceptor = Methylated Acceptor
TM
S-Adenosyl-Homocysteine
Choline
Betaine
15
1
3
Adenosine
Homocysteine
Serine
B6
4
Cystathione
B6
5
Cysteine
6
Proteins
Cystathione
7
Glutathione
Cysteine
8
Cysteinesulfinate
9
Cysteinesulfinate
10
Taurine
Suphate
Figure 2. Pathways of methionine metabolism
Illustration of the pathways of methionine metabolism in mammalian tissue via transmethylation (TM), transsulphuration (TS), and remethylation (RM). The numbers represent the following enzyme or reaction sequence: 1. L-methionine-s-adenosyl-transferase; 2. transmethylation reaction; 3. adenosylhomocysteinase; 4. cystathionine-β-synthase; 5. cystathionase; 6. γ-glutamylcysteine synthetase (GCS); 7. glutathione synthetase 8. cysteine dioxygenase (CDO); 9. cysteinesulfinate decarboxylase (CSDC); 10. aspartate aminotransferase; 11. betaine-homocysteine methyltransferase; 12. methyltetrahydrofolate homocysteine methyltransferase; 13. serine hydroxymethylase; 14. methylene tetrahydrofolate reductase; 15. choline dehydrogenase and betaine aldehyde dehydrogenase. (Adapted from Ball et al 2006).
poultry, pigs, cats, dogs), it has been shown that cysteine can reduce the amount of dietary methionine required (Baker 2006).

Functions of cysteine include protein synthesis, the biosynthesis of taurine, sulphate (Griffith 1987), and the antioxidant glutathione (Lyons, Rauh-Pfeiffer et al. 2000; Badaloo, Reid et al. 2002; Jackson, Gibson et al. 2004). The functions of cysteine regarding protein and glutathione synthesis and its relationship to methionine are of paramount importance to the TPN-fed human neonate. While it is well documented that the total sulphur amino acid could be adequately provided as methionine only in the adult human (DU Vigneaud 1944), and that cysteine is capable of providing a sparing effect on the methionine requirement in both adults (Rose and Wixom 1955) and neonates (Albanese 1949; Fomon, Ziegler et al. 1986; Shoveller, Brunton et al. 2003), controversy exists as to whether the TPN-fed human neonate is capable of adequate cysteine synthesis from methionine when the total sulphur amino acid is provided as methionine only.

2.1.1 Sulphur Amino Acid Metabolism Using Nitrogen Balance

The metabolic significance of the sulphur amino acids (SAAs) began with the classic work of William C. Rose and his colleagues in the first half of the 20th century. Using positive nitrogen balance as the criterion of adequacy, Rose et al. (Rose 1950), were the first to demonstrate qualitatively that methionine was an indispensable AA in humans. In a follow-up study, the quantitative methionine requirement was determined (Rose, Coon et al. 1955). Rose et al designated a minimal tentative requirement of
1.1 g day\(^{-1}\) and twice that; 2.2 g day\(^{-1}\) as the safe daily intake. Less than one year later, Rose and Wixom (Rose and Wixom 1955) published their report which has led to much controversy in the ensuing years. That report summarized the results of three experiments in which the methionine requirement was first determined in the absence of cysteine followed by the requirement determination in the presence of a set dietary excess of cysteine. Those results showed that L-cysteine was capable of replacing 80-89% of the methionine requirement of adult men and provided evidence for a regulatory mechanism not only in rats (Womack 1941), but also in humans. Those studies however, despite being ground breaking in nature, did not delineate the mechanisms, quantify substrate or isolate precursors in vivo.

## 2.1.2 Sulphur Amino Acid Metabolism Using Stable Isotope Tracers

Mudd et al. (Mudd and Poole 1975; Mudd, Ebert et al. 1980) were the first to establish a method (the methyl balance approach method) with which to identify and quantify different aspects of methionine metabolism in humans. That approach, although useful, measured turnover of substrates. Because these substrates turnover relatively slowly, measurement of turnover was insensitive since acute changes could go undetected. Therefore, that approach was considered problematic in terms of quantifying methyl group excretion and oxidation (Storch, Wagner et al. 1988).

In an effort to alleviate those problems, Storch et al. (Storch, Wagner et al. 1988) successfully developed a stable isotope tracer method for quantifying the various aspects of methionine metabolism in humans. In that study, various aspects of methionine metabolism were examined in both the fed and fasted states after a 5-day adaptation
period to an adequate diet in which methionine and cysteine were provided at intakes of ~ 30 and 29 mg.kg\textsuperscript{-1}.day\textsuperscript{-1} respectively. On the concluding morning of the 5\textsuperscript{th} day, the tracers were administered via a primed constant intravenous (IV) infusion of [methyl – \textsuperscript{2}H\textsuperscript{3}] and [1-\textsuperscript{13}C]methionine.

In the fed state, plasma methionine levels rose more significantly than that compared with the fasted state. Also, there was a significant increase in methionine flux during the fed state compared with the fasted state reflective of fed state increases in methionine intake. Feeding resulted in a decrease in methionine release from protein breakdown as well as an increase in transmethylation, transsulphuration and remethylation. Although transsulphuration was increased, this was accompanied by an increased efficiency of methionine recycling via remethylation relative to transsulphuration. Thus in the fed state, methionine metabolism is regulated towards anabolism with an increased flux partly accounted for by enhanced flow of methionine into transmethylation and remethylation relative to the fasted state.

In the fasted state, the rate of methionine utilization for protein synthesis was increased relative to its use for transmethylation. This suggests that in the fasted state, methionine is conserved through the process of protein synthesis and that protein synthesis takes precedence when sulphur amino acid (SAA) availability is low.

In a subsequent experiment, using the same design as above and a doubly labeled methionine (L-[1-\textsuperscript{13}C;methyl-\textsuperscript{2}H\textsuperscript{3}]methionine) instead of two different tracers, Storch et al (Storch, Wagner et al. 1990) went on the further explore other regulatory aspects of methionine metabolism; this time by altering both methionine and cysteine intakes. A
The further intent of the study was to explore the mechanism whereby cysteine might exert a sparing effect on the methionine requirement. Eight healthy adult males were adapted for 5 days to one of 3 diets providing either 25 mg.kg\(^{-1}\) methionine without cysteine (adequate diet), a TSAA free diet, or zero methionine and 20 mg.kg\(^{-1}\) cysteine. The isotope tracer study was performed on the 6\(^{th}\) day in the fed state.

Plasma methionine levels were significantly lower with ingestion of the SAA-free diet compared with the adequate diet whereas plasma cysteine did not differ significantly across diets. The response to the diet free of SAA showed a significant decrease in all aspects of the methionine cycle. There was a significant increase in the incorporation of methionine into protein synthesis relative to transmethylation confirming previous results that methionine is conserved via protein synthesis when SAA intakes are low or absent (Storch, Wagner et al. 1988). With the addition of cysteine to the SAA-free diet, there was a significant decline in the rates of transsulphuration with a trend toward increased remethylation relative to transsulphuration. One of the obvious suggestions from that study and a conclusion drawn by the authors is that the sparing effect of cysteine on the methionine requirement was achieved by a reduction in the transsulphuration rate.

These studies (Storch, Wagner et al. 1988; Storch, Wagner et al. 1990) are important as they provide valuable information on the different aspects of SAA metabolism \textit{in vivo}. It shows that in the presence of a SAA-free diet, methionine is highly directed toward protein synthesis relative to transmethylation, and that homocysteine is also partitioned toward remethylation relative to transsulphuration. These all serve to conserve methionine by decreasing oxidation (Ball, Courtney-Martin et al. 2006). However, it would have been of greater benefit if they had included a group in which
they studied the SAA metabolism at a low but not devoid methionine intake in the presence of excess cysteine.

2.1.3 Sulphur Amino Acid Metabolism Animal Studies

The elegant work of Finkelstein et al. (Finkelstein, Martin et al. 1986; Finkelstein, Martin et al. 1988) has served to provide some further clarification as well as confirmation on the mechanisms involved in the SAA metabolism. In an in vivo experiment (Finkelstein, Martin et al. 1988), rats were fed an adequate diet for 7 days with varying content of methionine and cysteine. In the first group, animals were fed a diet containing 1% methionine for 7 days (control group) or 1% methionine to which 0.8% cysteine was added (experimental group). The only statistically significant result from all of the parameters measured was a 30% lower level of S-adenosyl-homocysteine (AdoHcy) in liver. However, when the same cysteine supplement of 0.8% was added to a diet containing 0.25% methionine and 0.5% cysteine, there was a significant decrease in the hepatic concentration of s-Adenosylmethionine (AdoMet) and serine to the level of 28 and 33% respectively, and an 88% increase in cysteine. In addition, there was a significant increase in cystathione synthase activity.

Using an in vitro system, Finkelstein et al. (Finkelstein, Martin et al. 1986) demonstrated a 44% decrease in cystathionine synthesis, when 0.8% cysteine was supplemented to the 0.25% methionine and 0.5% cysteine diet. There was no change in transsulphuration when 0.8% cysteine was added to the 1% methionine diet.
These results demonstrate that cysteine supplementation to a diet marginal in TSAA (0.2 and 0.5 % methionine) resulted in decreased hepatic cystathionine synthase whereas there was no change in cystathionine synthase activity when a diet with excess methionine (1 %) was further supplemented with a 0.8% cysteine resulting in an excess of methionine and TSAA. A significant result was only observed when 0.8% cysteine was added to a marginal but adequate methionine and TSAA. This suggest that the availability of cysteine in order to spare methionine “must allow for the efficient conservation of a limited methionine pool by means of augmenting homocysteine remethylation and or decreased cystathionine synthesis (transsulphuration)” (Finkelstein, Martin et al. 1986).

The various aspects of methionine metabolism has been summarized by Finkelstein et al. (Finkelstein, Martin et al. 1988) as follows. (The reader is referred to Figure 2.2. p 10-11).

1. In growing animals, the net flow of methionine is in the direction of protein synthesis, which removes methionine from the cycle.
2. The utilization of Adomet (SAM) in the formation of poly amines is the second outlet.
3. The irreversible cystathionine synthase reaction is the final outlet since homocysteine used in this way is committed to transsulphuration- these 3 outlets represent the 3 essential functions of methionine.
4. The reactions of the cycle itself fulfill 3 additional requirements: (1) transmethylation reaction, (2) the recycling of
methyltetrahydrofolate and (3) the catabolism of choline (betaine) via remethylation.

5. Cysteine can spare methionine in only one of these functions; the synthesis of cysteine and its derivatives by means of transsulphuration.

6. The residual methionine requirement after cysteine supplementation represents the need for protein synthesis, the obligatory synthesis of cystathionine (if relevant) and methionine used in the process of remethylation secondary to inefficient conservation (since these two enzymes are utilized in homocysteine conservation).

The methionine-sparing effect of cysteine is based on the redistribution of homocysteine between competing reactions, notably an increase in remethylation relative to transsulphuration. While the absolute rates of remethylation remain unchanged, there is a marked decrease in transsulphuration as the rates of flow of metabolites through cystathionine synthase reaction decrease. The determinant of this metabolic pattern is represented by a reduction in the liver enzymes together with a decrease in Adomet which is an effector of cystathionine synthase (Finkelstein, Martin et al. 1988).

2.2 Sulphur Amino Acid Requirement of the Adult Human

2.2.1 Nitrogen Balance

Using nitrogen balance technique, the requirement for methionine was first determined by Rose et al. in 6 young men (Rose 1950). A range of 0.8 to 1.1 g of
racemic methionine in the absence of cysteine was required by these subjects to maintain positive nitrogen balance. As was his custom, Rose suggested the tentative minimum requirement as the maximum, amount (1.1g = 13.25 mg.kg$^{-1}$.day$^{-1}$) required to keep all subjects in positive nitrogen balance and with a suggestion of twice that amount (2.2 g.day$^{-1}$) to be taken as the safe intake level.

In a later study (Rose and Wixom 1955), Rose and Wixon varied the dietary content of D-L methionine on a set L-cysteine intake of 0.8 g.day$^{-1}$, and found the methionine requirement to be considerably reduced to 0.1 to 0.2 g.day$^{-1}$. The authors concluded that cysteine spared 89 and 80% of the methionine requirements of these two subjects.

Nitrogen balance technique was later applied by other investigators first in women (Reynolds, Steel et al. 1958), and later in men, (Clark, Howe et al. 1970), to study methionine and cysteine requirements by varying the intakes of both amino acids. The results were variable and disappointing largely because of the failure of these investigators to understand some of the fundamental principles governing the sparing effect of cysteine. A sparing effect can only occur when the methionine intake is within a certain range that is less than the total sulphur amino acid requirement but more than the minimal methionine requirement (Ball, Courtney-Martin et al. 2006). Failure to abide by those principles in the design of the above experiments led to results that were sometimes confusing, or to results from which the wrong conclusions were sometimes drawn.

The problems with nitrogen balance have been well reviewed (Young and Bier 1987; Fuller and Garlick 1994). A chief concern is that nitrogen balance underestimates losses and overestimates intake with on overestimation of true rates of nitrogen retention.
All of these errors result in an underestimation of the true requirement. In addition, a very small number of subjects were used in the studies by Rose and colleagues.

2.2.2 Sulphur Amino Acid Requirements by Stable Isotope Tracer Kinetic Method

2.2.2.1 Direct Indicator Amino Acid Oxidation Technique (DAAO)

The most important contribution to our current knowledge of the SAA requirement using stable isotope tracer kinetics have been by the MIT group headed by the late V.R. Young and his collaborators in India, namely Kurpad et al, and our group; the combined Toronto/Alberta group headed by P. Pencharz and R. Ball. These two groups have employed the techniques of DAAO and Indicator Amino Acid Oxidation (IAAO) to determine the total SAA requirement and the effect of cysteine on these requirements with similar results with respect to the total SAA requirement, but have come to seemingly opposite conclusions as to the existence of a sparing effect or lack thereof of cysteine on the methionine requirement.

This valuable series of investigations began with a study by Young et al. (Young, Wagner et al. 1991) designed with the purpose of exploring the methionine metabolism and body methionine balance of five young men fed a diet supplying methionine (without cysteine) at 13 mg.kg\(^{-1}\).day\(^{-1}\) which represents the current FAO/WHO/UNU recommended requirement for TSAA intake. As in their previous studies (Storch, Wagner et al. 1988; Storch, Wagner et al. 1990) L-[\(^2\)H\(_3\)-methyl-1\(^{13}\)C]methionine was used as tracer. The tracer was infused via the intravenous (IV) route on the 5\(^{th}\) and 7\(^{th}\) day in the fed and fasted states after a period of adaptation.
Compared to the data of Storch et al. (Storch, Wagner et al. 1990) in which the methionine intake was 25 mg.kg\(^{-1}\).day\(^{-1}\), the methionine flux was much lower in this study at a methionine intake of 13 mg.kg\(^{-1}\).day\(^{-1}\). Also synthesis rates were about 30% lower with breakdown being also lower ~16%. This decreased flux and breakdown along with the reduced synthesis suggest a conservation of methionine at an insufficient intake and invites the conclusion that 13 mg.kg\(^{-1}\).day\(^{-1}\) is lower than the true methionine requirement. In addition, the flow of methionine via the transmethylation and transsulphuration pathway was reduced when compared to the data of Storch et al (Storch, Wagner et al. 1990).

Based on their estimation of methionine balance which relies on a number of assumptions, one subject was in positive methionine balance, 2 were in negative balance and 2 were close to methionine equilibrium. The conclusion made by Young et al. (Young, Wagner et al. 1991) based on these results with particular emphasis on the methionine balance data was that “a methionine intake of 13 mg.kg\(^{-1}\).day\(^{-1}\) in the absence of dietary cysteine approximates the mean requirement for the healthy young adult male but the upper range of the requirement (RDA) is probably below ~25 mg.kg\(^{-1}\).day\(^{-1}\)”. Such a conclusion is flawed as it is based on one intake level of methionine; a design from which accurate estimation about the requirement of an amino acid cannot be made. In order to accurately determine an amino acid requirement, the amino acid of interest must be fed at intakes both below and above the requirement so that a change in endpoint could be accurately measured and the estimate so derived would be representative of the true requirement. The data presented in the Young et al study (Young, Wagner et al.
1991) is insufficient to make a conclusion regarding methionine requirement since it was
designed to test only one intake level of methionine.

2.2.2.2 Indicator Amino Acid Oxidation Technique (IAAO)

Using IAAO technique, our group has contributed six reports (Di Buono, Wykes et al. 2001; Di Buono, Wykes et al. 2001; Di Buono, Wykes et al. 2003; Shoveller, Brunton et al. 2003; Shoveller, Brunton et al. 2003; Shoveller, House et al. 2004) as part of our contribution to increasing the current knowledge on SAA metabolism. Three of those reports were conducted in adult males whereas the other 3 were conducted in the neonatal piglet which has been used as a surrogate for the neonatal human infant. This model has been found to be appropriate for the human infant (Roberts, Ball et al. 2001).

Using L-[1-13C]phenylalanine as an indicator, our group conducted our first study in 6 men who were randomized to receive 6 graded intakes of methionine in the absence of cysteine after a 2-day adaptation to a prescribed diet. This study was designed to determine the TSAA requirement as supplied by methionine only. Using F^{13}CO_{2} as the main outcome measure, we identified a breakpoint in the F^{13}CO_{2} oxidation curve by two phase linear regression analysis. The results showed that the mean total SAA requirement of adult males was 12.6 mg.kg^{-1}.day^{-1} with a safe population estimate (RDA) of 21 mg.kg^{-1}.day^{-1}.

This mean estimate was almost identical to that obtained by Rose et al (Rose 1950) using nitrogen balance and that confirmed by Young et al. (Young, Wagner et al. 1991) to at least represent a mean requirement. It was also however the suggested RDA
set by the FAO/WHO/UNO (FAO/WHO/UNU 1985). Such an RDA therefore, would have to be too low.

The second study of the series by our group was conducted in an effort to shed light on the topic of cysteine sparing of methionine (Di Buono, Wykes et al. 2001). The group considered that “to detect a sparing effect of cysteine on the methionine requirement, cysteine must be supplied in amounts adequate to completely or largely arrest the flow of methionine through the transsulphuration pathway, whereas methionine must be present in amounts adequate to meet all its other metabolic functions, including protein synthesis, transmethylation and remethylation necessary for folate and betaine metabolism”. We recognized that “unless the TSAA needs of all subjects are met, addition of cysteine will lead to an immeasurably small sparing effect on methionine requirement” as observed by others (Raguso, Ajami et al. 1997; Fukagawa, Yu et al. 1998).

With these concerns in mind, the group used a similar design as previously described (Di Buono, Wykes et al. 2001). We fed graded intakes of methionine on a set cysteine intake of 21 mg.kg⁻¹.day⁻¹ which represents the RDA for the TSAA requirement. In that study, a breakpoint was identified in the F¹³CO₂ in response to L-[1-¹³C]phenylalanine oxidation which was much less than the previous breakpoint estimated in the absence of cysteine. This present breakpoint was 4.5 mg.kg⁻¹.day⁻¹ which represented a decrease in the methionine requirement when an excess cysteine was present in the diet. The authors concluded that based on their data, dietary cysteine was able to exert a sparing effect on the methionine requirement to the extent of 64%.
A third study was conducted by our group to look at various aspects of sulphur amino acid metabolism when the ratio of methionine and cysteine were varied to represent the ratios present in common foods (Di Buono, Wykes et al. 2003). Using the model of Storch et al. (Storch, Wagner et al. 1988), five healthy men were fed 3 different diets in random order after a 56-hour adaptation period to a crystalline L-amino acid diet. Methionine kinetics was measured in the fed state using an orally administered L-[1-$^{13}$Cmethyl $^{2}$H$_{3}$]methionine. The 3 diets were diet A; 24 mg.kg$^{-1}$ methionine without cysteine, diet B; 13 mg methionine plus 11 mg.kg$^{-1}$ cysteine and diet C; 5 mg.kg$^{-1}$ methionine plus 19 mg.kg$^{-1}$ cysteine.

The results show a significant decrease in transsulphuration when the diets containing cysteine were given (diets B and C) compared to diet A. There was also a significant decrease in transmethylation rates between diet A and diet B and C. This decrease in transmethylation rates translated into a decreased availability of homocysteine for remethylation, which was also decreased, but only significantly so in the case of diet C when methionine was further decreased and cysteine intakes increased to levels almost equal to the RDA for the TSAA. There was an almost 40% decrease in transsulphuration rates compared to transmethylation which translated into a 2.5 fold increase in remethylation rate relative to transsulphuration.

The authors concluded from this study that the ratio of cysteine to methionine regulates whole body SAA metabolism in adult humans. When TSAA intake is adequate and held constant at 24 mg.kg$^{-1}$.day$^{-1}$, replacement of methionine with cysteine results in increased remethylation at the expense of transsulphuration, whereas at high methionine intakes, the methionine pool is regulated by high rates of transsulphuration.
2.2.2.3 Twenty four IAAO and Balance Technique

The MIT group together with their collaborators in India have extended the IAAO technique by infusion of the isotope over a 24-hour period instead of 8 hrs and measuring AA balance as have been done in the past.

Using the 24-hour IAAO and balance technique, Kurpad et al. (Kurpad, Regan et al. 2003; Kurpad, Regan et al. 2004) conducted two studies on SAA metabolism and requirement. In the 1st experiment, (Kurpad, Regan et al. 2003), 21 healthy Indian men were studied during 3 randomly assigned 7-day periods in which methionine intakes (without cysteine) were varied. Twenty four hour indicator AA oxidation studies were conducted on day 7 using an IV administration of L-$^{[13]$\text{C]}$leucine as the indicator AA). The results identified a breakpoint in the F$^{13}$CO$_2$ response curve of 14 mg.kg$^{-1}$.day$^{-1}$. However, using indicator AA balance, the suggested mean TSAA requirement in the absence of cysteine was 15 mg.kg$^{-1}$.day$^{-1}$. This was in agreement with the requirement estimate derived by Di Buono et al. (Di Buono, Wykes et al. 2001) using short term IAAO technique.

In order to assess a sparing effect of cysteine on this requirement, they went on to design a follow-up study (Kurpad, Regan et al. 2004) in which the effect of 2 different intakes of cysteine (5 and 12 mg.kg$^{-1}$.day$^{-1}$) were assessed. The design and isotope were similar to that used in the previous experiment (Kurpad, Regan et al. 2003).

With a cysteine intake of 5 mg.kg$^{-1}$.day$^{-1}$, the breakpoint was identified at a methionine intake of 20 mg.kg$^{-1}$.day$^{-1}$, whereas at a cysteine intake of 12 mg.kg$^{-1}$.day$^{-1}$, the methionine breakpoint was 10 mg.kg$^{-1}$.day$^{-1}$. This was compared to their previous study in which the methionine requirement without cysteine was 15 mg.kg$^{-1}$.day$^{-1}$. The authors
concluded based on the overall results obtained that cysteine may spare methionine requirement in healthy men but that the amount of sparing is difficult to quantify. A more likely explanation is that at a cysteine intake of 5 mg.kg\(^{-1}\).day\(^{-1}\), cysteine intake was inadequate to stop the flow of methionine through the transsulphuration pathway. At a cysteine intake of 12 mg.kg\(^{-1}\).day\(^{-1}\), the methionine breakpoint was less than their previous estimate, showing a small sparing effect of cysteine.

The fundamental problem with the above study is again a failure to appreciate the importance of the balance of the intake of methionine cysteine and the impact of this balance on cysteine’s ability to spare a portion of the methionine requirement. Since cysteine does not spare methionine on an equimolar basis, the amount of cysteine present has to be in excess of the methionine it is supposed to spare (Finkelstein, Martin et al. 1986; Di Buono, Wykes et al. 2001; Di Buono, Wykes et al. 2003).

2.3 Sulphur Amino Acid Requirement of the Human Infant

2.3.1 Nitrogen Balance

Using nitrogen balance and growth as the criteria of adequacy, Albanese et al. investigated the sulphur amino acid (SAA) requirement of five infants between the ages of 4 to 11 months (Albanese 1949). Infants were fed 100 kcals.kg\(^{-1}\) and protein of 3.5 g.kg\(^{-1}\).day\(^{-1}\) in the form of a casein hydrolysate. At the start of the experiment the subjects were maintained on a complete diet. The subjects were then fed a sulphur amino acid-deficient (15 and 25 mg.kg\(^{-1}\) cysteine and methionine respectively) diet adequate in all other nutrients for a total of 3 weeks. Methionine was added in graded amounts after
the first of the 3 week period followed by a period where the diet was supplemented with cysteine alone, then cysteine at 1% with graded intakes of methionine. This last part of the experiment was done to determine the sparing effect of cysteine on the total sulphur amino acid requirement.

The result of the study showed that the total SAA requirement when provided mainly as methionine was 85 mg.kg\(^{-1}\).day\(^{-1}\) and 15 mg.kg\(^{-1}\).day\(^{-1}\) cysteine. When cysteine was provided in the amount of 50 mg.kg\(^{-1}\).day\(^{-1}\), the amount of methionine required to restore adequate growth and nitrogen balance was 65 mg.kg\(^{-1}\).day\(^{-1}\). Thus the authors concluded that 35 mg of cysteine can spare approximately 20 mg of methionine and that in terms of the sulphur atom, 9 mg of cysteine sulphur are equivalent to about 4 mg of methionine sulphur. They also added that 22% of the methionine requirement of the infant can be met by cysteine (Albanese 1949).

In a later study, Snyderman et al. (Snyderman et al, 1964) estimated the total SAA requirement of the enterally fed human infant in the presence of cysteine. The diet was a completely synthetic diet and the amino acid composition was patterned after human milk. Seven male infants ranging in age from 2 weeks to 2 months were included in the study. Adequate growth and nitrogen balance was achieved at a methionine intake of 32 to 49 mg.kg\(^{-1}\).day\(^{-1}\) in the presence of cysteine about 50 mg.kg\(^{-1}\).day\(^{-1}\).

Subsequently, Fomon et al. (Fomon et al, 1986) estimated the total SAA requirement of the human infant fed a soy based formula by N balance and found it to be between 80-88 mg.kg\(^{-1}\).day\(^{-1}\). The methionine requirement alone (in the presence of cysteine) was estimated to be 36 - 39 mg.kg\(^{-1}\).day\(^{-1}\). This suggests that cysteine provided between 44 to 49 mg.kg\(^{-1}\).day\(^{-1}\) of the total SAA requirement.
It is difficult to estimate the sparing effect of cysteine from the design of the Fomon study (Fomon, Ziegler et al. 1986), however looking at the three studies presented, it’s safe to say that the enteral total SAA requirement is very similar across studies and is in the region of 80 mg.kg\(^{-1}\).day\(^{-1}\). Subsequent studies on SAA requirement if the enterally fed human infants have not been published.

2.3.2 Indicator Amino Acid Oxidation (IAAO)

One of the major scientific contributions of our group has been the development of the piglet model as a surrogate for the human neonate (Wykes, Ball et al. 1993) to study amino acid requirement and metabolism. Using the piglet model as a surrogate of the human neonate, Shoveller et al. (Shoveller, Brunton et al. 2003) determined the total SAA as methionine only (methionine in the absence of cysteine) of the enterally and parenterally fed neonatal piglet. Using IAAO technique and \(^{14}\)C-phenylalanine as the indicator, the mean methionine requirement (in the presence of zero cysteine) for the enterally and parenterally fed neonatal piglet were estimated to be 0.42 and 0.26 mg.kg\(^{-1}\).day\(^{-1}\) respectively. Importantly, the methionine requirement in the TPN fed piglet was 30% lower than the enteral requirement. Using these requirement estimates derived from the piglet model the total SAA requirement of the human neonate can be predicted. Since piglets grow at 5 times the rate of the human infant, the total SAA requirement estimates of the human neonate is predicted to be 84 and 52 mg.kg\(^{-1}\).day\(^{-1}\) for enteral and parenteral feeding respectively. The predicted enteral requirement of 84 mg.kg\(^{-1}\).day\(^{-1}\) using the piglet model is surprisingly similar to that derived by using nitrogen balance.
In a follow-up study looking at the minimum methionine requirement (methionine in the presence of excess cysteine), the methionine requirement was reduced to 0.25 and 0.18 g.kg\(^{-1}\).day\(^{-1}\) for enterally and parenterally fed piglets respectively (Shoveller, Brunton et al. 2003). Again, the requirement for the human neonate can be predicted from these estimates to be 50 and 36 mg.kg\(^{-1}\).day\(^{-1}\) for enteral and parenteral feeding respectively when cysteine is present in excess. In that study cysteine was fed in excess, and the intake of methionine was varied until requirement was reached. In the presence of an excess of cysteine the methionine requirement was reduced by 40 % in both the enterally and parenterally fed piglet (Shoveller, Brunton et al. 2003). This shows that dietary cysteine is equally effective in producing a sparing effect on the total SAA requirement whether fed enterally or parenterally.

Of note is that when the percent of the dose of phenylalanine oxidized was compared between the two studies (Shoveller, Brunton et al. 2003; Shoveller, Brunton et al. 2003), there was no difference in the amount of \(^{14}\)C-phenylalanine oxidized between the study when cysteine was absent from the diet (Shoveller, Brunton et al. 2003) or in the study when cysteine was provided in excess (Shoveller, Brunton et al. 2003). This provides evidence that cysteine is not an essential amino acid for the neonatal piglet and possibly for the human neonate as well.

As part of these series of investigations in the neonatal piglet, Shoveller et al. published a study in which they showed the impact of SAA intake and route of feeding on plasma homocysteine concentrations (Shoveller, House et al. 2004). Piglets were fed an elemental diet enterally or parenterally with or without cysteine. The methionine intake was then varied from deficient to excess and the plasma homocysteine
concentration measured at each intake level of methionine. Plasma homocysteine showed a linear response to increasing methionine intake in all treatment groups, whether diets were fed enterally or parenterally or whether methionine was increased in the presence or absence of cysteine. However, plasma homocysteine concentrations were highest in the group that was fed enterally with increase in methionine intake in the absence of cysteine. These data shows that both routes of feeding and dietary supply of methionine and cysteine affect plasma homocysteine concentration in the neonate. Since high plasma homocysteine is a risk factor for hemorrhagic and ischemic stroke in infants and children (van Beynum, Smeitink et al. 1999; Hogeveen, Blom et al. 2002) investigation in the human neonate is needed. The suggestion is also that providing the SAA as a balance between methionine and cysteine with the minimum amount as methionine for all the functions of methionine only, would be of benefit to the neonate. This is likely to be of benefit in light of evidence that excess cysteine results in decreased transmethylation and a decreased amount of homocysteine for remethylation (Di Buono, Wykes et al. 2003).

Certainly the primary outcome results of this study provide evidence that could have important implication for cardiovascular risk, as well as for children and adults that are dependant of enteral and parenteral nutrition. Therefore, the impact of route of feeding on SAA metabolism deserves further review.

2.4 Sulphur Amino Acid Metabolism: Effect of Route of Feeding

Data from Rose et al. (Rose and Wixom 1955) suggest that in the normal healthy adult, up to 90% of ingested methionine can be converted to cysteine via the transsulfuration pathway (fig 2.2), making cysteine a non essential amino acid in adult
humans. More recent studies using stable isotopes technology have shown approximately 65% of the total SAA requirement can be converted to cysteine via the transsulphuration pathway (Di Buono, Wykes et al. 2001; Di Buono, Wykes et al. 2001). The enzymes of transsulfuration are found mainly in the liver with some enzyme activity being present in the kidney and adrenals (Zlotkin and Anderson 1982). With the successful introduction of TPN feeding in the 60s, and its frequent use in patients, along with evidence that the enzymes of transsulfuration were underdeveloped in the human fetus, premature and newborn infant, Stegink et al. set out to evaluate whether route of feeding affected cysteine synthesis from methionine in healthy adults (Stegink and Den Besten 1972).

Eight healthy men participated in the study. They received a baseline normal diet for 3 days followed by a cysteine-free protein hydrolysate, and dextrose infusion, which was fat free for 2 weeks via the intravenous or enteral route in a random cross-over design. Plasma samples were obtained daily at the same time each day from each subject.

The results of the study showed that when the protein hydrolysate dextrose mixture was administered parenterally, plasma cysteine concentrations dropped markedly within 12 hours and remained depressed for the entire 2-week period. When the identical solution was administered enterally by nasogastric tube, the plasma cysteine rose immediately, and remained almost constant for the entire 2-week period. Nitrogen balance was not measured in that study. However, the weight of the subjects varied less than 1.5 kg during the entire 4 weeks of the study suggesting that subjects were in nitrogen balance throughout the study period. The results however, suggest that extra-hepatic tissues have a limited capacity to synthesize cysteine from methionine and that the transsulfuration pathway is mainly active in the liver of even adult humans. The
minimal changes in weight however, suggest that such limited synthesis of the extra hepatic tissues may still be adequate for maintenance of protein synthesis even though inadequate for normal plasma concentrations. Nevertheless, the question of whether adequate cysteine is synthesized for glutathione synthesis in the intravenously fed human is a legitimate one in this scenario. Regrettably, plasma homocysteine was not measured in the study mentioned above.

The results of the above study, though important, do not explain the various pathways of methionine metabolism that result in lower plasma cysteine concentration in the intravenously fed individual when compared to the enterally fed. Using stable isotope technology, Riedijk et al. were able to quantify the various aspects of methionine metabolism in the piglet’s gastrointestinal tract (Riedijk, Stoll et al. 2007). The results of the Riedijk’s study serves to provide some explanation as to why there was a difference in plasma cysteine concentration in the study by Stegink et al.

The goal of the Riedijk’s study was to investigate the metabolic fate of methionine in the gut and the contributions to the whole body rates of transsulphuration and transmethylation (Riedijk, Stoll et al. 2007). This was done using an established stable isotope tracer approach with [1-13C and methyl-2H3]methionine. Fourteen day old piglets were adapted for 7 days to a liquid milk based diet providing a daily intake of methionine and cysteine of 0.25 and 0.31g.kg⁻¹. day⁻¹ respectively. At 20 days of age the piglets were surgically implanted with intraduodenal (ID) and intravenous catheters. The piglets were fed via the ID catheters and the isotopes infusion was administered via the IV catheters. Isotope tracers were infused via a primed continuous infusion for a period
Portal blood flow was monitored by ultrasonography. Arterial and portal blood samples were collected starting at 0 hours and at varying time points up to 8 hours.

The results of the study (Riedijk, Stoll et al. 2007) show that 20% of the total methionine intake was utilized by the gut but that there was no first-pass metabolism of dietary methionine in the gut. Based on the ID methionine tracer absorbed into the portal blood after correction for recycling and arterial uptake, the results showed that the gut preferentially metabolized systemic rather than dietary methionine. Transmethylation as measured by the release of $[^{13}\text{C}]$homocysteine accounted for 67% of the methionine tracer uptake. Transsulphuration, measured by the $^{13}\text{CO}_2$ release by the portal drained viscera accounted for 38% of the methionine tracer uptake. The overall contribution of the gut to whole body methionine metabolism is as follows: twenty percent of the dietary methionine intake is taken up by the gut, 27 and 23% of the whole-body rates of transmethylation and transsulphuration occurs in the gut. On the other hand, 32 and 49% of dietary methionine intake was metabolized in the whole body.

These results demonstrate that the gut is a significant site of methionine transsulphuration and transmethylation, producing significant amounts of homocysteine for net release into the circulation. This data then, explains why enteral nutrition results in higher plasma homocysteine concentrations than TPN (Shoveller, House et al. 2004). This supports the idea that sulphur amino acids are best provided as a balance between methionine and cysteine, with only the minimum amounts of methionine being provided to provide for the particular functions of that amino acid.

In consideration of this form of SAA provision in the diet, it is important to be clear of the role of each of the SAA in particular cysteine as there has been many
suggestions in the literature that it might be conditionally indispensable especially in the neonate. This issue therefore needs further discussion.

2.5 Is Cysteine a Conditionally Essential Amino Acid in Human Neonates?

2.5.1 In Vitro Studies:

Sturman and Gaull were among the first to report on the absence of cystathionase activity in the livers of premature and newborn infants (Sturman, Gaull et al. 1970), and to make the suggestion that cysteine is an essential amino acid in the newborn until sometime after birth. Cystathionase is the second enzyme in the transsulfuration pathway (figure 2.2). When that enzyme is absent or underdeveloped, cystathionine concentrations are elevated. When the livers of fetuses and premature newborns were analyzed for cystathionase activity (Sturman, Gaull et al. 1970), it was completely absent. In addition, cystathionine concentration was very high when compared to controls (adults and children 2 ½ years old). On the other hand when the livers of mature newborns were analyzed for cystathionase activity it was found to be 7% of that present in controls (Sturman, Gaull et al. 1970). This led the authors to propose that cysteine is an indispensable amino acid in premature and new born term infants.

The proposed absence of cystathionase enzyme from fetal liver was further investigated by the same group (Gaull, Sturman et al. 1972; Pascal, Gillam et al. 1972), with similar conclusions drawn, that cystathionas activity was limited to absent from
human fetal liver making cysteine an indispensable amino acid for newborn infants especially those born prematurely.

Ten years later, this story was picked up by Zlotkin et al. (Zlotkin and Anderson 1982), who designed a study with the goal of quantifying the development of cystathionase activity in premature and full-term infants during the first year of life and to identify the factors affecting its maturation. They hypothesized that the preterm infant may have sufficient cystathionase capacity to produce cysteine in amounts adequate to meet estimated needs. This hypothesis was based on the results of a prior study by the same group (Zlotkin, Bryan et al. 1981) in which they found that premature infants on cysteine-free TPN had adequate growth and nitrogen retention which was not improved by adding cysteine to cysteine-free TPN formulation.

In order to measure cystathionase activity, 22 samples of human liver tissue were obtained during post-mortem examination of infants who died prior to 1 year of age; premature and full term. A control group consisted of samples of children who died older than 1 year of age (Zlotkin and Anderson 1982). The results of the study show that cystathionase activity in the liver is dependent of both gestational age and postnatal age. In addition, kidney and adrenals have considerable activity which is not affected by postnatal age. Cystathionase activity was also detected in the pancreas although to a lesser extent than kidneys and adrenals. In the full-term infant there is a gradual increase in liver cystathionase activity during the first few months of life whereas in the premature infant there is a more marked increase during the first 2 weeks of life. The authors concluded that cystathionase activity in the term and premature infant is considerably
greater than previously appreciated and that if the total SAA was provided as methionine only, cysteine may not be a concern.

Studies discussed so far have shown opposite and conflicting results on the presence of cystathionase activity in fetal liver. The group of Gaull et al. (Sturman, Gaull et al. 1970; Gaull, Sturman et al. 1972; Pascal, Tallan et al. 1972) has reported on the absence of cystathionase activity in premature infants thus raising the possibility of cysteine becoming an essential amino acid for premature and newborn infants. On the other hand, Zlotkin and Anderson (Zlotkin and Anderson 1982), reported that cystathionase activity may be sufficient to provide an adequate rate of cysteine synthesis from methionine making cysteine a nonessential amino acid.

In an effort to shed some light on this controversial topic, Vina et al. (Vina, Vento et al. 1995), designed a study to determine if low cystathionase activity was sufficient to maintain normal cysteine status in premature infants and to measure the in vitro rate of glutathione synthesis from methionine in erythrocytes of these infants. Infants were divided into 3 groups: group 1; ≤ 32 weeks gestation, group 2; 33-36 weeks gestation and a control group consisting of term infants. Venous blood samples were extracted in the first 24 hours of life before any oral and parenteral feeding for the determination of SAA concentrations. Glutathione synthesis was also determined from erythrocytes by incubating the cells with amino acid precursors of glutathione. Cells were incubated with glutamine, glycine, methionine and serine or with glutamine, glycine, and N acetyl cysteine. The rate of glutathione synthesis was calculated from the glutathione concentration at various incubating times.
The results of the study (Vina, Vento et al. 1995) showed that plasma cystathionine concentrations were higher in infants in group 1 than in group 2 or controls. On the other hand, plasma cysteine concentrations were lower in groups 1 and 2 premature infants than in mature infants. Also, erythrocytes from group 1 premature infants synthesized glutathione from L-methionine at a much slower rate that did erythrocytes from group 2 premature infants or full-term infants. However the rates of glutathionine synthesis from N-acetyl cysteine were similar in all three groups.

The results are nevertheless difficult to interpret as the current understanding of physiology states that the transsulphuration pathway for the conversion of methionine to cysteine is not present in erythrocytes. Testing of glutathione synthesis in erythrocytes may be the wrong medium from which to assess the functioning of the transsulphuration pathway and draw conclusions regarding the essentiality of cysteine in neonates. These confusing results suggest that cystathionase reaction may be limiting cysteine synthesis from methionine especially when the gestational age is \( \leq 32 \) weeks and if anything supports the observation by Zlotkin and Anderson (Zlotkin and Anderson 1982), that cysteine synthesis although slow in this study (Vina, Vento et al. 1995), may still be able to provide adequate cysteine for its functions. The observation that glutathione synthesis is also slower in group 1 premature infants raises a question as to whether premature infants will be more subject to oxidative stress as a result of deficient glutathione availability as a result of inadequate amounts of cysteine made via the transsulphuration pathway when the total SAA is provided as methionine only. If such is the case, neonates especially those that are premature who are fed cysteine free TPN could be at risk of increased oxidative stress or a decrease in their ability to recover from such stressors.
2.5.2 Plasma Amino Acids

There have been many published reports of low plasma cysteine in infants receiving TPN (Pohlandt 1974; Winters 1977; Kanaya, Nose et al. 1984), when compared to the plasma cysteine concentration of breastfed infants (Wu, Edwards et al. 1986). These observed low plasma cysteine concentrations in premature and newborn infants have led investigators to suggest that cysteine is an essential amino acid in the premature and newborn infant. It must be remembered that breast milk contains a relatively high cysteine content providing 60% of the total SAA in breast milk with methionine providing 40%. On the other hand, the plasma cysteine concentrations reported from these infants studied are based on the provision of a TPN solution that is usually devoid of cysteine or containing only minimal cysteine.

In 1974, Pohlandt designed a study to test the hypothesis that cysteine is an essential amino acid in newborn premature and full-term infants (Pohlandt 1974). He did this by measuring the plasma cysteine concentrations in response to a 5% dextrose infusion over the first 2 days of life and again in response to an L-amino acid mixture free of cysteine. The results show that in premature newborns, the plasma cysteine concentrations decreased markedly within the first 12 hours of life and remained low thereafter. Also for the full-term infants plasma cysteine concentrations decreased in a similar fashion. In response to the cysteine-free, methionine-containing amino acid solution, cysteine concentrations remained low in both term and premature infants, despite elevated methionine concentrations. This led Pohlandt to suggest that cysteine is a semi-essential amino acid in the newborn infant.
Earlier studies conducted by Stegink et al (Stegink and Baker 1971), also showed low plasma cysteine/cysteine concentrations in infants receiving intravenous parenteral solutions with protein source as casein and beef fibrin hydrolysates.

These reports along with others, have been used as evidence that cysteine is an essential amino acid in the newborn infant. Plasma amino acid concentrations have been shown to be relatively insensitive in both animal (Ball and Bayley 1984), and humans (Young, Tontisirin et al. 1972). It has been discussed that while plasma amino acids may give valuable information about some aspects of amino acid metabolism, it is not a sensitive endpoint from which the requirement for an amino acid should be determined.

2.5.3 Nitrogen Balance

In an effort to determine if cysteine is an essential amino acid for the intravenously fed newborn infant, growth, nitrogen balance and plasma sulphur amino acid concentrations were measured in term and preterm infants on cysteine free and cysteine supplemented TPN (Zlotkin, Bryan et al. 1981). Twenty-eight infants were studied; 17 premature and 11 full-term. Infants were alternatively assigned into a control and experimental group. Both groups received adequate protein and calories with the experimental group receiving cysteine of 77 mg.kg\(^{-1}\).day\(^{-1}\) (the only difference in the diet). Each study was conducted over a 5-day period, with the first 3 days serving as the adaptation days.

The results of the study (Zlotkin, Bryan et al. 1981) show that there was no difference in the nitrogen retention between the un-supplemented and cysteine-
supplemented group. Both groups showed similar positive nitrogen retention of 282 mg.kg⁻¹.day⁻¹ which was 56% of nitrogen infused. These retentions also paralleled the expected nitrogen *in utero* retention. In addition, there was no difference in the weight change between groups. As expected, plasma cysteine concentrations were higher in the cysteine supplemented group. Nitrogen retention and plasma cysteine were not affected by postnatal age.

*Zlotkin et al.* later published another study (*Zlotkin and Anderson 1982*) in which they reported on the sulphur balance in the cysteine supplemented and un-supplemented groups of infants discussed above (*Zlotkin, Bryan et al. 1981*). They were able to account for 99% of the sulphur infused into the un-supplemented group but only 95% of the sulphur infused into the supplemented group. Of the sulphur retained in the cysteine supplemented group 75% was calculated to be retained in lean tissue and total body sulphate whereas the other 25% remained unaccounted for. They interpreted the failure to account for all of the sulphate infused in the cysteine supplemented group as errors in the balance technique. This line of argument is not substantiated by their ability to account for all of the sulphate infused into the un-supplemented group. Rather, it is possible that the unaccounted for sulphate was packaged into glutathione as the supplemented cysteine was used for glutathione synthesis in that group of neonates (*Zlotkin and Anderson 1982*).

The results of *Zlotkin et al.* (*Zlotkin, Bryan et al. 1981*), were validated in a later study of similar design with a goal of determining if cysteine supplementation improved nitrogen retention and weight gain in neonates on TPN (*Malloy, Rassin et al. 1984*). Cysteine was supplemented at 72 mg.kg⁻¹.day⁻¹ (cysteine-HCL) at two different levels of
nitrogen intakes 240 and 400 mg.kg$^{-1}$.day$^{-1}$. The results, as that of Malloy et al., showed no difference in nitrogen retention or weight gain in the cysteine supplemented group when compared to the cysteine un-supplemented group. Unlike Zlotkin et al., the authors postulated that the lack of effect of cysteine supplementation, on nitrogen balance could be related to the concentration of cysteine being held as glutathione in the liver.

2.5.4 Stable Isotope Tracer Studies

In 1995, Miller et al. published a stable isotope tracer technique to assess human neonatal amino acid synthesis using D-[U-$^{13}$C]glucose (Miller, Jahoor et al. 1995). With this technique the conversion of glucose carbon into seven nonessential amino acids was assessed by measuring their isotopic enrichments in plasma using gas chromatography/mass spectrometry (GC/MS). Using this technique they were unable to detect significant $^{13}$C enrichment in plasma cysteine (Miller, Jahoor et al. 1995; Miller, Jahoor et al. 1995). This led them to suggest that cysteine is an essential amino acid in parenterally fed premature neonates.

Ten years later, the same group using the same method but a more sensitive end point, apo B-100, were able to detect $^{13}$C labeled cysteine in hepatically derived apo B-100 (Shew, Keshen et al. 2005). The tracer/tracee ratios of the M+1 isotopomer of cysteine derived from apo B-100 were significantly greater after the $[^{13}$C$_6]$glucose than at baseline. They also found a direct correlation between the increase in cysteine synthesis and birth weight. They concluded that a functional pathway exists for cysteine synthesis in premature neonates and that the minimum synthetic capacity of this pathway is directly
related to neonatal maturity. The question left to be answered is whether this minimum synthetic capacity is sufficient to sustain normal protein and glutathione homeostasis. From the data of Zlotkin (Zlotkin, Bryan et al. 1981; Zlotkin and Anderson 1982) and Malloy et al. (Malloy and Rassin 1984), it seems that the synthetic capacity for cysteine is sufficient to sustain normal protein homeostasis. The ability to promote and sustain glutathione homeostasis remains in question.

The latest study to address the question of the indispensability of cysteine was recently published and was conducted in the enterally fed preterm neonate (Riedijk, van Beek et al. 2007). The study was designed to determine the cysteine requirement of the enterally fed preterm neonate and was based on the assumption that cysteine was an essential amino acid in the preterm neonate. Twenty five formula fed neonates between the ages of 32 to 34 weeks gestation were randomized to receive graded intakes of cysteine ranging from 11 to 65 mg.kg\(^{-1}\) in the presence of a methionine intake of 70 mg.kg\(^{-1}\).day\(^{-1}\). The oxidation of \([1^{-13}C]\)phenylalanine was measured in response to graded intakes of cysteine. The results of the oxidation data showed that there was no change in the oxidation of phenylalanine in response to changes in the intake of cysteine. The results of this study provided clear evidence that cysteine is not a conditionally essential amino acid in the preterm neonate.

Since all of these data previously discussed used endpoints aimed at measuring cysteine adequacy for protein synthesis, there is clearly an identified need to measure the adequacy of cysteine for antioxidant status; namely glutathione synthesis. But first, a review of glutathione metabolism, functions and synthesis is clearly required.
2.6 Glutathione

2.6.1 Introduction to Glutathione Metabolism

The tripeptide glutathione (gama-glutamyl-cysteinyl-glycine:GSH) is synthesized \textit{de novo} within all cells from glycine, cysteine and glutamate (Reid and Jahoor 2000). Although synthesized within all cells, the liver is the major producer and exporter of GSH. It is synthesized primarily if not exclusively in the cytoplasm (Smith, Jones et al. 1996). Therefore, most of the cellular GSH (85-90\%) is also present in the cytosol. Unlike the synthesis of larger peptides, no RNA template is involved in GSH synthesis (Beutler 1989).

There are two steps in the synthesis of GSH: first the enzyme \( \gamma \)-glutamyl cysteine synthetase catalyzes the formation of a peptide bond between the \( \gamma \)-carboxyl group of glutamic acid and the amino group of cysteine (Figure 2.3). This is the rate limiting step in GSH synthesis (Meister and Anderson 1983). In the next step, glycine is joined to \( \gamma \)-glutamyl cysteine (figure 2.3) to form GSH. This reaction is catalyzed by glutathione synthetase (Beutler 1989). Glutathione has an inhibitory influence on the first enzyme \( \gamma \)-glutamyl cysteine synthetase, which acts as feedback control for the regulation of GSH synthesis. In rare cases of hereditary deficiency of glutathione synthetase, the second reaction in GSH synthesis is halted and large amounts of \( \gamma \)-glutamyl cysteine accumulates, which is then catabolized to 5-oxyproline and excreted in the urine (Beutler 1989).

Most of the functions of GSH require its reduced form, (figure 2.3) in which state it has a free sulfhydryl group and is designated GSH. However, the cysteine residue is
easily oxidized nonenzymatically to glutathione disulfide (GSSG). Because most of the functions of glutathione require its reduced form, an active enzyme mechanism exists, in the form of glutathione reductase for the reduction of GSSG to GSH (figure 2.3). This enzyme uses NADPH or NADH as the hydrogen donor. Hence the activity of glutathione is very dependant on the intake of riboflavin (Beutler 1989). Not only is the activity of GSH dependant on the intake of riboflavin, but it is worth noting that the concentration of GSH itself, as well as the enzymes involved in its metabolism, is markedly influenced by diet (Beutler 1989).

Glutathione disulfide consists of two glutathione molecules joined by a disulfide bond at the cysteine sulphydryl groups. This oxidized form of glutathione is designated GSSG (Figure 2.5).
Figure 2.3 Glutathione metabolism

Reaction 1. γ-Glutamyl synthetase; Reaction 2. Glutathione synthetase; Reaction 3. Oxidation of GSH by O2; conversion to GSH to GSSG is also mediated by free radicals; Reaction 4. GSH peroxidase; Reaction 5. GSSG reductase.
GLUTAMIC ACID
\[ \text{NH}_2 \]
HOOCCH\text{CH}_2\text{CH}_2\text{CO} - \text{NH} \quad \text{GLYCINE}
HSCH\text{CH}_2\text{CH}_2\text{CO} - \text{NHCH}_2\text{COOH} \quad \text{CYSTEINE}

Figure 2.4 Structure of reduced glutathione (GSH)
The tripeptide is composed of glutamic acid, cysteine and glycine.

Figure 2.5 Structure of glutathione disulfide (GSSH) (Oxidized form of GSH)
2.6.2 Functions of Glutathione:

The intracellular concentration of GSH in mammalian cells is in the millimolar range (0.5-10 mM) with 85-90% being present in the cytosol. The extracellular concentration (with the exception of bile acids which contain up to 10 mM L\(^{-1}\)) is typically in the micromolar range e.g. 2-20 µM L\(^{-1}\) (Meister and Anderson 1983). Glutathione is therefore regarded as the most prevalent intracellular thiol (Meister and Anderson 1983) and the most important endogenous antioxidant and scavenger (Wernerman and Hammarqvist 1999). The [GSH]:[GSSG] ratio is often used as an indicator of the cellular redox state and is >10 under normal physiological conditions.

Glutathione is consumed in the detoxification of electrophilic metabolites and xenobiotics, and is an effective free radical scavenger, protecting cells from the toxic effects of reactive oxygen compounds (Reid and Jahoor 2000). Through the enzyme glutathione peroxidase (figure 2.3), GSH removes peroxides that could oxidize sulfhydryls and participates in several reactions that serve to prevent oxidation of SH groups or to reduce them once they have become oxidized (Beutler 1989). This function is important to promote and protect the normal functioning of proteins.

Glutathione is also important for its role in detoxification, converting the first step in the conversion of formaldehyde (a toxic product of methanol oxidation) to formic acid (Beutler 1989). Glutathione is needed for the synthesis of leukotrienes, making GSH an important mediator of inflammation (Beutler 1989). Glutathione plays an important role in amino acid transport and is a source of cysteine reserve during food deprivation, and a major source of cysteine for lymphocytes (Malmezat, Breuille et al. 2000).
Irreversible cell damage occurs when the cell is no longer able to maintain its content of GSH (Reid and Jahoor 2000). Indeed poor prognosis is associated with decreasing GSH concentration in certain disease states. Consequently an understanding of GSH metabolism and kinetics with particular emphasis/knowledge of substrate needs for its synthesis is of importance in health as well as in disease states.

2.6.3 Glutathione: Turnover and Metabolism

Although GSH is synthesized primarily in the cytoplasm, it is utilized in other compartments (e.g. nucleus, mitochondria, endoplasmic reticulum and in the extracellular space) for a variety of functions as previously discussed (Smith, Jones et al. 1996).

Glutathione is supplied to the mitochondria through an energy dependant transport system that couples GSH uptake to efflux of metabolic anions (Smith, Jones et al. 1996). Supply to the nucleus involves passive diffusion whereas the supply to extracellular compartments like plasma depends on efflux of GSH and redox control mechanisms that allow extracellular reduction of GSSG (Smith, Jones et al. 1996). Extracellular GSH pools, particularly the plasma pool, have been studied extensively in humans with decreased GSH concentration observed in HIV, cirrhosis, malnutrition and premature infants (Vina, Vento et al. 1995; Bianchi, Bugianesi et al. 1997; Jahoor, Jackson et al. 1999; Bianchi, Brizi et al. 2000; Reid, Badaloo et al. 2000). The enzyme glutamyl transpeptidase is of major importance in GSH metabolism, as it initiates GSH degradation (Meister and Anderson 1983). This enzyme is located on the external surface of certain cells especially renal cells. Glutathione is translocated out of cells, and cells
that have membrane bound $\gamma$-glutamyl transpeptidase can utilize translocated GSH (renal 
cells) whereas GSH exported from cells that do not have appreciable transpeptidase (e.g. 
liver cells) enters the blood stream. Glutathione is removed from the plasma by the 
kidney and other cells that have transpeptidase.

In a study looking at GSH turnover and metabolism in rats, (Griffith and Meister 
1979) plasma and tissue levels of GSH were measured after acute as well as chronic 
treatment of rats with DL-buthionine-SR-sulfoximine (BSO) a potent selective inhibitor 
of $\gamma$-glutamyl- cysteine synthase and therefore GSH synthesis. Two hours after BSO 
injection, there was a 72%, 65%, 54% and 33% decrease in GSH concentration in the 
kidney, liver, pancreas and muscle respectively. The greater decrease in GSH 
concentration in the kidney compared to the liver reflects a more rapid turnover of GSH 
in kidney compared to liver. Despite a more rapid turnover of GSH in the kidney 
compared to liver, after the single dose of BSO the decline in GSH concentration in 
kidney and liver was smooth rather than biphasic. There was a more rapid return of the 
GSH content in the liver which may reflect an increased rate of enzyme synthesis or an 
increased capacity of liver to metabolize BSO. Nevertheless, the similar pattern of 
response in GSH concentration provides evidence for a single pool of GSH between the 
tissues which turnover at very similar rates, or rather different pools between tissues 
which turn over at similar rates (Griffith and Meister 1979).

In addition to decreased levels of tissue GSH, the plasma levels of GSH also 
decreased by 67% 2 hrs after BSO injection. This rapid turnover of plasma GSH supports 
the conclusion that the plasma pool of GSH is derived from the tissue in which GSH 
turns over rapidly and which have relatively little transpeptidase (Griffith and Meister
The GSH levels of brain, lung and spleen were not significantly different 2 hrs after BSO injection when compared to controls. This led the authors to conclude that the results reflected the high erythrocyte content of these tissues because erythrocyte GSH has a very low rate of turnover. This statement should be viewed only in the context in which it is made and that is; erythrocyte GSH turnover compared with hepatocyte or renal cell GSH turnover. On the other hand the unchanged GSH level in brain and lung may be only partly, if at all related to the erythrocyte content of these tissues.

Nevertheless, it must be noted that this statement is contrary to results in human studies (Lyons, Rauh-Pfeiffer et al. 2000) in which the FSR of GSH in healthy adult males was 65%, suggesting that all the GSH is completely replaced by 1.5 days. This translates into a half life of 18 hrs suggesting that erythrocyte GSH has a relatively high turnover rate.

In that same study (Griffith and Meister 1979) mice were given BSO orally for 15 days to look at the effect of chronic administration of BSO on GSH metabolism. The tissue GSH levels were much lower than those found after 2 hrs: with the exception of liver which showed a 44% decrease compared to the 65% decrease previously shown after the 2 hr BSO treatment.

In an effort to prove the importance of the kidney in GSH metabolism the same authors in a separate experiment (Griffith and Meister 1979) measured the plasma GSH concentrations at baseline, and again in unilaterally and bilaterally nephrectomized rats. The results were 17-18 µM, 22 µM and 33 µM respectively. When bilaterally nephrectomized animals were given a transpeptidase inhibitor, the plasma GSH was increased to 170 µM. This suggests that the transpeptidase present in extra renal tissues uses the GSH released into the plasma from various tissues. Through a series of
experiments the authors calculated that 2/3 of the plasma GSH is removed by the kidney with the remaining 1/3 being removed by the extrarenal transpeptidase.

It is clear from these experiments that the highest turnover rate of GSH occurs in the kidney, followed by liver, pancreas and skeletal muscles. Nevertheless, the liver possesses a higher synthetic capacity for GSH.

2.6.4 Glutathione Kinetic Measurement:

2.6.4.1 Concentration Measurement

In the biological compartments, changes in GSH concentration are affected if there is a difference between the rates of synthesis and the rates of disposal of GSH (Reid and Jahoor 2000). A single concentration measure therefore, while it gives a static measure of previous kinetics and of amounts of GSH available or lacking in that compartment and possible surrounding tissues, tells us nothing about the rates of synthesis and loss of GSH. Consequently, kinetic measures using radio and stable isotope tracers provide an opportunity to make meaningful interpretation of concentration measurements.

2.6.4.2 Kinetic Measurement

The first report in which GSH kinetics was measured was by (Dimant, Landsberg et al. 1955). They used the rate of incorporation of orally administered $^{15}$N glycine to estimate GSH synthesis in erythrocytes. This provided the first evidence that erythrocytes synthesize GSH de novo. Recent reports suggest that erythrocyte contribute up to 10% of
whole body GSH synthesis in humans (Wu, Fang et al. 2004). Subsequent to the Dimant report in 1955, kinetic studies used intravenous (IV) injections of supraphysiologic doses of GSH with measurement of loss from the plasma compartment; still others measured incorporation of radio labeled precursors of GSH into \textit{in vitro} systems. These methods were flawed because they suggested that plasma GSH reflected inter-organ, particularly hepatic GSH efflux. It must be noted that venous plasma GSH concentration is higher than arterial GSH concentration suggesting a limited role for plasma GSH in inter-organ GSH homeostasis (Reid and Jahoor 2000). In addition, plasma GSH \textit{in vivo} is much less than intracellular concentration (µmol versus mmol) (Reid and Jahoor 2000), not to mention the fact that plasma GSH is highly unstable, readily undergoing auto-oxidation to GSSG or protein GSH disulphides (Reid and Jahoor 2000).

In 1995, with the development of a stable isotope precursor product model for measuring GSH synthesis \textit{in vivo}, (Jahoor, Wykes et al. 1995), there began an opportunity for the more effective characterization of various aspects of GSH metabolism and improvement in this body of knowledge.

\textbf{2.6.4.3 The Precursor Product Model}

The precursor product model for measuring GSH kinetics was developed by Jahoor \textit{et al.}, 1995 (Jahoor, Wykes et al. 1995). The minimum requirement for the calculation of the rate of synthesis of a protein or peptide with this model is the measurement of the isotopic enrichment at two time points during the quasilinear portion of the exponential increase in peptide-bound amino acid labeling (Reid and Jahoor 2000). In addition, an estimate of the enrichment of amino acid (AA) tracer in the precursor pool
(the free pool of the tissue being studied) is necessary since the AA tracer should be at isotopic steady state before a measurement of its incorporation into the protein/peptide is made.

2.6.4.3.1 Infusion Protocol:

A primed continuous infusion (CI) of either $^{13}$C$_2$ or $^2$H$_2$ glycine is administered intravenously or intragastrically for 6 hrs in neonates and 7 - 8 hrs in adults (Reid and Jahoor 2000). $^{13}$C$_2$ glycine is used for glycine flux measurements because of the loss of one or two of the deuterium when $^2$H$_2$ glycine is used as the tracer (Reid and Jahoor 2000). Blood samples are collected at baseline and hourly during the infusion, with sampling restricted to the last 3 hrs of infusion in neonates and small children (Reid and Jahoor 2000). The rate of synthesis of erythrocyte GSH is obtained from the rate of incorporation of $^{13}$C$_2$ or $^2$H$_2$ glycine into the GSH. Erythrocyte-free glycine isotopic enrichment is used to represent the enrichment of the glycine precursor pool from which erythrocytes make GSH (Reid and Jahoor 2000).

2.6.4.3.2 Sample Analysis:

As part of the sample analysis for the method developed by Jahoor et al., 1995, the erythrocyte GSH is first isolated on HPLC. This is done by an elution process using a fraction collector. The eluted GSH molecule is then dried and hydrolyzed to each of its individual amino acids. The glycine (or the amino acid tracer used) is then converted to the $n$-propyl ester, heptafluorobutyramide derivative and the tracer to tracee ratio.
determined by GCMS. This process is very complex, involving several steps and more than one instrument for analysis.

Recently our collaborator, Dr. Linda Wykes, developed a simpler method in our laboratory for analyzing the complete tripeptide molecule, which bypasses the isolation and fractionation steps. For this method she was able to analyze the derivatized GSH molecule as isolated from erythrocytes by LCMS/MS (Tandem mass spectrometry). Since the whole molecule is analyzed, the use of an M+2 isotope tracer is not possible due to the high background enrichments. Therefore, an M + 3 tracer has to be used if the samples are to be analyzed by this method.

2.6.4.3.3 Calculations:

- The fractional synthesis rate (FSR) of erythrocyte GSH is calculated as follows:
  - \[ FSR_{GSH} \text{ (\%/h)} = \frac{PE_{t2} - PE_{t1}}{IE_{pl}} \times \frac{1}{(t_2-t_1)} \times 100 \]
  - Where \( PE_{t2} - PE_{t1} \) = the increase in the enrichment of GSH bound glycine over the period \( PE_{t2} - PE_{t1} \) of the infusion.
  - \( IE_{pl} \) = isotopic enrichment at plateau of erythrocyte- free glycine

- Absolute synthesis rate (ASR) of GSH is calculated as follows
  - \[ ASR = GSH_{\text{mass}} \times FSR_{GSH} \]
  - Where \( GSH_{\text{mass}} \) = the product of the cell volume (or cell number or cell protein) and the concentration of GSH in the cell.
2.6.5 Glutathione Metabolism and Synthesis Rates:

2.6.5.1 In Healthy States:

The availability of cysteine is believed to be the most important nutrient rate-limiting factor for GSH synthesis. To determine the importance of dietary cysteine availability for GSH synthesis Lyons et al. (Lyons, Rauh-Pfeiffer et al. 2000) measured the fractional and ASR of GSH during 2 separate 10-day periods following a 10-day adaptation to an L-AA diet supplying adequate (1 g.kg\(^{-1}\)) protein (AP) or a sulphur amino acid (SAA) (methionine and cysteine) free mixture. After the adaptation period, GSH kinetics was measured in the fasted state via a primed continuous infusion (CI) of L \([1-^{13}C]\)cysteine at 3 μmol.kg\(^{-1}\).h\(^{-1}\) for 6 hrs. The result of that study (Lyons, Rauh-Pfeiffer et al. 2000) showed that cysteine oxidation accounted for 15% of the cysteine disposal when the AP diet was consumed falling to 5% with the SAA free diet reflecting, supposedly, a conservation of methionine and cysteine.

The FSR of GSH was 0.65 for the AP diet and 0.49 for the SAA free diet. The ASR was 747 ± 216 and 579 ± 135 μmol. L\(^{-1}\).day\(^{-1}\) for the adequate and SAA free diet respectively. This represented a significant difference between the groups for fractional and ASR. Nevertheless there was no difference in GSH concentration between the groups with GSH concentration being 1142 ± 242 and 1216 ± 162 μM in the adequate and SAA free diet respectively. This demonstrates that in healthy adult subjects, a restricted SAA intake slows the rate of GSH synthesis. However the decrease in GSH synthesis did not translate into a decrease in GSH concentration. This therefore suggests that there was decrease in GSH turnover at the deficient sulphur amino acid intake and provides further
evidence that GSH concentration alone cannot be used as an isolated marker of GSH metabolism, since it does not reflect changes in GSH synthesis within the cell.

In addition to decreasing synthesis of GSH with a SAA free diet, a decrease in GSH synthesis has also been observed in response to 30% decrease in protein intake in healthy adults (Jackson, Gibson et al. 2004). Using a prime CI of $^{13}$C$_2$ glycine tracer (20 µmol.kg$^{-1}$ and 15 µmol.kg$^{-1}$.hr$^{-1}$) the erythrocyte GSH synthesis was measured in 12 young adults (6 males, 6 females). Each subject underwent a 7- hour infusion protocol to measure the rate of erythrocyte GSH synthesis on 3 occasions: at baseline while consuming their habitual protein intake (1.13g.kg$^{-1}$.day$^{-1}$), and on day 3 and 10 of consumption of a diet that provided the safe WHO-recommended amount of dietary protein of 0.75 g.kg$^{-1}$.day$^{-1}$ (FAO/WHO/UNU 1985). The results showed that FSR on day 3 was significantly lower than at baseline and remained decreased at day 10. Similarly ASR decreased significantly (22%) from baseline on day 3 but was not different from baseline on day 10 (Jackson, Gibson et al. 2004). Similar to the findings by Lyons et al., 2000, the mean GSH concentration on day 10 had recovered to baseline levels and was not significantly different on day 10 from that observed at baseline. However, the GSH concentration on day 3 was significantly lower than that observed at baseline. It is unclear if these changes occurred in the study by Lyons et al., 2000 because GSH concentration was only measured on day 10. There was no significant changes in enzymes associated with GSH cycling and the concentrations of amino acids (AA) on day 3. However, by day 10 erythrocyte concentrations of glycine, glutamate and glutamine increased significantly. These findings suggest that despite the body’s ability to adapt and possibly regain nitrogen (N) equilibrium by day 10, low protein and hence
low AA intake may cause functional loss leading to metabolic consequences such as decreased ability of the host to synthesize important non-protein substances like GSH (Jackson, Gibson et al. 2004). The results also provide evidence that the WHO recommended protein intake of 0.75 g.kg\(^{-1}\) is too low for the healthy adult. This is in agreement with a study recently published by our group which shows that the protein requirement of the adult human is 1.0 g.kg\(^{-1}\).day\(^{-1}\) (Humayun, Elango et al. 2007).

The results of Jackson’s study (Jackson, Gibson et al. 2004) begs the question whether the cysteine requirement for protein synthesis is a fraction of the total required for the synthesis of protein, plus non-protein substances like GSH? This question was partly answered in a study by Hiramatsu et al., (Hiramatsu, Fukagawa et al. 1994) in which the tracer-derived estimate of cysteine flux was considerably higher than that predicted from estimates of protein turnover. In order to further understand the components of cysteine flux, the same group conducted a follow-up study (Fukagawa, Ajami et al. 1996), in 7 healthy adults (3 males, 4 females). The subjects received three different isotope tracer infusions of L- [methyl-\(^2\)H\(_3\);1-\(^{13}\)C] methionine, L [3,3-\(^2\)H\(_2\)] cysteine and L – [methyl -\(^2\)H\(_2\)]Leucine for almost 8 hrs. After a 3 hour baseline period, GSH was infused at 32 \(\mu\)mol. kg\(^{-1}\). h\(^{-1}\) until the end of the study. In the final hour of GSH administration, cysteine flux was increased significantly by \(~60\%) from 55 to 88 \(\mu\)mol. kg\(^{-1}\). h\(^{-1}\). In addition, remethylation rates increased whereas transsulfuration rates were significantly reduced during GSH administration. The authors concluded that, based on the data, GSH breakdown accounts for 50\% of cysteine flux in the basal state and for all of the increase in measured cysteine turnover during exogenous GSH infusion (Fukagawa, Ajami et al. 1996). This study also shows that in healthy adults, the
endogenous disappearance rate of GSH (into cysteine) is \( \sim 26 \mu\text{mol.kg}^{-1}\text{.hr}^{-1} \), supporting the view that GSH is a major transport/storage form of cysteine in the body.

In a study designed to validate the method of using urinary sulphur (S) excretion to measure whole body SAA catabolism and explore the use of whole body nitrogen/sulphur (N/S) balance ratio as a non-invasive indicator of non-protein SAA storage under different nutritional conditions in growing pigs (Hou, Wykes et al. 2003), animals were randomized to receive an adequate protein (AP) or a low protein (LP) diet (50% NRC requirements) for 10 days. On day 10, L-methionine (1.2 mmol.kg\(^{-1}\)) was infused into the animals over a 2 hour period. In that study, (Hou, Wykes et al. 2003) the rate of weight gain in the pigs was not significantly different between groups 79.4 g.kg\(^{-1}\).day\(^{-1}\) and 70.5 g.kg\(^{-1}\).day\(^{-1}\) in the AP and LP group respectively. Sulphate excretion from methionine infusion was significantly lower in the LP than the AP group. Before methionine infusion, nitrogen (N) balance was significantly lower in the LP than in the AP group and further decreased slightly in both groups following methionine infusion. However, in the AP group the S balance and N/S balance ratio was not different from baseline (before infusion) after the methionine infusion whereas in the LP group the S balance became more positive after methionine infusion with a further decrease in the N/S balance ratio. The results also suggest that the composition of body weight gain was different in the LP and AP fed piglets. Whereas 79% of weight gain in the AP piglets was lean tissue, only 36% of the weight gain in the LP piglets was lean tissue. In the AP piglets the entire methionine load was catabolized and excreted in the urine whereas only 69% of the infused methionine was excreted in the LP piglets. This S retention was not due to increased methionine uptake for protein synthesis because there was no increase in
N balance in the face of the increased S balance. This indicates that an improvement in SAA balance can be achieved even in the presence of worsening N balance suggesting that methionine was retained in a non protein form, possibly GSH (Hou, Wykes et al. 2003).

2.6.5.2 In Stress/Disease:

It has long been known that GSH concentration is reduced in several disease states including HIV infection (Jahoor, Jackson et al. 1999), liver cirrhosis, (Bianchi, Bugianesi et al. 1997; Bianchi, Brizi et al. 2000), diabetes (Ghosh, Ting et al. 2004), Sickle cell disease (Reid, Badaloo et al. 2006) and Alzheimer’s disease (Liu, Harrell et al. 2005). Glutathione concentration is also found to be reduced in surgical trauma (Luo, Hammarqvist et al. 1998) septic patients (Lyons, Rauh-Pfeiffer et al. 2001), premature infants (Vina, Vento et al. 1995) as well as in children with severe protein energy malnutrition (Reid, Badaloo et al. 2000; Badaloo, Reid et al. 2002). The mechanism surrounding this decreased concentration was believed to be increased utilization. However, protein deficient animals subject to the stress of inflammation are unable to maintain GSH homeostasis and ASR, while piglets fed adequate protein maintained GSH homeostasis even when subjected to the stress of inflammation (Jahoor, Wykes et al. 1995). In addition, cysteine and methionine supplementation was shown to modulate the effect of TNF-α on protein and GSH synthesis in animals fed a low protein diet (Hunter and Grimble 1994). Additionally, survival of guinea pig pups subjected to oxidative stress was improved by feeding nutritional substrate for GSH synthesis (Chessex, Lavoie et al. 1999).
To determine the mechanism of decreased GSH concentration in HIV a primed, continuous infusion (CI) of $^2$H$_2$ glycine was used to measure fractional and ASR of GSH in 5 healthy and 5 symptom-free HIV infected subjects before and after supplementation with N-acetylcysteine (NAC) (Jahoor, Jackson et al. 1999). N-acetylcysteine was supplemented at 20 mg.kg$^{-1}$.day$^{-1}$ (15 mg.kg$^{-1}$.day$^{-1}$ cysteine). The $^2$H$_2$ glycine was infused by a primed (15 µmol. kg$^{-1}$) and CI (15 µmol. kg$^{-1}$) for 5 hours. After one week of supplementation, there was a significant increase in the erythrocyte cysteine concentration in the HIV group (NAC supplemented), to a value not different from control group. Baseline plasma GSH concentration which was lower in the HIV group than control group significantly increased after supplementation with NAC but not to a value that was higher than the control group. Although the baseline ASR of GSH was lower in the HIV than in the control group, after one week of NAC supplementation, both fractional and ASR of GSH experienced a significant increase in the HIV group. The authors concluded that the GSH deficiency in HIV infection is due in part, to reduced synthesis, secondary to cysteine deficiency (Jahoor, Jackson et al. 1999).

A study by Reid et al., (Reid, Badaloo et al. 2000) in a group of malnourished Jamaican children showed that those with edematous protein energy malnutrition (PEM) had significantly lower plasma and erythrocyte GSH concentrations than those with non-edematous PEM. In order to delineate the mechanism of this GSH deficiency, a prime continuous infusion of $^2$H$_2$ glycine (40 µumol kg$^{-1}$ and 40 µumol.kg$^{-1}$.hour$^{-1}$) was infused over 6 hours via nasogastric infusion. Three isotope infusion studies were conducted: study1, shortly after admission to hospital, study 2, 7-10 days after admission and study 3, at recovery, just before discharge. Children with edematous malnutrition had
significantly lower ASR of erythrocyte GSH than children with nonedematous malnutrition, both shortly after admission and at day 10, than at recovery. Plasma cysteine concentrations were also significantly lower in the edematous group at studies 1 and 2 than at recovery. In contrast, erythrocyte GSH concentration, rates of GSH synthesis and plasma and erythrocytes free glycine concentrations of the nonedematous group were similar at all times and greater at studies 1 and 2 than in the edematous group (Reid, Badaloo et al. 2000).

In a follow-up study conducted by the same group in a similar population of patients in the same hospital and country (Jamaica), Badaloo et al., (Badaloo, Reid et al. 2002), used a similar study design, except for the introduction of cysteine supplementation in the form of NAC at 81.6 mg.kg\(^{-1}\).day\(^{-1}\) of NAC (~60 mg.kg\(^{-1}\).day\(^{-1}\) cysteine) or alanine (control group) which was started immediately after period 1 and continued until recovery. They (Badaloo, Reid et al. 2002) found that GSH concentration almost doubled from period 1 to period 2 in the NAC supplemented group and that the ASR also doubled in the NAC supplemented group. Importantly, the time taken to lose the edema was significantly less (9 days) in the NAC supplemented group compared to control group (14 days). This study shows that increasing the substrate for GSH synthesis promotes not only an increased GSH synthesis, but that increased synthesis and therefore availability of GSH leads to a quicker recovery at least in children with edematous PEM. It would seem also from these results that there is a partitioning of available resources between protein synthesis and GSH synthesis such that only in the presence of additional substrate is there an increase in GSH synthesis with decreased morbidity measured by shorter recovery time. This is obvious because in the control group, positive N balance
was attained (weight gain) with no significant difference in weight gain between either
groups in either periods. Nevertheless only in the group receiving NAC supplementation
was there increased GSH synthesis and shortened recovery time. Hence, an adequate
protein intake to promote N balance and protein synthesis may not be all that is necessary
for the total well being of the host.

This hypothesis was partly addressed in a study conducted by Hunter & Grimble,
(Hunter and Grimble 1994) in rats. The study was designed to examine the essentiality of
an adequate supply of SAA during a response to TNF-α. Rats were fed a LP diet (8%)
supplemented with cysteine and alanine, methionine and alanine or alanine alone, or a
normal diet (20% protein), for 8 days before injection of TNF or saline. A second
experiment was conducted to serve as the control group in which animals were fed the
same but received no treatment. The results showed that supplementation of the low
protein diet with either cysteine or methionine improved growth and increased liver and
lung GSH concentration, zinc concentration, protein concentration and protein synthesis
compared with the alanine supplemented group. Most importantly the changes in the
protein synthesis and GSH concentration of the liver in response to the TNF showed that
SAA may be partitioned to a greater extent into hepatic protein than into GSH when SAA
intake is low (Hunter and Grimble 1994).

The results from all the above studies suggest an increased need for SAA
particularly cysteine during illness, infection, disease. This increased need for cysteine
can be partly explained by the increased need for GSH synthesis; a lack of which can lead
to slower recovery time and prolonged illness (Badaloo, Reid et al. 2002).
Malmezat et al., (Malmezat, Breuille et al. 2000) conducted a study in rats to determine GSH concentration and kinetics as well as enzyme activity in various tissues of infected rats. Pair-fed rats were used as controls because of knowledge that infection leads to anorexia in these animals. Rats were fed *ad libitum* for 5 days and then injected with live *E. Coli* or saline. Glutathione synthesis rates were determined by a 4 or 6 hr $^{15}$N glycine infusion. Animals were sacrificed and blood, liver, spleen, lung, heart, small and large intestine and muscle from hind leg were excised and analyzed. The results show that GSH concentration was significantly greater in septic rats in all tissues than in pair-fed controls except for small intestine and blood (Malmezat, Breuille et al. 2000). There was no difference in small intestine GSH concentration between the two groups whereas blood GSH concentration was significantly lower in infected rats than in pair-fed rats. Glutathione synthesis rates were significantly greater in liver, spleen, large intestine, lung, muscle and heart of infected rats compared with pair-fed controls. The activity of $\gamma$-glutamyl cysteine synthetase, and glutathione reductase were significantly greater in livers of infected rats than of pair-fed controls. Cysteine flux was also greater in infected rats. Based on the results the authors estimated that GSH synthesis accounts for at least 40% of the enhanced cysteine utilization during inflammation, and that increased utilization may be the primary cause for an increased cysteine requirement during infection (Malmezat, Breuille et al. 2000).

This increased cysteine requirement in infection was further substantiated in another study by the same group (Malmezat, Breuille et al. 2000), in which they found increased methionine transsulfuration during sepsis in rats using a similar model as that discussed above. Infection also increased methionine flux by 16% but less than cysteine
flux which was increased 38%. The plasma cysteine flux was also higher than predicted form estimates of protein turnover based on methionine data. This was similar to the findings of Hiramatsu et al., 1994. The present authors (Malmezat, Breuille et al. 2000), suggested that the increased cysteine flux was probably due to enhanced GSH turnover, a logical conclusion when assessed in conjunction with their previous findings and that of Hiramatsu et al., 1994 as well as Fukagawa et al., 1996.

2.7 Cysteine Metabolism

Cysteine is required for protein synthesis and for the synthesis of nonprotein compounds including taurine, sulphate and glutathione (GSH). The key regulatory enzymes of cysteine metabolism are cysteine-sulfinate decarboxylase (CSDC), cysteine dioxygenase (CDO) and γ-glutamyl-cysteine synthetase (GCS) (Bella, Hahn et al. 1999; Stipanuk, Dominy et al. 2006) (Figure 2.6).

2.7.1 In Vitro Studies

In order to explore the utilization of cysteine relative to methionine for the formation of its metabolites, a study was conducted in cultured rat hepatocytes (Stipanuk, Coloso et al. 1992). Rat hepatocytes were cultured with graded concentrations of cysteine and methionine and the production of glutathione, sulphate and taurine were measured in response. The results show that the production of each metabolite was affected by cysteine concentration and availability. Glutathione formation was favoured when cysteine availability was low whereas sulphate and taurine were favoured when cysteine availability was high (Stipanuk, Coloso et al. 1992).
Figure 2. 6 Pathways to cysteine metabolism

Reaction 1; \( \gamma \)-Glutamylcysteine Synthetase (GCS), Reaction 2; Cysteine Dioxygenase (CDO), Reaction 3; Cysteinesulfinate Decarboxylase (CSDC), Reaction 4; Aspartate Aminotransferase.
Cysteine supplied as such and cysteine formed from methionine was equally partitioned demonstrating that methionine is not a superior substrate to cysteine for hepatic glutathione synthesis. The results also demonstrated that cysteine concentration is an important factor in the determine the partitioning of cysteine into varying substrates (Stipanuk, Coloso et al. 1992).

To further understand the particular sulphur amino acid or their metabolites that act as a signal for regulatory changes in enzyme activity, rat hepatocytes were cultured with methionine, cysteine or one of their metabolites (Kwon and Stipanuk 2001). Enzyme activity, expression and mRNA analysis were conducted on the key enzymes of cysteine metabolism (figure 2.6). Rat hepatocytes cultured for 3 days in basal medium had low levels of CDO and high levels of GCS. When the medium was supplemented with 2 mmol.L\(^{-1}\) methionine or cysteine, CDO activity and CDO protein increased by >10-fold. In addition, CDO mRNA increased 1.5 to 3.2 fold. On the other hand, GCS activity decreased to 51 or 29% of basal. GCH heavy subunits (GCS-HS) protein decreased 89 or 58% of basal and GCS mRNA decreased to 79 or 37% of basal for methionine or cysteine supplementation respectively. The results show that supplementation with cysteine consistently resulted in a greater decrease than supplementation with the same amounts of methionine.

These results are in agreement with the previous results by Stipanuk and Coloso (Stipanuk, Coloso et al. 1992). Taken together the suggestion is that cysteine is an important signal for upregulation of CDO and downregulation of GCS.

Cysteine dioxygenase therefore has been shown to play a dominant role in cysteine catabolism. It catalyzes the oxidation of the sulphhydryl group of cysteine to form
cysteinesulphinate which is the precursor for synthesis of taurine as well as substrate for transamination to yield pyruvate and inorganic sulphate (figure 2.6). \(\gamma\)-Glutamylcysteine synthetase (GCS) catalyzes the rate-limiting step in GSH synthesis and therefore competes with CDO for cysteine as a substrate (Stipanuk, Londono et al. 2002).

### 2.7.2 In Vivo Studies

In order to further explore the role of CDO, CSD and GCS on cysteine metabolism the effect of diet on the regulation of these enzymes was investigated in liver as well as non-hepatic tissues namely kidney, lung and brain (Stipanuk, Londono et al. 2002). Rats were adapted to a casein base diet for 1 week before being fed the experimental diet. After 1 week rats were randomized to receive a low protein (LP), high protein (HP), low protein plus cysteine (LP +C), or low protein plus methionine (LP +M) diet for 2 weeks. At the end of the 2 weeks rats were killed and liver, kidney, brain and lung removed for analysis. CDO, CSD and GCS activities, concentrations and mRNA levels and the concentrations of cysteine, taurine and GSH were measured in the collected tissue. The results (Stipanuk, Londono et al. 2002) show that all 3 enzymes in liver responded to the differences in protein and sulphur amino acid intake. However, only CSD in the kidney and none of the enzymes in the lung and brain responded. Based on the results from the liver, the authors concluded that the change in cysteine concentration was consistent with cysteine-mediated regulation of hepatic CDO activity and changes in cysteine concentration and CDO activity. Changes in renal and lung cysteine, taurine and GSH concentrations were not associated with similar pattern or change in CDO, CSD or GCS activity. The authors concluded that the results confirm the
importance of the liver in the maintenance of cysteine homeostasis (Stipanuk, Londono et al. 2002).

In order to confirm the *in vitro* findings that cysteine mediates upregulation of CDO and downregulation of GCS, rats were fed a LP basal diet (100g casein.kg⁻¹ diet) with or without supplemental sulphur amino acids (8 g cystine, 9 g homocysteine, or 10 g methionine.kg⁻¹ diet) and with or without proparglycine (PPG) and irreversible inhibitor of GCS (Cresenzi, Lee et al. 2003). Rats were fed the diets for 2 days after which they were killed and the livers collected. The results show that rats fed the PPG containing diets had hepatic GCS activities that were ~16% lower than those that were not fed PPG. PPG treatment lowered the CDO activity and increased the GCS activity in the homocysteine and methionine supplemented groups but not in the cysteine supplemented groups or the unsupplemented groups. In addition, the GSH concentrations were not affected by PPG treatment in any of the groups. The authors concluded that the results are consistence with a role for cysteine as opposed to a precursor or metabolite of cysteine, in the metabolic signaling responsible for diet-induced regulation of CDO and GCS (Cresenzi, Lee et al. 2003).

To determine the role of non-sulphur compared to sulphur amino acids in the regulation of cysteine metabolism rats were fed a basal diet or a diet supplemented with a mixture of nonsulphur amino acids, sulphur amino acids, or both for 3 weeks after which they were killed and the hepatic CDO, CSDC, GCS activity, concentration and mRNA abundance measured (Bella, Hahn et al. 1999). Based on the results, supplementation of the basal diet with SAA with or without nonsulphur AA resulted in a higher CDO concentration (32 – 45 times basal), a lower CSDC mRNA levels (49-65% of basal) and a
lower GCS-HS of mRNA level (70-76%). This suggests a partitioning of SAA towards sulphate production when intakes are high. Both SAA and nonsulphur AA played a role in the regulation of the enzymes of cysteine metabolism but SAA had the dominant effect and effects of nonsulphur AA was not observed in the absence of SAA (Bella, Hahn et al. 1999).

In order to examine the time-course of changes in CDO and GCS activity, catalytic subunits and protein and mRNA levels were assessed in rats previously adapted to a low or high protein (HP) diet, switched to the opposite diet and then followed for 6 days after which they were killed and the above parameters measured in liver (Lee, Londono et al. 2004). The results show that hepatic CDO activity and amount but not mRNA level increased in response to higher protein diet and that the t$_{1/2}$ of the change for CDO activity or protein level was 22 hours for rats switched from the low protein (LP) to the HP diet and 8 hours for rats switched from HP to LP diet. This suggests that the HP diet decreased the turnover of CDO (Lee, Londono et al. 2004). Regarding GCS, its activity, catalytic subunits and mRNA levels decreased in response to a HP intake. The change in GCS activity in rats switched from a LP to a HP diet was faster that that for rats switched from a HP to a LP diet (16 vs 7 hours). Liver cysteine and GSH concentrations reached new steady states within 12 hours in rats switched from a LP to a HP and 24 hours in rats switched from a HP to a LP. CDO activity in the current study was regulated at the level of protein, possibly by decreased turnover of CDO in response to HP intake or cysteine levels whereas GCS activity appeared to be regulated at the mRNA level in response to changes in protein and cysteine availability. These findings support the role of cysteine as a mediator in its own metabolism. When cysteine
concentrations and availability is high, cysteine is partitioned towards catabolism whereas when availability is low cysteine is partitioned towards GSH synthesis.

In order to evaluate the mechanisms regulating hepatic CDO, GCS and CSDC activities in response to dietary protein or sulphur amino acids, enzyme activity and mRNA analysis were conducted on liver samples from rats fed a basal LP diet or diets with graded levels of protein or methionine for 2 weeks (Bella, Hirschberger et al. 1999). The results show that higher levels of CDO activity and CDO protein but not of CDO mRNA were observed in liver of rats fed the protein or methionine supplemented diets. This suggests that CDO activity is regulated by changes in enzyme concentration. Additionally, lower concentrations of GCS-HS, mRNA and protein as well as lower GCS-HS were observed in rats fed protein or methionine supplemented diets. The suggestion is that GSC is regulated by both pre-translational and post-translational mechanisms.

Regarding CSDC, there was lower activity; lower protein concentration and lower mRNA observed in the livers of rats fed the highest level of protein. The authors concluded that regulation of CSDC appeared to be related to changes in mRNA concentrations.

The overall summation from this experiment is again showing a similar pattern as that previously observed. Essentially cysteine metabolism responds to changes in protein, methionine and cysteine intake and the enzymes of central importance CDP, GCS and CSDC respond based on cysteine availability with a greater proportion being partitioned towards oxidation and sulphate production in the presence of high cysteine concentrations and a greater proportion being channeled towards GSH synthesis when
cysteine concentrations are lower. Thus GSH acts a storage form of cysteine and can be broken down to supply cysteine to the free amino acid pool when intake is low (Cho, Sahyoun et al. 1981). It is therefore clear that the liver plays a most important role in cysteine metabolism with the key enzymes acting to keep cysteine concentrations within a tightly regulated normal range. The purpose of keeping cysteine within tightly controlled levels has been looked at from two perspectives; the first is to support the need for protein synthesis and the production of other essential molecules including GSH, the other it to keep cysteine below the level of cytotoxicity (Stipanuk, Dominy et al. 2006).
3 THESIS SCOPE AND MOTIVATION

3.1 Rationale

An appropriate amino acid pattern for the TPN fed neonate that promotes optimal protein synthesis and growth while minimizing overload of the immature catabolic enzyme pathways has yet to be developed. The pattern of amino acid in currently available TPN solutions is based on human milk, egg protein or cord blood and reflects the amino acid requirement of the enterally fed neonate. In the neonatal piglet model it has been demonstrated that the requirement for several amino acids are lower with parenteral feedings compared to enteral feedings. One such amino acid methionine, has demonstrated toxic effects on the livers, having been shown to promote cholestatic changes in animal models similar to that observed in neonates on TPN. If methionine is hepatotoxic to the human neonate it might be possible to design a TPN solution that is non-toxic with the correct balance of methionine and cysteine especially in light of recent evidence that 40% of the methionine requirement can be replaced by cysteine.

Glutathionine, the most important intracellular scavenger and antioxidant is synthesized from the amino acids glutamate acid, cysteine and glycine. Of these three amino acids, cysteine is found to be the amino acid which has the greatest impact on limiting GSH synthesis.

Decreased GSH synthesis rates have been shown in healthy adults consuming diets low in sulphur amino acids as well as low in dietary protein. This decreased synthesis is observed in parallel with a lack of change in GSH concentration; an observation which suggests a decrease in GSH turnover.
Low concentrations of GSH have been observed in humans with different diseases. In addition, animal data show that in protein and amino acid deficiency animals are unable to maintain GSH status despite an increase in synthesis rates. Animal data also show an overall increase in cysteine requirement during stress/disease/illness. This increased requirement is partly explained by the increase in the synthesis of GSH under these circumstances. This increase in cysteine requirement seems to be at a level higher than that present in protein for nitrogen balance.

Because it is common knowledge that current TPN solutions are limiting in cysteine there is a need to measure GSH synthesis rates in the TPN fed neonate. In addition, since the TPN fed neonate is stressed, due to a variety of illnesses, or congenital anomalies requiring surgical intervention, it seems logical to suggest that such an individual may have an increased requirement for cysteine. It is important therefore to measure the amount of cysteine required for adequate GSH synthesis in the TPN fed neonate.

Additionally, since current methods of determining amino acid requirement measures the need for protein synthesis, it is important to determine if the sulphur amino acid required for protein synthesis is also equivalent to that required for provision of adequate antioxidant status. Since the requirement for total sulphur amino acid in healthy adults is the same as that required in chronically malnourished adults, a determination of whether the previously derived total sulphur amino acid requirement is also sufficient to maintain antioxidant status in healthy adults will be important to determine in order to provide a starting point of reference for further determination in the TPN fed human neonate.
3.2 Hypothesis and Objectives

In order to address the gaps mentioned above, this thesis was divided into four main studies. The first study was to develop a TPN solution *de novo* in our laboratory that was sterile, pyrogen free and stable to be used for the conduction of the studies in the TPN-fed human neonate. The second, third and fourth parts are the three specific studies designed to answer the questions raised herein. The objectives and hypothesis are outlined below.

**Experiment 1:** The development of an amino acid solution suitable for use in the determination of amino acid requirement and metabolism in the TPN fed human neonate

**Hypothesis**

- A TPN solution that is sterile, pyrogen free and stable for at least six weeks could be developed for use in the TPN fed neonate for the determination of amino acid requirement and metabolism.

**Objectives**

- To obtain a suitable grade amino acid which was sterile and pyrogen free
- To prepare an amino acid under sterile conditions to avoid contamination
- To test the solutions once prepared, using nitrogen analysis and HPLC to verify stability of the nitrogen and amino acid content.

**Experiment 2:** The total sulphur amino acid requirement and metabolism of the TPN-fed post-surgical neonate.
Hypothesis

• The total sulphur amino acid requirement of the TPN-fed post-surgical neonate will be approximately 52 mg.kg\(^{-1}.d^{-1}\).

Objectives

• To determine the total sulphur amino acid requirement (methionine in the absence of cysteine) of the TPN-fed human neonate using the indicator amino acid oxidation technique
• To measure the concentration of the amino acids involved in sulphur amino acid metabolism in response to feeding graded intakes of methionine

Experiment 3: Methionine-adequate cysteine-free diet does not limit glutathione synthesis in young healthy adult males

Hypothesis

• The total sulphur amino acid required to achieve the highest level of glutathione synthesis would be higher than that required for protein synthesis

Objectives

• To measure the concentration, fractional and absolute synthesis rates of glutathione in erythrocytes of healthy adult males
• To determine if feeding graded intakes of cysteine will affect the parameters listed above.
Experiment 4: Does the addition of cysteine to the experimentally derived total sulphur amino acid requirement (methionine only) increase glutathione synthesis in erythrocytes of the TPN-fed human neonate: a pilot study.

Hypothesis

- The provision of additional cysteine to the TPN, (in addition to the total sulphur amino acid requirement) will not increase erythrocyte GSH synthesis in the TPN fed stable post surgical human neonate.

Objectives

- To measure erythrocyte GSH concentration, fractional and absolute synthesis in the TPN fed human neonate in response to cysteine free methionine adequate TPN and in response to methionine-adequate, cysteine-supplemented TPN.
- To determine if cysteine supplementation increase GSH synthesis and concentration in the TPN-fed human neonate.
The following chapter has been submitted to the European Journal of Clinical Nutrition

Glenda Courtney-Martin, Karen P. Chapman, Ronald O. Ball, and Paul B. Pencharz. The development of an amino acid solution suitable for use in the determination of amino acid requirement and metabolism in parenterally fed humans
THE DEVELOPMENT OF AN AMINO ACID SOLUTION SUITABLE FOR USE IN THE DETERMINATION OF AMINO ACID REQUIREMENT AND METABOLISM IN PARENTERALLY FED HUMANS

4.1 Abstract

Current available amino acid solutions used for parenteral nutrition (PN) feeding contain mixtures of amino acid that are in set, predetermined concentrations. On the other hand, an amino acid solution appropriate for use in the determination of amino acid requirement and metabolism must allow for the adjustment of the level of the amino acid being studied. Such a solution has never been developed for use in humans and is not available for current use. The purpose of the current manuscript is to present the methods used in the development of amino acid solutions suitable for use in the determination of amino acid requirement and metabolism in the PN-fed, human subject. The criteria as established a-priori for these solutions were sterility and pyrogen free, and stability of individual amino acids over a 6-week to 3-month period. Pharmacy grade amino acid powders were obtained and solutions prepared under sterile conditions in our research laboratory. Solutions were filter sterilized and tested for pyrogens using standard procedures. Nitrogen analysis and high performance liquid chromatography (HPLC) testing were conducted to verify amino acid concentrations and stability. Solutions were found to be sterile and pyrogen free and individual amino acids were stable for up to 3 months in solution. The results of this study confirmed the possibility of safely and accurately making amino acid solutions for use in the conduct of amino acid requirement and metabolism studies in PN fed subjects.
4.2 Introduction

The amino acid profiles of current commercial amino acid solutions used for parenteral nutrition (PN) feeding are patterned after reference proteins consumed enterally (Endres, Lewandowski et al. 1982; Bertolo, Pencharz et al. 1999), or based on the amino acid composition of umbilical cord blood (Rigo and Senterre 1987), and plasma amino acids concentrations (Heird, Dell et al. 1987; Roberts, Ball et al. 1998). During the early 1990s, our group initiated a program of research in which we developed a piglet model (as a surrogate for the human neonate) for the determination of amino acid requirements in parenteral nutrition (PN) feeding (Wykes, Ball et al. 1993). Using this model, we determined the amino acid requirement for most of the essential amino acid (House, Pencharz et al. 1997; House, Pencharz et al. 1997; Bertolo, Chen et al. 1998; Elango, Pencharz et al. 2002; Shoveller, Brunton et al. 2003; Shoveller, Brunton et al. 2003; Cvitkovic, Bertolo et al. 2004). In parallel, we determined the enteral as well as parenteral requirement for the same amino acid in the same group of piglets (Bertolo, Pencharz et al. 1999; Elango, Goonewardene et al. 2004). This valuable extension to our existing model allowed us to make a most interesting discovery: that the amino acid requirement of enterally fed piglets was between 30 to 50% higher than that of PN fed piglets. Our observations were supported by the work of Stoll et al. who showed that approximately 30% of enterally fed dietary essential amino acids are consumed by the gut on first pass metabolism (Stoll, Henry et al. 1998). Thus we concluded that the current PN solutions that are available on the commercial market may be inadequate (Brunton, Ball et al. 2000) for the PN fed neonate.
The suitability of the piglet model as an appropriate surrogate for the human neonate was partially validated in a study on tyrosine requirement in the PN-fed human neonate (Roberts, Ball et al. 2001). The piglet model proved to be appropriate in that the requirement so derived was as predicted using the requirement estimate from the piglet study (House, Pencharz et al. 1997). Initial studies used commercial amino acid mixtures. Subsequently, we moved to parenteral amino acid mixtures made from crystalline amino acids and hence were able to vary as needed the amino acid under study (the test amino acid). Our previous extensive body of work in the neonatal piglet provided the knowledge and experience needed to pursue our goal of determining all the essential amino acid requirements of the PN-fed human neonate: with an ultimate goal of generating a scientifically derived amino acid pattern for PN feeding for that population.

We recently published a report in which we determined the first ever sulphur amino acid requirement in the PN fed human neonate using amino acids mixtures made from crystalline amino acids in a research laboratory (Courtney-Martin, Chapman et al. 2008). In order to be able to determine the total sulphur amino acid requirement as methionine only, we needed to prepare amino acid solutions devoid of all sulphur amino acids and then vary the methionine intake at levels both below and above the estimated requirement. Before we could do that we needed to develop amino acid solutions that met the requirements for human as stipulated by the Research Pharmacy at The Hospital for Sick Children, Toronto. Specifically, solutions needed to be sterile, pyrogen free and stable for at least six weeks.

The goal of the current manuscript therefore is to present the details of the methods used in the development of amino acid solutions for determination of the total
sulphur amino acid requirement and metabolism in the PN fed human neonate (Courtney-Martin, Chapman et al. 2008). We anticipate that other groups interested in the study of amino acid requirement and metabolism will benefit from our experience.
4.3 Methods

There were a number of steps to be followed to accomplish the goals of this study. The various tasks to be undertaken were as follows:

1. Obtain individual amino acid powders that were of a pharmacy grade, sterile and pyrogen free and suitable for use in the making of an amino acid solution that was stable for at least a six week period. Amino acids once obtained needed to be stored in a cool place away from direct light.

2. Making of the amino acid solution under sterile conditions

3. Test the solutions for sterility and pyrogenicity

4. Nitrogen analysis for the confirmation of the nitrogen content of the amino acid solution

5. Six-week and three-month high performance liquid chromatography (HPLC) stability testing for the identification and quantification of each individual amino acid present in the solutions (observed from HPLC) compared with amount added (expected amount).

6. The isotopes that were going to be used (L-[1\textsuperscript{13}C]phenylalanine, and [U\textsuperscript{13}C\textsubscript{2}–\textsuperscript{15}N]glycine) in future amino acid requirement studies required additional testing as requested by The Research Pharmacy at The Hospital for Sick Children. These testing were required to ensure limited exposure of the babies in the study to heavy metals. In addition every 6 months, we were required by pharmacy to conduct HPLC testing on the isotopes to check for breakdown products of phenylalanine and glycine. Isotopes were delivered to the Research Pharmacy immediately upon
receipt from the manufacturer in sealed unopened bottles. Pharmacy prepared all isotopes and was responsible for the storage under refrigerator conditions at 4°C.

4.3.1. Obtain Individual Amino Acid Powders:

Individual L- amino acids were obtained from Ajinomoto (Ajinomoto Aminoscience LLC, 4020 Ajinomoto Drive, Raleigh, North Carolina 27610), through its distributor L.V Lomas Ltd (75 Summerlea Road, Brampton ON, L6T 4V2, Canada). Each amino acid was received in individually wrapped air tight containers, with a specific lot number, manufacture and expiry date. Amino acids were then stored in airtight containers, in a cool environment (18.5 to 21°C) away from direct sunlight. Individual certificates of analysis were received with each amino acid and were reviewed and approved by the research pharmacy at The Hospital for Sick Children before the amino acid powder was used.
4.3.2. Preparation of the amino acid solution

The composition of the amino acid solution used was patterned after cord blood (Primene, Baxter Laboratories, Mississauga, Ontario); the pediatric amino acid solution used for PN feeding of neonates at The Hospital for Sick Children. Specific amino acid composition could then be adjusted to accommodate the changes in the amino acid under study. A bulk amino acid solution was made along with individual stock solutions of the test amino acid as well as alanine, which is the amino acid used to balance the nitrogen content of the PN solution.

Each solution was prepared under sterile conditions in glass containers that had been previously autoclaved. All utensils used in the weighing of amino acids were also autoclaved. Utensils and glass containers were autoclaved at 270 - 273°C for four minutes, followed by a cooling period of 30 minutes. Amino acids were added and dissolved in sterile water (Baxter Corporation, Toronto, ON). Amino acids were added individually, from the least to the most soluble and stirred continually with stirring magnets until dissolved. The prepared solution was then poured into autoclaved volumetric flasks and sterile water added to the desired volume.

Parenteral nutrition solutions were prepared by two different individuals. One individual weighed and added the powder to the sterile water while the other individual checked the lot number, expiry date, weight and addition of each amino acid to the sterile water. Each individual wore sterile gown, gloves mask and hair nets during solution preparation.
Once the preparation of the solution was completed it was then taken to the Research Pharmacy in the sterile volumetric flask for sterilization, pyrogen check and aliquoting for future use.

### 4.3.3. Test the solutions for sterility and pyrogenicity

Solutions were filter sterilized in the Research Pharmacy at The Hospital for Sick Children by passing through a 0.22 μm filter. Solutions were subsequently demonstrated to be sterile and free of bacterial growth over 7 days in culture and to be proven pyrogen-free by the limulus amebocyte lysate test (Pearson 1979).

### 4.3.4. Nitrogen analysis

After the preparation of the PN bulk and test solutions in the laboratory, a sample of each of the solutions was analyzed for determination of the total nitrogen content within 24 to 48 hours after preparation. The ANTEK 7000 Elemental Analyzer, (Mandel Scientific Company Ltd. Houston, TX) was used to determine the nitrogen content of each sample. Nitrogen concentrations were determined against a standard curve prepared using a know concentration of nitrogen. For each sample analyzed, the concentration observed from the machine was checked against the expected concentration of nitrogen in the total sample using the formula: (Expected – Observed / Expected)*100. A difference in nitrogen concentration between expected and observed of ≤ 5% was the pre-determined accepted range.
4.3.5. HPLC stability testing

In order to determine the shelf-life of each of the PN solutions made (bulk and test solutions), samples were analyzed by HPLC at 6 weeks and again at 3 months to determine the stability of each individual amino acid in the solution over time. Individual amino acid concentration was analysed by reverse phase HPLC using the PICO.TAG (Waters, Milford, MA), pre-column derivitization method with phenylisothiocyanate as the derivative. The amino acid concentrations were determined by using an external standard. Each observed amino acid concentration was then compared to the expected concentration using the formula: (Expected – Observed/ Expected)* 100. A predetermined difference of ± 5% was the acceptable range.

4.3.6 Heavy metal testing and testing for breakdown products of isotopes

The testing for heavy metal on the isotope was performed by the manufacturer at an additional cost.

Regular, six-month testing for the presence of “breakdown products” of phenylalanine and glycine in the isotope were conducted on HPLC. The isotope was judged to be free of breakdown products by the absence of one or more unidentifiable peaks in the chromatogram and a quantification of the concentration of the isotope to ± 5% difference between expected and observed concentrations.
4.3.7 Statistical analysis

The acceptable difference between expected and observed concentrations of nitrogen and amino acids were set a priori at ± 5%. The expected nitrogen concentration in each amino acid solution was determined by finding the sum of the total nitrogen content of each amino acid in the solution. The difference in nitrogen and amino acid concentrations as measured by nitrogen and HPLC analysis respectively was calculated using the following equation:

\[
\left(\frac{\text{expected concentration} - \text{observed concentration}}{\text{expected concentration}}\right) \times 100.
\]

All calculations were performed using Microsoft EXCEL for Windows Version 2002 (Microsoft Corporation). Mean and SD were calculated on all samples analyzed by nitrogen analysis. These calculations were done on the results obtained from the injection of each sample three times into the nitrogen analyzer.

4.4. Results

All samples prepared except for one initial batch passed the sterility and pyrogenicity tests. The total nitrogen content of the solutions as measured within 24 to 48 hours after preparation was confirmed to be within ± 5% of the expected nitrogen content. The observed nitrogen content of the bulk solution prepared was -0.9 ± 1.7 (mean ± SD) mg nitrogen/litre. The observed nitrogen content of the test amino acids methionine and alanine were also within the expected acceptable range. The observed nitrogen content for methionine and alanine were -0.4 ± 2.5 and -0.2 ± 3.1 (mean ± SD)
mg. nitrogen/litre. Also the observed nitrogen content of the (L-[1-\textsuperscript{13}C]phenylalanine used in the study was \(-0.9 \pm 2.0\) (mean \pm SD) mg. nitrogen/litre.

The amino acids were found to be stable in solution at 6 weeks and again after 3 months as verified by a predetermined difference between expected and observed concentration of \(\pm 5\%\). Solutions were therefore given an initial shelf life of 6 weeks which was extended to a 3 month shelf life after the 3-month testing. The results of a typical batch of samples tested by HPLC after 6 weeks and 3 months are presented in Tables 4.1 and 4.2.

The isotope powder met the specifications required by the Research Pharmacy with regard to heavy metal content, and was deemed acceptable for use in human infants. The isotope was tested for over 60 metals including silver, nickel, tin, gold and lead. In addition, there were no breakdown products observed from the isotope during the periodic 6 month testing by HPLC. **Figures 4.1** presents a typical chromatogram obtained from HPLC for (L-[1-\textsuperscript{13}C]phenylalanine.

There were no adverse events associated with the use of any of the solutions in any of the babies studied.
Table 4.1  HPLC stability analysis of TPN bulk solution

Results of HPLC analysis on bulk PN solution made for conduction of stability analysis:

Typical 6-week and 3-month results

<table>
<thead>
<tr>
<th>Amino acid Name</th>
<th>Total amino acid Expected (nmoles)</th>
<th>Total amino acid Observed (nmoles)</th>
<th>Percentage (%) difference between expected and observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine</td>
<td>127.7</td>
<td>128.74</td>
<td>-0.82</td>
</tr>
<tr>
<td>Leucine</td>
<td>188.69</td>
<td>194.33</td>
<td>-2.99</td>
</tr>
<tr>
<td>Valine</td>
<td>162.19</td>
<td>163.15</td>
<td>-0.59</td>
</tr>
<tr>
<td>Lysine</td>
<td>186.4</td>
<td>189.43</td>
<td>-1.62</td>
</tr>
<tr>
<td>Methionine(^2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine(^2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>36.32</td>
<td>36.13</td>
<td>0.53</td>
</tr>
<tr>
<td>Tyrosine(^2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycyl-tyrosine</td>
<td>42.77</td>
<td>42.62</td>
<td>0.35</td>
</tr>
<tr>
<td>Threonine</td>
<td>38.83</td>
<td>38.58</td>
<td>0.62</td>
</tr>
<tr>
<td>Tryptophan(^3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>61.23</td>
<td>63.39</td>
<td>-3.53</td>
</tr>
<tr>
<td>Arginine</td>
<td>60.28</td>
<td>57.35</td>
<td>4.86</td>
</tr>
<tr>
<td>Glycine</td>
<td>45.22</td>
<td>46.22</td>
<td>-2.22</td>
</tr>
<tr>
<td>Alanine</td>
<td>110.84</td>
<td>115.98</td>
<td>-4.64</td>
</tr>
<tr>
<td>Aspartate</td>
<td>56.35</td>
<td>59.11</td>
<td>-4.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>----------</td>
<td>----------</td>
<td>------</td>
</tr>
<tr>
<td>Glutamate</td>
<td>84.11</td>
<td>85.42</td>
<td>-1.55</td>
</tr>
<tr>
<td>Proline</td>
<td>65.14</td>
<td>66.63</td>
<td>-2.29</td>
</tr>
<tr>
<td>Serine</td>
<td>47.58</td>
<td>49.24</td>
<td>-3.50</td>
</tr>
<tr>
<td>Taurine</td>
<td>5.99</td>
<td>5.70</td>
<td>4.92</td>
</tr>
</tbody>
</table>

1. Original PN bulk solution prepared as a 5% solution. During sample preparation, solution diluted to a 1 in 2 and 1 in 4 solution, derivitized, then 2.5, 5.0, or 10ul injected into the HPLC.

2. These amino acids not used in making the bulk solution. These amino acids were initially made as separate test solutions. Tyrosine was replaced by glycyl-tyrosine.

3. Not accurately quantifiable on our HPLC because tryptophan is light sensitive and our HPLC uses a UV detector.
Table 4.2 HPLC stability analysis of individual amino acids

Results of HPLC analysis on individual amino acids prepared for conduction of stability analysis: Typical 6 weeks and 3 month results

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Original concentration (mg/ml)</th>
<th>Dilution parameters</th>
<th>Amino acid expected in diluted sample (nmoles)</th>
<th>Amino acid observed from HPLC sample (nmoles)</th>
<th>Percentage difference (%) between expected and observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>50</td>
<td>1 in 200</td>
<td>19.64</td>
<td>19.31</td>
<td>1.71</td>
</tr>
<tr>
<td>Methionine</td>
<td>20</td>
<td>1 in 50</td>
<td>18.77</td>
<td>18.91</td>
<td>-0.75</td>
</tr>
<tr>
<td>(L-[1-^{13}C]phenylalanine</td>
<td>15</td>
<td>1 in 40</td>
<td>15.80</td>
<td>16.39</td>
<td>-3.74</td>
</tr>
</tbody>
</table>
Figure 4.1 HPLC chromatogram

Typical chromatogram obtained from HPLC during the testing for breakdown products of (L-[1-13C]phenylalanine. Testing done at 3 and 6 months after making solution throughout the duration of the shelf life of the product as designated by the manufacturer.

Chromatogram from HPLC analysis of phenylalanine showing HPLC buffer gradient for aqueous and organic buffer used by HPLC program.

NL = Norleucine (internal standard)
Phe = Phenylalanine
4.5 Discussion

Herein we have presented the details of the methods used to successfully prepare PN solutions from scratch in a research laboratory in order to determine the total sulphur amino acid requirement in the PN fed human neonate (Courtney-Martin, Chapman et al. 2008). This is the first report to our knowledge in which PN solutions have been successfully made de novo in a research laboratory for use in humans for the study of amino acid requirements and metabolism in PN feeding. Our previous work in the PN neonatal piglet model (Wykes, Ball et al. 1993; Wykes, House et al. 1994; House, Pencharz et al. 1997; House, Pencharz et al. 1997; Bertolo, Chen et al. 1998; Bertolo, Pencharz et al. 1999; Elango, Pencharz et al. 2002; Cvitkovic, Bertolo et al. 2004; Elango, Goonewardene et al. 2004) provided us with the knowledge and experience required to embark on such an endeavor.

In order to determine the requirement for an amino acid, a study should be so designed that the amino acid of interest is fed at varying intake levels from deficient to excess. (Di Buono, Wykes et al. 2001; Ball, Courtney-Martin et al. 2006). The determination of amino acid requirement in subjects that are PN fed therefore requires the development of a PN solution in which the intake of the amino acid of interest as well as a dispensable amino acid used for balancing the nitrogen content of the solution (usually alanine) could be varied across a wide range.

The results of the current study show that it is possible to design amino acid solutions de novo in a laboratory for use in PN feeding that are sterile and pyrogen free, as well as stable. Of all the solutions made, only one batch failed sterility testing, which accounted for about a 4% failure rate. The solution was promptly discarded and an
evaluation of the preparation process carried out. This happened very early on in the process and was most likely due to the failure of one of the individuals making the solution to wear sterile gloves. This was rectified in the making of subsequent batches and all subsequent solutions passed sterility and pyrogenicity testing. In addition, there were no adverse effects in any of the babies in response to the PN solutions used in the study.

We undertook a very rigorous and painstaking process to assess stability and preparation accuracy of the solutions. Thus we performed nitrogen analysis on all samples within 24 to 48 hours after preparation in order to assess the accuracy of weighing of all individual amino acids. The observed results of the nitrogen analysis were within the predetermined acceptable range and provided proof of accuracy of the weighing process.

Nitrogen analysis however, was not considered adequate to judge the stability of individual amino acids in solution after prolonged periods, therefore subsequent analysis were conducted using HPLC to determine the concentration of each individual amino acid in solution after 6 weeks and again after 3 months. The results in tables 4.1 and 4.2 typifies the results obtained from each batch of solution prepared and provided the evidence required to judge the stability of the individual amino acids and assign a shelf life and expiry date to each batch of solution made.

Also to judge the stability of the isotopes, HPLC testing was conducted to look for breakdown products of (L-[1-13C]phenylalanine. There was never any breakdown products found as judged from the lack of additional unidentified peaks in the
chromatograms (figure 4.1), and accurate quantification of the observed concentrations to within ± 5% of expected.

The current report clearly shows that if amino acid powders are obtained from a credible source providing powders that are pharmacy grade, and the powders are weighed carefully and made into solutions using sterile water, under sterile technique, it is possible to make safe and appropriate solutions for conducting studies in PN-fed subjects. This information herein are novel, and provided the base from which we were able to conduct the first ever amino acid requirement study in the PN fed human neonate using an amino acid solution made from scratch in a research laboratory (Courtney-Martin, Chapman et al. 2008). They also provide a basis from which all future studies on amino acid requirements and metabolism can be conducted in PN-fed human subjects.
The following study presented in chapter 5 has been published in The American Journal of Clinical Nutrition.

5. TOTAL SULPHUR AMINO ACID REQUIREMENT AND METABOLISM IN THE PARENTERALLY-FED POST-SURGICAL HUMAN NEONATE.

5.1 Abstract

Except for tyrosine, the amino acid requirements of the parenterally (PN) fed, human neonate have never been experimentally derived. The objectives were to determine the total sulphur amino acid requirement (TSAA) requirement (methionine in the absence of cysteine) of the PN-fed post-surgical human neonate using the Indicator amino acid oxidation (IAAO) technique with L-[1-13C]phenylalanine as the indicator. Fifteen post-surgical neonates were randomly assigned to receive one of 18 levels of methionine intake ranging from 10 to 120 mg. kg\(^{-1}\).d\(^{-1}\); delivered in a customized, cysteine-free amino acid solution. Breath and urine samples were collected for \(^{13}\)CO\(_2\) and amino acid enrichment. Blood samples were collected at baseline and after the test methionine infusion for measurement of plasma methionine, homocysteine, cystathionine, and cysteine concentrations. Using breakpoint analysis the mean and 95% CI of the TSAA requirement were determined to be 47.4 (38.7 – 56.1) and 49.0 (39.9 – 58.0) mg. kg\(^{-1}\).d\(^{-1}\), using oxidation and F\(^{13}\)CO\(_2\) respectively. This is the first study to report on the TSAA requirement of the PN fed post-surgical human neonate. The estimated methionine requirement expressed as a proportion of the methionine content of current commercial pediatric PN solutions was 90% (range 48 – 90%) of that found in the lowest methionine-containing PN solution.
5.2 Introduction

Except for tyrosine, the amino acid requirements for parenteral feeding have never been experimentally determined in humans. Instead, the amino acid profile of current commercial amino acid solutions are patterned after reference proteins consumed enterally (Endres, Lewandowski et al. 1982; Bertolo, Pencharz et al. 1999), or on the amino acid concentrations of plasma or cord blood (Heird, Dell et al. 1987; Roberts, Ball et al. 1998).

Our laboratory developed a neonatal piglet model (Wykes, Ball et al. 1993) to study amino acid requirement which was later validated in the human neonate for the determination of the tyrosine requirement during parenteral (PN) feeding (Roberts, Ball et al. 2001). Results from the piglet studies have shown that the requirements for several amino acids are lower during parenteral feeding compared with enteral feeding (Bertolo, Chen et al. 1998; Elango, Pencharz et al. 2002; Shoveller, Brunton et al. 2003). This suggests that the amino acid profiles of some currently available commercial amino acid solutions are potentially too high. In addition, existing evidence suggest that the inadequate amino acid profile of current parenteral nutrition (PN) solutions contributes to the liver cholestasis observed in neonates on long term PN feeding (Belli, Fournier et al. 1987; Brown, Thunberg et al. 1989; Moss, Das et al. 1993).

Methionine is an indispensable sulphur amino acid which donates its sulphur atom to form cysteine during the process of transsulfuration (Stipanuk 1986). Methionine is also the most important methyl donor in vivo and a precursor for DNA and RNA synthesis (Griffith 1987). However, methionine has been shown to be the most toxic of
all amino acids (Hardwick, Applegarth et al. 1970; Benevenga 1974); producing cholestatic changes in the liver of animals similar to that observed in human neonates receiving PN feeding (Moss, Haynes et al. 1999). Commercial amino acid solutions have a high methionine content; this in an effort to provide cysteine via the transsulfuration pathway because of the instability of cysteine in solution. This practice, although resulting in high plasma methionine, has not served to normalize the plasma cysteine of the PN fed neonate, which appears low based on current data (Dale, Panter-Brick et al. 1976; Malloy, Rassin et al. 1984; Miller, Jahoor et al. 1995). In addition, data from our piglet studies show that the methionine requirement of the PN-fed neonate is 30% less than the enteral requirement (Shoveller, Brunton et al. 2003).

The goals of this study were to determine the total sulphur amino acid requirement (methionine in the absence of cysteine) in the PN fed human neonate using the indicator amino acid oxidation technique (IAAO) and to measure the plasma amino acid concentrations of several amino acids involved in sulphur amino acid metabolism in response to feeding graded intakes of methionine. Based on results from our piglet data, we predicted that the total sulphur amino acid requirement of the PN fed human would be about 52 mg.kg⁻¹.d⁻¹.
5.3. Subjects and method

5.3.1 Subjects

Fifteen neonates treated during the months of July 2005 to December 2006, in the Neonatal Intensive Care Unit, at The Hospital for Sick Children, Toronto, Canada were enrolled in this study. The following inclusion criteria were used to determine eligibility: born at $\geq 34$ weeks gestation and $\leq 28$ days chronological age at the time of the study, birth weight and length appropriate for gestational age, medically stable as determined by normal blood results and lack of a fever or infection, at least 3 days post operatively, and on PN providing adequate protein and calories as determined by attending physicians and dietitians. Exclusion criteria included: mechanical ventilation, small for gestational age status, presence of disease or on medications known to affect protein and amino acid metabolism, documented infection, fever, unstable medical condition, and receiving enteral feeding providing greater than 10% of protein intake.

Neonates were studied at least 3 days post operatively for a number of reasons. (1) Jones et al (Jones, Pierro et al. 1993) have shown that the increase in resting energy expenditure (REE) experienced by post surgical neonates peaks at 2 to 4 hours postoperatively and returns to baseline by 12 to 24 hours post surgery. They also showed that substrate utilization was not altered by operation. Whereas they showed that the increase in REE was greater in infants having a major surgery, there still was a return to baseline by 24 hours. (2) Zlotkin et al. (Zlotkin, Bryan et al. 1981) have shown that provision of protein and energy intakes of 2.7 to 3.5 g.kg$^{-1}$ and 81 kcals.kg$^{-1}$ respectively resulted in nitrogen retention and growth rates similar to in utero values in post surgical
premature infants when studied 4 days post surgery. (3) We had previously reported
similar amino nitrogen flux rates in post surgical infants (1 to 4 days post surgery) (Duffy
and Pencharz 1986) on TPN as that observed in a similar group of infants on TPN who
had not undergone surgery (Duffy, Gunn et al. 1981). Based on this evidence, the
suggestion is that at 3 days post operatively, protein metabolism of the post surgical
neonate should not differ from non post surgical neonates.

Ethical approval for the study was obtained from The Research Ethics Board at
The Hospital for Sick Children. Permission was obtained from the attending physician
before approaching parents, and written informed consent was obtained from at least one
parent before enrolling subjects into the study. Study characteristics of the neonates
included in the study are presented in Table 5.1.
Table 5.1 Subject characteristics of TPN fed neonates

Subject Characteristics of parenterally fed neonates who received varying methionine intakes and zero cysteine during the determination of the total sulphur amino acid requirement.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n )</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>2.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Birth Length (cm)</td>
<td>47.5</td>
<td>25</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>32.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Gestational age (wk)</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>Postnatal age (wk)</td>
<td>1.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Postconceptional age (wk)</td>
<td>39</td>
<td>2</td>
</tr>
<tr>
<td>Gender (F:M)</td>
<td>4:11</td>
<td></td>
</tr>
<tr>
<td>Study weight (kg)</td>
<td>2.8</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Diagnoses:

1 Esophageal atresia/tracheoesophageal fistula; \( n = 2 \), jejunal atresias; \( n = 1 \), duodenal web; \( n = 1 \), gastroschisis; \( n = 5 \), multiple small bowel atresias; \( n = 1 \), duodenal atresia; \( n = 1 \), meconium plug syndrome; \( n = 1 \), omphalocele; \( n = 1 \).
5.3.2 Experimental Design

The indicator amino acid oxidation (IAAO) technique (Zello, Wykes et al. 1995) was used for the determination of the total sulphur amino acid requirement TSAA requirement. The indicator amino acid oxidation (IAAO) technique is based on the observation that the partitioning of any indispensable amino acid between oxidation and protein synthesis is sensitive to the level of the most limiting amino acid in the diet (Ball and Bayley 1986). Since amino acids are not stored in the body, when an indispensable amino acid is limiting in the diet for protein synthesis, all other amino acids are in excess and are therefore oxidized. Therefore, increasing the level of the limiting amino acid in graded amounts from deficient to excess will also increase the uptake of all dietary amino acids for protein synthesis. As their uptake for protein synthesis is increased, the portion of the indicator that is oxidized will decrease until the requirement of the test amino acid (in this case methionine) is reached. Once the requirement of the test amino acid is reached, further increase in its intake will have no further effect on the indicator amino acid. The first point after which increasing the test amino acid has no further effect on the oxidation of the indicator is the breakpoint or the mean requirement; the point at which the test amino acid is no longer limiting for protein synthesis. The inverse relationship between phenylalanine oxidation and protein synthesis is supported by the experiment of Ball and Bayley (Ball and Bayley 1986) in which L-[1-\textsuperscript{14}C]phenylalanine was used as an indicator of protein requirement in young pigs. The results show that the recovery of radioactivity in breath was inversely related to the recovery of radioactivity in liver tissue when the dietary protein was increased from deficient to adequate, demonstrating that the
oxidation of the indicator was inversely related to protein synthesis. In addition, we have demonstrated in humans that the requirement for tyrosine determined by the IAAO method using lysine as an indicator was similar to that determined by measuring the hydroxylation of phenylalanine to tyrosine in Apo-B100 (Rafii, McKenzie et al. 2008).

Blood samples were drawn from each subject for the determination of amino acid concentration. Each study lasted for a total of 48 hours. During the first 24 hours, each subject received a commercial amino acid solution (Primene, Baxter Laboratories, Mississauga, Ontario) (Table 5.2), plus dextrose, and a 20% lipid solution (Intralipid; Fresenius Kabi, Uppsala, Sweden) for provision of adequate protein, and non protein energy. Standard amounts of vitamin and minerals were provided in the form of a liquid supplement (Multi-12/K1, providing a mixture of fat and water-soluble vitamins) formulated for use in intravenous feeding. All vitamins and minerals met current DRI recommendations. The sulphur amino acid intake of subjects during the first 24 hours (day 1) was 52.97 ± 9.04 and 69.42 ± 4.17 (mean ± SD) for cysteine and methionine respectively, for a total of 122.39 ± 8.93 (mean ± SD) mg.kg\(^{-1}\).day\(^{-1}\) total sulphur amino acid intake. The base amino acid solution as indicated above was chosen for a number of reasons; (1) it is the amino acid used in the NICU at our hospital (The Hospital for Sick Children, Toronto), and has been the amino acid solution in use for over 10 years. (2) It is also used in NICUs in Europe (Van Goudoever, Sulkers et al. 1994; Saenz de Pipaon, Quero et al. 2005). When the plasma amino acid concentration of neonates on the amino acid solution used in this study were compared to that of breast fed infants (Wu, Edwards et al. 1986), most of the amino acids were within the reference range with only two indispensable amino acids; valine, and lysine being higher and tyrosine being lower (Van
Gouдоever, Sulkers et al. 1994). This low tyrosine concentration suggest that tyrosine is limiting in the amino acid solution used, an observation which was confirmed by our group (Roberts, Ball et al. 1998). Subsequently we determined the tyrosine requirement of the TPN-fed human neonate (Roberts, Ball et al. 2001) which formed the basis for the amount of tyrosine used in the solutions made for this study. We used glycyl-tyrosine as our tyrosine source instead of N-acetyl-tyrosine because glycyl-tyrosine has been found to be an available source of tyrosine for the neonate (Roberts, Ball et al. 2001) unlike N-acetyl-tyrosine (Heird, Dell et al. 1987; Heird, Hay et al. 1988). Additionally, we used data from our piglet work to make adjustments to the arginine content of the amino acid base solution. Our piglet data suggest that the arginine requirement is greater with IV compared with enteral feeding (Bertolo, Brunton et al. 2003).

During the second 24 hour period, subjects were randomly assigned to receive one of 18 levels of methionine intake ranging from 10 to 120 mg. kg$^{-1}$.day$^{-1}$, (with zero cysteine). Hence a separate amino acid solution was prepared for this part of the study (Table 5.2). In order to control the methionine intake, both methionine and cysteine were removed from this solution. Methionine was added back on the study day in an amount consistent with the intake level being studied on that day. Again, grams of fat, protein and total calories delivered were determined by the attending physician and dietitians. Each subject was intravenously fed via a central line and received a fluid intake between 140 to 160 mL. kg$^{-1}$.day$^{-1}$.
Table 5.2 Amino acid composition of PN solution administered to neonates
Amino acid concentration of parenteral solutions administered to neonates on day 1 (adaptation day) and day 2 (study day).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/100 g</td>
<td>g/100 g</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.9</td>
<td>9.9</td>
</tr>
<tr>
<td>Valine</td>
<td>7.6</td>
<td>7.6</td>
</tr>
<tr>
<td>Lysine (lysine-HCL)</td>
<td>10.9</td>
<td>13.62</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.4</td>
<td>Variable (based on test intake level)</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1.9</td>
<td>0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.2</td>
<td>3.7</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.9</td>
<td>0</td>
</tr>
<tr>
<td>Glycyl-tyrosine</td>
<td>0</td>
<td>5.25</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Arginine</td>
<td>8.4</td>
<td>9.66</td>
</tr>
<tr>
<td>Amino Acid</td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>-------------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Glycine (total)</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.9</td>
<td>7.9 plus additional amounts to make the solution isonitrogenous</td>
</tr>
<tr>
<td>Aspartate</td>
<td>6.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Glutamate</td>
<td>9.9</td>
<td>9.9</td>
</tr>
<tr>
<td>Proline</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Serine</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Taurine</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Ornithine</td>
<td>2.2</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total AA (grams/100g)</strong></td>
<td><strong>100.00</strong></td>
<td><strong>94.15</strong>¹</td>
</tr>
</tbody>
</table>

¹Solution on day 2 represented 94.15% of the total protein/total amino acid.

Phenylalanine provided in parenteral solution and as isotope. 1.9 g.100 g⁻¹ provided in parenteral solution, 1.8 g.100 g⁻¹ provided as isotope.
5.3.3 Study Diets

The base amino acid profile of the amino acid solutions used on day 1 (adaptation period) and day 2 (test period) is presented in Table 5.2. The amino acid profile of the base amino acid solution used was based on the amino acid composition of cord blood. The amino acid solution used on day 2 (test period) was prepared using human parenteral grade amino acids (Ajinomoto Company Inc., Japan via L.V Lomas, Brampton, Ontario) in our research laboratory, under sterile conditions. The profile of the base amino acid, was followed with some modifications; methionine was varied to meet the level of the test intake for each neonate, cysteine was removed, tyrosine was provided in excess as the dipeptide glycyl-tyrosine at a level of 4 g.100 g\(^{-1}\) (Roberts, Ball et al. 2001). The excess tyrosine was provided to facilitate the channelling toward oxidation of any tyrosine synthesized from phenylalanine (Shiman and Gray 1998) thereby making phenylalanine a more sensitive indicator. Phenylalanine was provided at a total amount of 3.7 g.100 g\(^{-1}\) and the amount used for tracer infusion was subtracted from the base solution. Arginine was increased from 8.4 to 9.66 g.100 g\(^{-1}\) (Bertolo, Brunton et al. 2003; Wilkinson, Bertolo et al. 2004) and aspartate was decreased from 6.0 g.100 g\(^{-1}\) to 5.0 g.100 g\(^{-1}\) to accommodate for the increased amount of nitrogen provided by arginine. Alanine was used to balance the nitrogen and make the solution isonitrogenous. Methionine and alanine were prepared as separate solutions in concentrations of 20 and 50 mg.mL\(^{-1}\) respectively. All prepared solutions were filter sterilized in the Research Pharmacy at The Hospital for Sick Children by being passed through a 0.22 μm filter. Solutions were subsequently demonstrated to be sterile and free of bacterial growth over
7 days in culture and to be proven pyrogen-free by the limulus amebocyte lysate test (Pearson 1979). The chemical composition of the solutions was verified by amino acid analysis using HPLC and analysis of total nitrogen.

On each study day, vitamins and minerals were added to the solutions before delivery to the baby. All vitamins were supplied in a commercial solution Multi-12K1 (Baxter Corporation, Mississauga Ontario), which provides a combination of fat and water-soluble vitamins, formulated for use in pediatric parenteral solutions. Co-factors involved in methionine metabolism; vitamin B-12, B-6 and folic acid were provided in the Multi-12K1 solution at 1.2 to 1.5 times requirement (Medicine 2006). The mineral solution provided calcium, phosphorus, magnesium, zinc, copper, manganese, iodine, chromium and selenium. Nutrient intake for each individual neonate was prescribed by the attending physician and dietitian. All subjects were receiving adequate protein and calories (Zlotkin, Bryan et al. 1981; Zlotkin 1984). Non-protein calories were provided as dextrose and a 20% lipid solution Table 5.3.

The study began with each neonate receiving the base TPN/lipid solution for a total of 24 hours to ensure that all neonates started the test infusion with similar amino acid profiles. For the 2nd 24 hour period, neonates received the test solution containing a randomly assigned level of methionine ranging from 10 to 120 mg.kg⁻¹.day⁻¹ with zero cysteine. The test level of methionine was added to the test solution on the study day. All neonates received the test TPN solution until the end of the study at which time they were returned to the TPN they were receiving prior to the study.

Baseline blood work performed for clinical monitoring was reviewed on each subject before the start of the study. All subjects had normal sodium, potassium, calcium,
phosphorous and pH. Five subjects had high chloride levels between 107-113, (normal range 96-106). This was corrected by replacing the chloride with acetate in the TPN solution.
Table 5.3 Nutrient intake of neonates who participated in methionine requirement study

Individual subject methionine, amino acid, lipid, carbohydrate and total energy intakes on Study day (day 2)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Study</th>
<th>Methionine (mg·kg⁻¹·day⁻¹)</th>
<th>Amino Acid (g·kg⁻¹·day⁻¹)</th>
<th>Lipid (g·kg⁻¹·day⁻¹)</th>
<th>CHO (g·kg⁻¹·day⁻¹)</th>
<th>Energy (KJ·kg⁻¹·day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>118.7</td>
<td>2.86</td>
<td>2.98</td>
<td>14.44</td>
<td>346.94</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>9.5</td>
<td>2.64</td>
<td>2.96</td>
<td>13.89</td>
<td>355.3</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>99.86</td>
<td>2.91</td>
<td>2.94</td>
<td>11.65</td>
<td>338.58</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>29.95</td>
<td>2.91</td>
<td>2.94</td>
<td>11.65</td>
<td>334.4</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>31.8</td>
<td>2.7</td>
<td>2.98</td>
<td>13.4</td>
<td>380.38</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>49.14</td>
<td>2.7</td>
<td>3.3</td>
<td>13.69</td>
<td>372.02</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>77.6</td>
<td>2.83</td>
<td>2.88</td>
<td>14.56</td>
<td>367.84</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>14.87</td>
<td>2.84</td>
<td>2.88</td>
<td>14.32</td>
<td>367.84</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>39.1</td>
<td>2.98</td>
<td>3.0</td>
<td>13.9</td>
<td>338.58</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>19.16</td>
<td>2.93</td>
<td>3.0</td>
<td>13.9</td>
<td>363.66</td>
</tr>
<tr>
<td>11</td>
<td>11</td>
<td>55.6</td>
<td>2.71</td>
<td>3.0</td>
<td>13.38</td>
<td>367.84</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>61.51</td>
<td>2.91</td>
<td>2.45</td>
<td>14.2</td>
<td>353.3</td>
</tr>
<tr>
<td>13</td>
<td>13</td>
<td>107</td>
<td>2.8</td>
<td>3.15</td>
<td>11.52</td>
<td>334.4</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>86.3</td>
<td>3.36</td>
<td>2.9</td>
<td>14.05</td>
<td>372.02</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>61.5</td>
<td>3.1</td>
<td>2.98</td>
<td>14.05</td>
<td>351.12</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>33.9</td>
<td>3.1</td>
<td>3.3</td>
<td>13.78</td>
<td>380.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>15</td>
<td>17</td>
<td>24.5</td>
<td>2.84</td>
<td>2.93</td>
<td>12.84</td>
<td>351.12</td>
</tr>
<tr>
<td>18</td>
<td>43.7</td>
<td>2.9</td>
<td>3.07</td>
<td>11.87</td>
<td>338.58</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>2.89</td>
<td>2.98</td>
<td>13.39</td>
<td>356.46</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td>0.17</td>
<td>0.18</td>
<td>1.03</td>
<td>15.63</td>
<td></td>
</tr>
</tbody>
</table>
5.3.4 Tracer Protocol

The tracer protocol was started at the beginning of the second 24 hour period, at the time the test bag of PN was hung. Phenylalanine was used as the indicator amino acid with L-[1-13C]phenylalanine, [99 atom percent excess (APE), Cambridge Isotope Laboratories, Woburn, MA] as the isotope for the measurement of phenylalanine kinetics. An intravenous priming dose of L-[1-13C]phenylalanine was given at 15.6 μmol. kg⁻¹ over 15 minutes followed by a continuous intravenous infusion of 13 μmol. kg⁻¹.hr⁻¹ for the remainder of the 23.75 hours. The amount of L-[1-13C]phenylalanine given was subtracted from the phenylalanine provided in the PN solution to maintain a total phenylalanine intake of 111 mg. kg⁻¹.day⁻¹ (3.7 g.100g⁻¹). Tyrosine was provided at 120 mg. kg⁻¹.day⁻¹ (to ensure an excess of tyrosine) (House, Pencharz et al. 1997; House, Pencharz et al. 1997). L-[1-13C]phenylalanine was analyzed by LCMS/MS using a chiral column for the presence of D-[1-13C]phenylalanine. There was no detectable D-[1-13C]phenylalanine in the tracer.

The infusion time of the tracer was extended to 23.75 hours for practical reasons. Given our goal to make these studies as least invasive as possible, an extended tracer infusion time was necessary to allow enough time for urine collection since babies do not void on demand. We had previously found that we needed at least 3 hours to get a urine sample. Although some babies in our study had lots of urine, it took up to 4 hours to get the required sample in other babies.
The phenylalanine solutions were prepared in the Manufacturing Pharmacy at the Hospital for Sick Children, and were sterilized and tested for bacteria and pyrogens as described above. Solutions were kept at 4ºC until use.

5.3.5 Sample Collection and Analysis

Urine samples were collected from cotton pads placed on the inside of the diapers, for the measurement of baseline and steady state tracer amino acid enrichment. Three baseline urine samples were collected before the start of the intravenous isotope infusion. Three to 5 samples were collected beginning 12 hours after the start of the isotope infusion until the end. Urine was stored at -20ºC until analysis.

Expired CO₂ was collected at baseline and at isotopic steady state from a ventilated hood system using a portable CO₂ analyzer (1400 series, Servomex, Westech Industrial Ltd., Mississauga, Ontario) and mass flowmeter (5860 series, Brooks, Trillium Measurement and Control, Stouffville, Ontario). Briefly, a clear plastic hood was placed over the baby’s head through which the baby had access to room air. The CO₂ concentration in the hood was monitored and expired CO₂ was sampled when the concentration was between 0.2 to 0.5%. In order to minimize the variability in the ratio of ¹²CO₂: ¹³CO₂ we kept the CO₂ concentration within the hood between 0.35 to 0.45% during the measurements. Three baseline samples were collected before the start of the intravenous isotope infusion and five samples were collected at 2-hour intervals, starting from 12 hours after the start of the isotope infusion. Each sample was collected over a period of 10 minutes by bubbling the sampled CO₂ into 10 mL of NaOH through a reflux
condenser to form NaHCO₃. The NaHCO₃ was then injected into Vacuutainers (Beckton Dickson and Company, Franklyn Lakes, NJ) and stored at -20°C until analysis. Carbon dioxide production rate (VC₀₂) was measured between 18 to 23 hours after the start of the intravenous isotope infusion.

Two 0.5 ml samples of blood were collected; one at the end of the first 24 hour period before the start of the test PN solution and the other, at the end of the 2nd day when subjects had received the test methionine intake. Blood was analyses for plasma amino acids, homocysteine, and cystathionine concentration.

The enrichment of L-[1-¹³C]phenylalanine in urine was analyzed using a triple quadrupole mass analyzer (API 4000; Applied Biosystems/MDS SCIEX, Concord, Canada) coupled to an Agilent 1100 HPLC system (Agilent, Mississauga, Canada), as previously described (Turner 2006). We used a Chirobiotic T, Chiral Column purchased from Sigma-Aldrich in order to separate the D-[1-¹³C]phenylalanine from L-[1-¹³C]phenylalanine. Isotopic enrichment was expressed as mole percent excess and was calculated from peak area ratios at isotopic steady state at baseline and plateau. The use of urine as a surrogate of plasma enrichment has been used by our group in an effort to make our studies less invasive for vulnerable groups like women, children and neonates. The urine enrichments have been found to be highly correlated with the plasma enrichments (Bross, Ball et al. 1998) and gives similar results except in cases where the presence of the D-isomer in the isotope infused is in excess of 0.2% or within any detectable range (Darling, Bross et al. 1999).

For the analysis of ¹³C enrichment in breath, samples were prepared by liberating the CO₂ from the NaHCO₃ by mixing 200μL of sample (NaHCO₃) with 200μL of H₃PO₄
into an evacuated tube. Enrichment of $^{13}$C in breath was analyzed by continuous-flow isotope ratio mass spectrometer (20/20 isotope analyzer; PDZ Europa Ltd, Cheshire, United Kingdom). Enrichments were expressed as APE compared with a reference standard of compressed CO$_2$ gas.

Plasma was separated from whole blood within 10 minutes of the blood being drawn from each subject. Plasma amino acid concentration was analysed by reverse phase HPLC using the PICO.TAG (Waters, Milford, MA), pre-column derivitization method with phenylisothiocyanate as the derivative. The amino acid concentrations were determined by using an external standard. Plasma total homocysteine, total cysteine and cystathionine concentrations were determined by LCMS/MS, using a bench top triple quadrupole mass spectrometer API 4000 (Applied Biosystems/MDS SCIEX) operated in positive ionization mode with the TurboIonSpray ionization probe source (operated at 5.8KV). This was coupled to an Agilent 1100 HPLC system (Rafii, Elango et al. 2007).

### 5.3.6 Tracer Kinetics

The model of amino acid kinetics used in this study is based on the model of Matthews et al. (Matthews, Motil et al. 1980). Isotopic steady state in the L-[1-$^{13}$C]phenylalanine enrichment in the urine and $^{13}$CO$_2$ in breath, was represented by unchanging values in each of the data points at baseline and plateau. Phenylalanine flux (in $\mu$mol.kg$^{-1}$.hour$^{-1}$) was calculated from the dilution of the intravenously administered L-[1-$^{13}$C]phenylalanine into the metabolic pool at isotopic steady state using urinary enrichment of L-[1-$^{13}$C]phenylalanine as a representation of plasma enrichment (Bross,
Ball et al. 1998). The rate of phenylalanine oxidation, represented by the rate of appearance of $^{13}$CO$_2$ in breath ($F^{13}$CO$_2$), in ($\mu$mol.kg$^{-1}$.hour$^{-1}$) was calculated according to the model of Matthews et al. (Matthews, Motil et al. 1980). A correction factor of 0.82 was used to account for the retention of $^{13}$CO$_2$ in the body’s bicarbonate pool in the fed state (Van Aerde, Sauer et al. 1985). This individual correction factor was used instead of the algorithm because we calculated the $F^{13}$CO$_2$ using the algorithm as well as using the set correction factor of 0.82 and found the results to be the same. Since the range in energy intakes was narrow, we judged it simpler if we presented our data using a constant correction factor.

Phenylalanine oxidation (in $\mu$mol.kg$^{-1}$.hour$^{-1}$) was calculated from the $F^{13}$CO$_2$ data and the urinary L-[1-$^{13}$C]phenylalanine enrichment (Matthews, Motil et al. 1980).

5.3.7 Statistical analysis

The first and second level of methionine intake studied were the highest and lowest intake levels as a means of bracketing the predicted breakpoint to determine if the correct range of intakes were chosen. Subsequent levels of methionine intake were completely randomized with methionine serving as the main treatment effect.

The effect of methionine intake on phenylalanine flux, oxidation and $F^{13}$CO$_2$ was tested using ANOVA with the PROC GLM procedure (SAS version 9.1; SAS Institute Inc, Cary, NC).

Determination of the methionine mean requirement (breakpoint) was performed using a two-phase linear regression crossover model (Seber 1977) as previously
described (Zello, Pencharz et al. 1990). Regression analysis variables were dietary methionine intake as the independent variable, and F\(^{13}\)CO\(_2\) and phenylalanine oxidation as the dependent variables. Selection of the best model was determined by factors relating to fit (significance of the model and r\(^2\)) and estimate of variation about the model (coefficient of variation and SE of the estimate). The safe population intake was estimated by determining the upper 95% confidence limits of the breakpoint estimate (Seber 1977).

The effect of methionine intake on plasma amino acid concentration was tested using ANOVA. Because the baseline plasma amino acid concentration were variable and the babies did not each receive the same protein intake as was planned, we chose to use the difference in amino acid concentration between baseline and after subjects received the test methionine intake as the main dependent variable. Independent variables tested were age, subject and methionine intake.

All statistical analyses were performed by SAS version 9.1 for Windows (SAS Institute Inc, Cary, NC). Statistical significance was established at p \(\leq\) 0.05.

5.4 Results

5.4.1 Clinical Characteristics and nutrient intake

Clinical characteristics and diagnoses for the 15 subjects studied are presented in Table 5.1. Four of the fifteen infants were above birth weight, 10 infants had just regained birth weight, and 1 infant who was one week old had not yet regained birth
weight. Three subjects received 2 intake levels of methionine for a total of 18 indicator oxidation studies in 15 subjects. Since parenteral nutrition was prescribed by the attending physician and dietitian, the exact nutrient intakes were dependent on the total volume of parenteral nutrition infused. The average energy and protein intakes were 356.46 ± 15.63 kJ.kg⁻¹.day⁻¹ (85 ± 3.74 kcal.kg⁻¹.day⁻¹) and 2.89 ± 0.17 g.kg⁻¹.day⁻¹, respectively (Table 5.3). Average grams of lipids and carbohydrate provided were 2.98 ± 0.18 and 13.39 ± 1.03 (mean ± SD) respectively (Table 5.3).

5.4.2 Urinary amino acid and expired CO₂ enrichment

Isotopic steady state (plateau) was achieved for all neonates by 12 hours after the start of the isotope infusion and was defined by the absence of a significant slope between the data points at plateau. The variation in urinary L-[1-¹³C]phenylalanine at plateau was <10% whereas the variation in expired ¹³CO₂ enrichment within the plateau was <1%.

5.4.3 Phenylalanine Kinetics

The phenylalanine flux of these TPN fed human neonates was 108.7 ± 9.8 μmol.kg⁻¹.hour⁻¹ (mean ± SD). Using ANOVA, there was no significant relationship between methionine intake and phenylalanine flux (p = 0.34). This lack of change in flux in response to changes in methionine intake indicates that differences observed in phenylalanine oxidation and F¹³CO₂ in response to methionine intake were related to a
partitioning of amino acids between oxidation and protein synthesis. One of the strengths and key criterion of the indicator amino acid oxidation (IAAO) method is that the flux of the indicator amino acid (phenylalanine in this study) does not change in response to feeding graded intakes of the test amino acid, in this case methionine. The flux of the indicator amino acid remains constant because the total intake remains constant at all levels of intake of the test amino acid. We kept phenylalanine intake exactly the same at all levels of methionine intake by deducting the amount of the tracer from the total dietary intake and making up the difference in the TPN solution. Because the intake of the indicator amino acid remains constant at all levels of intake of the test amino acid the pool size does not change. This is an advantage over the direct amino acid oxidation (DAAO) method in which the free amino acid pool size of the test amino acid changes with each increment of the test amino acid resulting in variable dilution of the tracer within the pool, thus increasing the variation and decreasing the sensitivity of the estimate.

Methionine intake had a significant effect on both phenylalanine oxidation and $^{13}$CO$_2$ ($p < 0.001$). As methionine intake increased from 9.5 to 47.4 mg.kg$^{-1}$.day$^{-1}$, phenylalanine oxidation decreased ($p < 0.001$); further increases in methionine intake did not affect phenylalanine oxidation Figure 5.1. Similarly, there was a decrease in the $^{13}$CO$_2$ in response to methionine intake to the level of 49.0 mg.kg$^{-1}$.day$^{-1}$ of methionine, after which further increase in methionine intake produced no change in the $^{13}$CO$_2$ Figure 5.2. The decline in phenylalanine oxidation and $^{13}$CO$_2$ until the breakpoint, were similar for both endpoints used and reflects the sensitivity of the urine measurement when compared to the more direct $^{13}$CO$_2$ estimate. The lack of change in phenylalanine
oxidation after the breakpoint indicates that, under the conditions of the study, the test amino acid (methionine) was no longer limiting for protein synthesis. It is possible that other amino acids were then limiting for protein synthesis. However, as detailed in the method section, the amino acid concentrations of the TPN solutions used in the current study met or exceeded the concentrations derived from our piglet studies (Bertolo, Chen et al. 1998; Elango, Pencharz et al. 2002; Bertolo, Brunton et al. 2003; Wilkinson, Bertolo et al. 2004) and our prior human neonatal studies (Roberts, Ball et al. 1998; Roberts, Ball et al. 2001).

To determine the methionine requirements, the data were partitioned between two distinct regression lines (Figures 5.1 and 5.2) using the two-phase linear regression cross-over model. A breakpoint was identified in phenylalanine oxidation at 47.4 mg.kg\(^{-1}\).day\(^{-1}\) (p < 0.001, \(r^2 = 0.81\); Figure 5.1) and \(^{13}\)CO\(_2\) at 49.0 mg.kg\(^{-1}\).day\(^{-1}\) (p < 0.001, \(r^2 = 0.82\); Figure 5.2). The 95% confidence limits of phenylalanine oxidation and \(^{13}\)CO\(_2\) were determined to be 38.7 to 56.1 and 39.9 to 58.0 mg.kg\(^{-1}\).day\(^{-1}\) respectively (Figures 5.1 and 5.2).
Figure 5.1 Parenteral methionine requirement. The effect of methionine intake on phenylalanine oxidation in the TPN fed human neonate

Parenteral methionine requirement. The effect of increasing methionine intake on phenylalanine oxidation in the TPN-fed human neonate (n = 18). By ANOVA, methionine intake had a significant effect on phenylalanine oxidation (p < 0.001, r² = 0.81). Using a two-phase linear regression crossover model the breakpoint (mean methionine requirement) was estimated to be 47.4 mg.kg⁻¹.day⁻¹. The safe population intake estimated by determining the upper 95% confidence limits of the breakpoint estimate was 56.1 mg.kg⁻¹.day⁻¹ with a lower confidence interval of 38.7 mg.kg⁻¹.day⁻¹.
**Figure 5.2** Parenteral methionine requirement. The effect of increasing methionine intake on $^{13}$CO$_2$ in the TPN fed human neonate.

Parenteral methionine requirement. The effect of increasing methionine intake on $^{13}$CO$_2$ in the TPN-fed human neonate (n = 18). By ANOVA, methionine intake had a significant effect on phenylalanine $^{13}$CO$_2$ ($p < 0.001$, $r^2 = 0.82$). Using a two-phase linear regression crossover model the breakpoint (mean methionine requirement) was estimated to be 49.0 mg.kg$^{-1}$.day$^{-1}$. The safe population intake estimated by determining the upper 95% confidence limits of the breakpoint estimate was 58.0 mg.kg$^{-1}$.day$^{-1}$ with a lower confidence interval of 39.9 mg.kg$^{-1}$.day$^{-1}$. 


5.4.4 Plasma amino acid concentration

Using ANOVA, with the difference between plasma methionine concentrations at baseline, and in response to graded intakes of methionine as the dependant variable, methionine intake had a significant effect on plasma methionine concentration ($p = 0.0439$). The difference in plasma methionine showed a linear response to methionine intake; at lower intakes of methionine, plasma methionine decreased but with graded intakes of methionine, plasma methionine concentration showed a linear increase ($r^2 = 0.277$) Figure 5.3.

The difference between plasma homocysteine concentration at baseline and in response to graded intakes of methionine was significantly affected by methionine intake ($p < 0.0001$). Plasma homocysteine concentration increased linearly in response to graded intakes of methionine, with 68% of the difference in homocysteine concentration being accounted for by changes in methionine intake ($r^2 = 0.677$) Figure 5.4.

There was a significant effect of methionine intake on the difference in plasma cystathionine concentration between baseline and in response to graded intakes of methionine ($p = 0.0003$). The difference in plasma cystathionine concentration also showed a linear response to graded intakes of methionine with 59% of the difference in plasma cystathionine concentration being explained by changes in methionine intake ($r^2 = 0.586$) Figure 5.5.

Plasma cysteine concentration significantly decreased from baseline in response to providing the total sulphur amino acid intake as methionine only ($p < 0.0001$). The decrease in plasma cysteine concentration ranged from -51.5 μmol/L at the lowest methionine intake to -141.0 μmol/L at the highest methionine intake, with a mean
decrease of -65.5 μmol/L. However, there was no effect of graded intakes of methionine on plasma cysteine concentration.
Figure 5.3 Plasma methionine concentration in response to graded intakes of methionine

Difference between plasma methionine concentration at baseline and in response to graded intakes of methionine (n=15 due to technical problems in 3 studies with the methionine measurement). Using ANOVA with the Proc GLM procedure methionine intake had a significant effect on the difference in plasma methionine concentration (p = 0.0439), with 28% of the difference in methionine concentration being explained by methionine intake ($r^2 = 0.277$). The regression equation for the calculation of the difference from baseline in plasma methionine concentration in response to increasing methionine intake is given by $y = -18.39 + 0.206x$. The 95% confidence intervals of the estimate were -13.43 to -0.65.
Figure 5.4 Plasma homocysteine concentration in response to graded intakes of methionine

Difference between plasma homocysteine concentration at baseline and in response to graded intakes of methionine (n=18). Using ANOVA with the Proc GLM procedure methionine intake had a significant effect on the difference in plasma homocysteine concentration (p < 0.0001). There was a linear increase in the difference in plasma homocysteine concentration in response to graded intakes of methionine with 68% of the difference in plasma homocysteine concentration being explained by the changes in methionine intake ($r^2 = 0.677$). The regression equation for the calculation of the difference from baseline in plasma homocysteine concentration in response to methionine
intake is given by $y = -1.104 + 0.055x$. The 95% confidence interval of the estimate was 0.76 to 2.94.
Figure 5.5 Plasma cystathionine concentration in response to graded intakes of methionine

Difference between plasma cystathionine concentration at baseline and in response to graded intakes of methionine (n=18). Using ANOVA with the Proc GLM procedure methionine intake had a significant effect on the difference in plasma cystathionine concentration (p < 0.0003), with 59% of the change in plasma cystathionine concentration being explained by changes in methionine intake ($r^2 = 0.586$). The regression equation for the calculation of the difference from baseline in plasma cystathionine concentration in response to methionine intake is given by $y = -0.69 + 0.01x$. The 95% confidence interval of the estimate was -0.36 to 0.07.
5.5 Discussion

This is the first report in which complete parenteral amino acid (AA) solutions were prepared in a research laboratory for use in human infants to determine amino acid needs. Our test AA solution was based on one particular pediatric solution whose AA balance differs in part from others on the market (Brunton, Ball et al. 2000), hence our results must be viewed in light of the test conditions used. Our experience in making such solutions for parenterally fed neonatal piglets (Bertolo, Chen et al. 1998; Elango, Pencharz et al. 2002; Shoveller, Brunton et al. 2003) provided the knowledge required to embark on these complex human studies. We chose to study total sulphur amino acid needs as methionine, because methionine is considered the most toxic of parenteral amino acids (13). Earlier we showed in piglets that parenteral methionine requirements were 69% of those in enterally fed piglets (Shoveller, Brunton et al. 2003). The total sulphur amino acid requirement estimated from our piglet study was 0.26 g.kg\(^{-1}\).day\(^{-1}\) (Shoveller, Brunton et al. 2003). Piglets were fed at a protein intake of 15 g.kg\(^{-1}\).day\(^{-1}\). Because piglets grow at approximately 5 times the rate of human neonates we estimated that at a protein intake of 3 g.kg\(^{-1}\).day\(^{-1}\), the total sulphur amino acid requirement of the PN fed human neonate would be approximately 52 mg.kg\(^{-1}\).day\(^{-1}\).

The mean and 95% confidence estimates (RDA) of the total sulphur amino acid requirement were determined in the current experiment to be 49 and 58 mg.kg\(^{-1}\).day\(^{-1}\) respectively. Given the inherent errors of converting piglet estimates to human infants, mean predicted requirement estimates of 52 mg.kg\(^{-1}\).day\(^{-1}\) (piglet) and 49 mg.kg\(^{-1}\).day\(^{-1}\) (infant) must be considered as remarkably similar. Because the values obtained for the infant are so close to the values we predicted from the piglet studies we believe that this
further validates the use of the piglet TPN model to determine the pattern of amino acids needed to optimize protein synthesis and hence growth in the human neonate (Roberts, Ball et al. 1998; Brunton, Ball et al. 2000; Roberts, Ball et al. 2001).

The lack of change in phenylalanine flux with varying methionine intakes in the current study provides evidence that the differences in oxidation and F$^{13}$CO$_2$ reflects a shift in the partitioning of amino acids between oxidation and protein synthesis. This indicates that at a mean methionine intake of 49 mg kg$^{-1}$ day$^{-1}$, methionine was no longer limiting for protein synthesis. Indeed, in previous studies in which cysteine was supplemented to cysteine free TPN, cysteine supplementation did not enhance nitrogen retention in infants (Zlotkin, Bryan et al. 1981; Malloy, Rassin et al. 1984). In addition, in our piglet studies in which methionine requirements were determined with and without cysteine (Shoveller, Brunton et al. 2003; Shoveller, Brunton et al. 2003), a comparison of phenylalanine oxidation between the two studies showed no difference in oxidation, demonstrating that there was no difference in whole body protein synthesis with or without dietary cysteine. These data along with those of others (Shew, Keshen et al. 2005; Riedijk, van Beek et al. 2007) clearly show that cysteine is not a dietary indispensable amino acid in either the human neonate or the piglet.

The mean plasma methionine concentration of 34.8 ± 12.5 μmol/L observed when babies were adapted to the baseline TPN solution was very similar to that previously reported in breast and TPN fed babies (Zlotkin, Bryan et al. 1981; Wu, Edwards et al. 1986; Heird, Dell et al. 1987). Plasma methionine showed an overall significant decrease in response to graded intakes of methionine, which was mainly due to the decrease observed below the methionine requirement. Once requirement was reached, the
difference in plasma methionine was closer to zero (figure 5.3). This pattern of response was similar to that observed by Tontisirin et al (Tontisirin, Young et al. 1973), when plasma tryptophan was used to determine tryptophan requirement in the elderly.

The increase in plasma homocysteine in response to graded intakes of methionine (figure 5.4) was similar to that observed in our piglet study (Shoveller, House et al. 2004). These and our piglet data (Shoveller, House et al. 2004), clearly show that methionine intake in TPN feeding has a significant positive effect on plasma homocysteine concentration. Plasma homocysteine is of concern because mean homocysteine concentration of >8.5 \( \mu \text{mol.L}^{-1} \) has been linked to ischemic and hemorrhagic stroke in infants and children (van Beynum, Smeitink et al. 1999; Hogeveen, Blom et al. 2002). Although the mean plasma homocysteine concentration did not reach such high levels in the current study, it is possible that PN solutions with higher methionine content could result in more undesirable homocysteine concentrations. The provision of the total sulphur amino acids as a balance of methionine and cysteine is recommended because the provision of a portion of the total sulphur amino acids as cysteine was found to decrease transmethylolation of methionine to homocysteine and increase remethylation of homocysteine to methionine (Di Buono, Wykes et al. 2003).

More importantly, provision of cysteine reduces the total amount of methionine required in the diet (Di Buono, Wykes et al. 2001).

Plasma cystathionine also responded linearly to graded intakes of methionine; however the difference in cystathionine concentration from baseline was only positive at the higher intakes of methionine. At methionine intakes below 61.5 mg.kg\(^{-1}\).day\(^{-1}\), the difference in plasma cystathione was negative (figure 5.5). This was surprising because
it is generally accepted that the enzyme cystathionase is underdeveloped in the human neonate (Gaull, Sturman et al. 1972; Zlotkin and Anderson 1982) and elevations in plasma cystathionine has been reported in the preterm (<32 weeks gestation) neonate (Vina, Vento et al. 1995). The plasma cystathionine concentration observed in the current study however is consistent with that observed in a cohort of over 4000 neonates, which ranged from 0.2 to 1.53 μmol/L (Refsum, Grindflek et al. 2004).

Plasma cysteine concentration showed a significant decrease from baseline in response to feeding the test TPN solution devoid of cysteine. The decrease in plasma cysteine especially at the higher methionine intakes is likely due in part to the mode of feeding since it has been found that the gastrointestinal tract is a significant site of transsulfuration (Riedijk, Stoll et al. 2007), and that extrahepatic tissues have limited capacity for transsulfuration (Stegink and Den Besten 1972). However, the observed concentrations were higher than that of the term breastfed infant in whom concentrations of 153 μmol/L were reported (Wu, Edwards et al. 1986). Zlotkin et al (Zlotkin, Bryan et al. 1981) reported plasma methionine and cysteine concentrations of 105.0 and 59.6 μmol/L in neonates receiving cysteine free PN providing 127mg.kg⁻¹ of methionine. The lower cysteine concentrations observed by Zlotkin et al (Zlotkin, Bryan et al. 1981) could be related to the use of amino acid automated amino acid analysis method which has been shown to underestimate cysteine concentrations in plasma and urine (Malloy, Rassin et al. 1983).

Although cysteine was not provided in this study, our flux oxidation and F¹³CO₂ data provide evidence that at a mean intake of 49 mg.kg⁻¹.day⁻¹ of methionine, protein synthesis was no longer limiting in the subjects and hence cysteine availability was most
likely adequate. This is supported by evidence that neonates fed cysteine free TPN are able to maintain adequate growth and nitrogen balance (Zlotkin, Bryan et al. 1981; Malloy, Rassin et al. 1984).

We conclude that the TSAA (as methionine only) requirement of the PN fed post-surgical human neonate is lower than in current commercially available PN solutions. We observed that plasma methionine and homocysteine concentrations increase in response to feeding high intakes of methionine. Since methionine produces cholestatic changes in the liver of animals, and high homocysteine concentration is implicated in stroke in infants and children, we believe that the current commercial PN solutions need to be reconsidered and revised to include a lower SAA content.
The following study presented in chapter 6 has been published by The Journal of Nutrition.

6 METHIONINE-ADEQUATE CYSTEINE-FREE DIET DOES NOT LIMIT ERYTHROCYTE GLUTATHIONE SYNTHESIS IN YOUNG HEALTHY ADULT MALES

6.1 Abstract

Most methods of determining amino acid requirements are based on endpoints that determine adequacy for protein synthesis. However, the sulfur amino acid (SAA) cysteine is believed to be the rate-limiting substrate for synthesis of the most abundant intracellular antioxidant; glutathione (GSH). Our objectives were: to determine if supplementation of cysteine to a diet containing adequate SAA for protein synthesis, as methionine, increased GSH synthesis, by measuring the fractional and absolute synthesis rates, and if concentration of glutathione changed in response to feeding 5 graded intakes of cysteine (0, 10, 20, 30 and 40 mg.kg⁻¹.day⁻¹), in a random order with a fixed methionine intake of 14 mg.kg⁻¹.day⁻¹, and a protein intake of 1 g.kg⁻¹.day⁻¹. Each subject received a multivitamin and choline supplement during the study. Four healthy adult males each underwent five isotope infusion studies of 7 hour duration after a 2-day adaptation to the level of cysteine intake being studied on the isotope infusion day. The isotope used was [U⁻¹³C₂⁻¹⁵N]glycine. Analyses included erythrocyte glutathione synthesis rates and concentration and urinary sulfate excretion. The glutathione synthesis rates and concentration, measured at a methionine intake of 14 mg.kg⁻¹.day⁻¹, did not change with increasing intakes of cysteine. Urinary sulfate excretion showed a significant positive relationship with cysteine intake. In conclusion, this study provides preliminary
evidence that consumption of SAA adequate to meet the requirement for protein synthesis does not limit GSH synthesis in healthy adult males receiving an otherwise adequate diet.

### 6.2 Introduction

Methods for determining amino acid (AA) requirements, including nitrogen balance and carbon oxidation methods, use endpoints that reflect uptake of the amino acid for protein synthesis. In addition to their roles for protein synthesis, the sulfur amino acids (SAA); methionine and cysteine are required for DNA and RNA methylation (Stipanuk 1986; Griffith 1987), creatine, epinephrine and carnitine synthesis (methionine), and for maintenance of the body’s redox status, which is mediated by the body’s most abundant antioxidant and free radical scavenger; glutathione (GSH) (Meister and Anderson 1983) (cysteine).

Glutathione status has been shown to be very sensitive to changes in cysteine intake (Stipanuk, Coloso et al. 1992; Kwon and Stipanuk 2001) and cysteine is considered the rate-limiting substrate for GSH synthesis (Lyons, Rauh-Pfeiffer et al. 2000; Badaloo, Reid et al. 2002; Jackson, Gibson et al. 2004). Cysteine deficiency, as measured by changes in GSH synthesis and concentration, has been observed in healthy adults consuming a SAA free diet (Lyons, Rauh-Pfeiffer et al. 2000), as well as healthy adults consuming a calorie-adequate, low-protein diet.

In addition, cysteine deficiency has been observed in stress and disease states including children with edematous malnutrition (Reid, Badaloo et al. 2000), adults with...
HIV (Jahoor, Jackson et al. 1999), and Sickle Cell Disease (Reid, Badaloo et al. 2006). The cysteine deficiency, as reflected by decreased GSH synthesis and concentrations in those studies, was mainly attributed to an increase in cysteine requirement due to an increased utilization of cysteine for GSH synthesis. The short term supplementation of N-acetyl-cysteine to subjects in those studies (Jahoor, Jackson et al. 1999; Badaloo, Reid et al. 2002) led to significant improvement in GSH synthesis and concentration.

There is some evidence from animal and human data that GSH synthesis is not adequate at SAA intakes adequate for protein synthesis and maintenance of nitrogen balance (Stipanuk, Coloso et al. 1992; Hunter and Grimble 1994; Badaloo, Reid et al. 2002; Jackson, Gibson et al. 2004). These data have been interpreted to mean that protein synthesis has a higher priority for cysteine than does GSH synthesis (Stipanuk, Dominy et al. 2006). The findings by Kurpad et al (Kurpad, Regan et al. 2004), that undernourished Indian men have the same requirement for total SAA (as provided by methionine alone without cysteine) as their well nourished Indian counterparts and well nourished North American men, therefore begs the question as to whether the SAA requirements as determined using currently available methods, which reflect the needs for protein synthesis, underestimates the SAA intake necessary to maintain a normal GSH status.

The goal of the present study was to determine if GSH synthesis would increase following supplementation of the level of TSAA intake that supported maximum protein synthesis. This was determined by measuring erythrocyte GSH fractional and absolute synthesis rates as well as erythrocyte GSH concentration in healthy adult males fed an
diet providing 1g.kg\(^{-1}\) protein in the presence of the mean population requirement for TSAA (as methionine only) of 14 mg.kg\(^{-1}\).day\(^{-1}\) and a varying additional cysteine intake.

### 6.3 Subjects and Method

#### 6.3.1 Subjects

Ethical approval for the study was obtained from The Research Ethics Board at The Hospital for Sick Children. Written informed consent was obtained from each subject after the protocol was explained to them fully. Four young healthy adult males participated in this study. To participate in the study, each subject had to be in good health as determined by medical history and blood test which included normal cell count and no evidence of anemia as determined by white and red cell count, hemoglobin, hematocrit, mean cell volume and mean cell hemoglobin concentration within the normal range for age. Exclusion criteria were: presence of diseases known to affect GSH concentration (e.g. HIV and diabetes), anemia, medications known to affect protein and amino acid metabolism (e.g. steroids), significant weight loss during the past month, consumption of weight reducing diets, inability to tolerate the experimental diet, unwillingness to have blood drawn from a venous access during the study, significant caffeine consumption (equivalent to more than 2 cups of coffee per day), or significant alcohol consumption (more than one drink per day, e.g.1 beer, \(\frac{1}{2}\) glass of wine).
Before the commencement of the study, height, weight and body composition were measured for each individual. The subject’s characteristics are presented in Table 6.1.
Table 6.1 Subject characteristics of adult men

Physical characteristics of the 4 adult male subjects who participated in the study

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>20.3</td>
<td>1.5</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>67.8</td>
<td>5.6</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.73</td>
<td>0.1</td>
</tr>
<tr>
<td>BMI (kg.m$^2$)</td>
<td>22.7</td>
<td>1.6</td>
</tr>
<tr>
<td>Lean Body mass (kg)</td>
<td>51.2</td>
<td>5.5</td>
</tr>
</tbody>
</table>

6.3.2 Study design and dietary intervention

The 4 subjects each underwent 5 stable isotope infusion studies of 7-hour duration, in a repeated measures design, to measure glutathione synthesis in response to 5 different intakes of dietary cysteine, assigned in random order. The decision to use 4 subjects and 5 levels of cysteine intake per subject was carefully made. A repeated measures design, in which each subject acts as their own control, is a powerful design to reduce the impact of subject variability, and this approach is also more sensitive to treatment differences than the alternative designs. In addition, we had predicted, based upon observations reported in the literature, that a curvilinear response was probable. The choice of 5 cysteine intakes was made to ensure that we could adequately detect and prove a curvilinear response, if it occurred. Alternatively, with 5 increasing intakes of cysteine per subject, if there was a linear response in any of our measured parameters this would be clearly established.

Each individual study was carried out over a period of 3 days and individuals completed all their studies within a 3 month period. The first 2 days were the adaptation days during which subjects were adapted to the level of cysteine administered for that study. The third day was the isotope infusion study day. A two-day adaptation period was chosen because Lyons et al. (Lyons, Rauh-Pfeiffer et al. 2000) showed that the fractional glutathion synthesis rate in blood was 65%, suggesting that the half-life of GSH in red blood cells was 18 hours. In addition, data from Stipanuk’s group (Lee, Londono et al. 2004), showed that the activity of the rate limiting enzyme for GSH synthesis reached a new steady state in liver within 16 hours of changing from a low to a high protein diet.
and that liver GSH and cysteine concentrations reached a new steady state within 12 hours. In addition, Jackson et al. (Jackson, Gibson et al. 2004) were able to show a significant decrease in GSH FSR on the 3rd day after switching healthy subjects from their habitual protein intake to the WHO recommended intake of 0.75 g.kg⁻¹.day⁻¹. In the same study, after a nine day adaptation on the WHO 0.75 g.kg⁻¹.day⁻¹ protein intake, the FRS was the same as that measured on the 3rd day of the diet. The suggestion is that the amino acid pool had already undergone an adequate adaptation after 2 days. Based on these data, a 48 hour adaptation period was judged to be adequate to achieve equilibration of GSH in erythrocyte after each individual change in cysteine intake.

The diet was provided as an experimental formula and protein-free cookies developed for amino acid kinetic studies (Zello, Pencharz et al. 1990). Briefly, a liquid formula (protein-free powder, Product 80056, Mead Johnson, Evansville, IN) flavored with orange and fruit crystals (Tang and Kool-Aid respectively; Kraft Foods, Toronto, Canada) and protein-free cookies supplied the main source of energy in the diet. The composition of the protein free powder and fruit crystals is presented in Table 6.2. The nitrogen content of the diet was provided as a crystalline amino acid mixture (1.0 g.kg⁻¹.day⁻¹), based on the amino acid pattern of egg protein (Geigy 1970). The reason for using egg protein composition is because most studies on amino acid requirement have used this pattern. Certainly, all studies from which the total sulphur amino acid requirement was based for this current study used egg protein. For consistency and to allow comparisons to be drawn, the same amino acid pattern was chosen (Geigy 1970). The adaptation diet for the two days before the tracer infusion study provided calorie content for resting energy expenditure (REE) x 1.7, whereas the energy content of the
diet on the isotope infusion day was provided as REE x 1.5. REE was measured by open
circuit indirect calorimetry (Deltatrac; SensorMedics, Yorba Linda CA USA). The
macronutrient content of the experimental diet, expressed as a percentage of dietary
energy, was 53% carbohydrate, 37% fat and 10% protein. The diet was weighed
(Sartorius Balance model BP110 S; Sartorius, Mississauga, ON, Canada) and prepared in
the research kitchen at The Hospital for Sick Children.

Subjects also consumed a daily multivitamin supplement (Centrum Forte;
Whitehall Robins Inc, Mississauga, ON, Canada) containing 0.6 mg folic acid, 5 mg
Vitamin B-6, and 20μg Vitamin B-12 beginning two weeks before the start of the study
and continuing for the entire duration of the 5 studies. In addition, a choline supplement
of 500 mg was provided daily beginning one week before the start of the first experiment
and continuing for the entire three month study duration. The multivitamin supplement
was provided to ensure adequate supply of all cofactors involved in sulphur amino acid
metabolism. The choline supplement was provided because the experimental diet is low
in choline about 150 to 200 mg choline.day⁻¹, while the adequate intake (AI) for choline
is 550 mg.day⁻¹ for adult males (Food and Nutrition Board 1998). It also provided
consistency among studies on sulphur amino acid metabolism as previous studies had
also given 500 mg choline.day⁻¹ to the subjects (Storch, Wagner et al. 1988; Kurpad,
Regan et al. 2003).

During the adaptation days, the diet was provided as four equal meals per day to
be consumed at the same time each day. Subjects were allowed water in their desired
quantity but caffeinated beverage, alcohol, or any other drinks except that provided by
the diet were not allowed. On the isotope infusion day the diet was provided as 10
isoenergetic, isonitrogenous meals each representing 1/12 of the subject total daily requirement. Subjects had free access to water on the study day.

Methionine was provided at an intake of 14 mg.kg^{-1}.day^{-1}, which is an average of the two mean published estimates (using carbon oxidation techniques) for TSAA requirement in adult humans (Di Buono, Wykes et al. 2001; Kurpad, Regan et al. 2003). We chose this level of methionine because it represented the mean estimated total sulphur amino acid requirement for protein synthesis as estimated by nitrogen balance (Rose, Coon et al. 1955), indicator amino acid oxidation technique (Di Buono, Wykes et al. 2001), and 24-hour indicator oxidation-balance technique (Kurpad, Regan et al. 2003). The levels of cysteine studied were 0, 10, 20, 30 and 40 mg.kg^{-1}.day^{-1}. The 0 mg.kg^{-1}.day^{-1} allowed for the estimation of GSH kinetics at the mean TSAA requirement of 14 mg.kg^{-1}.day^{-1}. The cysteine intake of 10 mg.kg^{-1}.day^{-1} combined with the methionine of 14 mg.kg^{-1}.day^{-1} provided the total sulfur amino acid intake of 24 mg.kg^{-1}.day^{-1}; the estimated RDA for total sulfur amino acid requirement (Di Buono, Wykes et al. 2001; Kurpad, Regan et al. 2003). The cysteine intake of 20, 30 and 40 mg.kg^{-1}.day^{-1} provided cysteine in an amount typically consumed in the western diet by individuals consuming a mixed protein diet providing protein in an amount > 1.0 g.kg^{-1}.day^{-1} (Jahoor, Jackson et al. 1999; Jackson, Gibson et al. 2004). These intakes also represented 30, 50 and 65%, respectively, of the amount given in a supplementation study of children with malnutrition (Badaloo, Reid et al. 2002). The 40 mg.kg^{-1}.day^{-1} of cysteine was chosen to represent the possible highest level beyond which increases in GSH synthesis would be unlikely in healthy subjects.
Glycine was provided at an intake of 69.5 mg.kg\(^{-1}\).day\(^{-1}\) which is twice that found
in the high quality egg protein at a protein intake of 1 g\(^{-1}\).kg\(^{-1}\). This was judged to be
adequate to prevent it being deficient for GSH synthesis. The amount of \([^{15}\text{N} – ^{13}\text{C}_2]\)glycine provided on the study day was subtracted from the dietary provision to
maintain the intake at 69.5 mg.kg\(^{-1}\).day\(^{-1}\). The glycine content of the diet was increased to
ensure glycine was not limiting for GSH synthesis. The glycine content of egg protein is
3.8% whereas the glycine content of human tissue protein is 7.2%. We chose to increase
the glycine content to make it more comparable to the composition in human tissue. The
amino acid composition of the amino acid mixture used in the study is presented in Table
6. 3.
### Table 6.2 Composition of protein free powder and flavoured crystals

Composition of protein free powder and flavored crystals used in the diet of subjects

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Protein–free powder Per 100g Powder</th>
<th>Flavored crystals Per 100g Powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, g</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Fat, g</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate, g</td>
<td>72</td>
<td>94</td>
</tr>
<tr>
<td>Linoleic acid, g</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>Vitamins:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin A, µg</td>
<td>545</td>
<td></td>
</tr>
<tr>
<td>Vitamin D, µg</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Vitamin E, (α-Tocopherol), mg</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Vitamin K, µg</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Thiamin, µg</td>
<td>450</td>
<td></td>
</tr>
<tr>
<td>Riboflavin, µg</td>
<td>540</td>
<td></td>
</tr>
<tr>
<td>Vitamin B6, µg</td>
<td>360</td>
<td></td>
</tr>
<tr>
<td>Vitamin B12, µg</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Niacin, µg</td>
<td>7200</td>
<td></td>
</tr>
<tr>
<td>Folic acid, µg</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Pantothenic acid, µg</td>
<td>2700</td>
<td></td>
</tr>
<tr>
<td>Biotin, µg</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Vitamin C, mg</td>
<td>47</td>
<td>60</td>
</tr>
<tr>
<td>Choline, mg</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Nutrient</td>
<td>Amount</td>
<td>RDA</td>
</tr>
<tr>
<td>-----------------------</td>
<td>--------</td>
<td>---------</td>
</tr>
<tr>
<td>Inositol, mg</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Minerals:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium, mg</td>
<td>540</td>
<td>60</td>
</tr>
<tr>
<td>Phosphorus, mg</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Magnesium, mg</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Iron, mg</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>Zinc, mg</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Manganese, μg</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>Copper, μg</td>
<td>540</td>
<td></td>
</tr>
<tr>
<td>Iodine, μg</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Selenium, μg</td>
<td>16.1</td>
<td></td>
</tr>
<tr>
<td>Sodium, mg</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Potassium, mg</td>
<td>340</td>
<td></td>
</tr>
<tr>
<td>Chloride, mg</td>
<td>135</td>
<td></td>
</tr>
</tbody>
</table>
Table 6. 3 Amino acid composition

Amino acid composition of the amino acid mixture used in the study.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td></td>
</tr>
<tr>
<td>L-Arginine-HCL</td>
<td>74.5</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>33.0</td>
</tr>
<tr>
<td>L-Aspartic Acid</td>
<td>33.0</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td></td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>56.2</td>
</tr>
<tr>
<td>L-Glutamic Acid</td>
<td>56.2</td>
</tr>
<tr>
<td>L-Glycine</td>
<td>76.0</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>22.5</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>62.4</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>82.6</td>
</tr>
<tr>
<td>L-Lysine-HCL</td>
<td>75.12</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>14.0</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>54.2</td>
</tr>
<tr>
<td>L-Proline</td>
<td>41.6</td>
</tr>
<tr>
<td>L-Serine</td>
<td>83.2</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>46.7</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>15.5</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>40.4</td>
</tr>
<tr>
<td>L-Valine</td>
<td>69.7</td>
</tr>
</tbody>
</table>

1 Alanine content of AA mixture varied to make mixture isonitrogenous with varying cysteine intakes

2 L-cysteine intake varied based on dietary cysteine intake being studied

3 L-Glycine intake made up from diet and isotope on isotope infusion day and from dietary glycine only on adaptation days.
6.3.3 Tracer protocol

[U\(^{13}\)C\(_2\)\(^{15}\)N\)]glycine (98% \(^{13}\)C\(_2\), 98% \(^{15}\)N\)), was purchased from Cambridge Isotope Laboratories, Andover, MA. Stock solutions were prepared in 0.9 % sodium chloride (10 mg.ml\(^{-1}\)) by the Research Pharmacy at the Hospital and were confirmed to be sterile and pyrogen free. We chose to use an \(M+3\) glycine tracer as the GSH precursor to determine its incorporation into the GSH molecular ion by LCMS/MS. In preliminary studies with \(M+2\) glycine we were unable to accurately detect the enrichment in the whole GSH molecule above background because of the high baseline \(M+2\) GSH enrichment.

Each tracer infusion study was conducted on day 3, after completion of the 2-day adaptation on the liquid amino acid based diet, subjects arrived at the Clinical Research Centre at the Hospital for Sick Children after a 12 hour overnight fast. Ten hourly, isoenergetic, isonitrogenous meals were consumed beginning 3 hours before the start of the intravenous isotope infusion. The cysteine content of each meal was dependent on the test level being studied. Because the amount of cysteine in the diet was manipulated, L-alanine was adjusted to keep the nitrogen content of the diet constant.

After consumption of the first two meals, intravenous catheters were inserted into superficial veins of both arms, one for continuous infusion of the tracer solution and the other for repeated blood sampling. Baseline blood (2.0 mL) was collected after the 3\(^{rd}\) hourly meal. At the beginning of the 4\(^{th}\) meal, a priming dose of [U\(^{13}\)C\(_2\)\(^{15}\)N\)]glycine (40 \(\mu\)mol.kg\(^{-1}\)) was given over a 15 minute period, followed immediately by a continuous infusion of [U\(^{13}\)C\(_2\)\(^{15}\)N\)]glycine (15 \(\mu\)mol.kg\(^{-1}\).hour\(^{-1}\)) for 7 hours. Blood samples (1.5
mL) were taken hourly from the 3rd hour of the infusion until the 5th hour and then every 0.5 hour until the end. To ensure arterialized blood, the hand was heated inside a thermostatic chamber maintained at 60 ºC for ≥ 15 min before the blood was sampled (Zello, Smith et al. 1990).

Urine samples were collected after each void for the 10 hours of the study day. Samples were pooled and 2 x 2 mL aliquots were stored at -20 ºC for urinary sulphate analysis in order to relate changes in cysteine intake to sulphate excretion.

6.3.4 Sample analysis

Blood (0.5 mL) for hematocrit was collect into tubes containing Na2EDTA and immediately sent to the Clinical Biochemistry lab at the Hospital for Sick Children for analysis. Briefly, the red cells were analyzed using an Abbott CELL-DYN Sapphire Hematology Analyzer. Hematocrit was then calculated using the formula; HCT (L/L) = (RBC x MCV) / 1000, where HCT = hematocrit, RBC = red blood cell and MCV = mean corpuscular volume.

6.3.4.1 Erythrocyte glutathione concentration and enrichment

All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, Ontario. A 0.5 mL aliquot of each blood sample collected in Na2EDTA was centrifuged for 2 minutes at 13,000 x g (Beckman Microfuge-TM 11, Beckman Coulter Canada Inc.) within 5 minutes of collection. Each tube was weighed before and after blood collection
to determine the volume of blood because GSH concentration was normalized to hematocrit. After centrifugation the plasma was immediately removed. Two hundred µL of 100 mmol/L N-ethylmaleimide, and 20 µL of 5 mmol/L gamma-glutamyl-leucine (internal standard) were added to the separated red blood cells. The sample was then capped, vortexed and left for 10 minutes at room temperature. Cells were then lysed with 50 µL 0.4 M ZnSO₄, and the protein precipitated with 1 mL ice cold methanol. The sample was then vortexed, centrifuged for 2 minutes, the supernatant removed and stored at -80 °C until analysis.

Glutathione concentration and enrichment were analyzed using a triple quadrupole mass spectrometer API 4000 (Applied Biosystems/MDS SCIEX, Concord, Canada) operated in positive ionization mode with the Turbo Ion Spray ionization probe source (operated at 5.8 KV). This was coupled to an Agilent 1100 HPLC system (Agilent, Mississauga, Canada). All aspects of system operation and data acquisition were controlled using The Analyst NT v 1.4.1 software. Glutathione concentration was measured using an external standard curve and the ratio of the analyte (GSH) to the internal standard (Gamma-glutamyl-leucine). The parent to daughter transition measured for GSH and the internal standard were 433.4 to 304.3 and 261.4 to 132.0 respectively. GSH concentration was determined in the whole GSH molecule (as the tripeptide). There was no fractionation and hydrolysis step prior to measurement of enrichment. Glutathione enrichment was calculated as a ratio of the (enriched) $M+3$ to (unenriched) $M$ peaks of the tripeptide molecule of glutathione by measuring the transition of parent to daughter ions of 436.4 to 307.0 (m+3) and 433.4 to 304.3 (M+0) and was expressed as mole percent excess calculated from peak area ratios at isotopic steady state of erythrocyte
glycine in the last 2 hours of isotope infusion. Inter-assay precision for GSH concentration was between 2.3 to 4.8% whereas inter-assay precision for GSH enrichment was $3.7 \pm 7.3\%$. The accuracy of the instrument for GSH concentration was measured by spiking samples with a known amount of GSH and comparing to the unspiked sample. Concentrations were determined using a standard curve. Accuracy of GSH concentration was between 90 to 108% of expected. Accuracy for GSH enrichment was measured using enrichment curves. Enrichment curves were linear within the ranges of expected sample enrichment. The results of the enrichment curve was $y = 0.94x + 0.097$, $r^2 = 0.99$.

### 6.3.4.2 Erythrocyte free glycine enrichment

Each sample was collected and centrifuged as above. Plasma was quickly removed and the cells washed twice with 300 μL iced cold saline on each occasion. Samples were vortexed between each wash. Cells were then lysed and de-proteinated as above, vortexed, centrifuged for 2 minutes at 13,000 x g and the supernatant stored at -80 °C until analysis.

Fifty μL of each sample was then dried under nitrogen at 35 °C. One hundred μL of butanol.HCl (Sigma-Aldrich Canada Ltd., Oakville, Ontario), was then added and the sample vortexed, topped with nitrogen and heated for 20 minutes at 55 °C. The sample was again dried under nitrogen, and reconstituted in 0.1% formic acid (Sigma-Aldrich Canada Ltd., Oakville, Ontario). Samples were then analyzed using a triple quadrupole mass analyzer as described above. Glycine enrichment was calculated as the ratio of the
(enriched) M+3 to (unenriched) M peaks of glycine after derivitization with butanol.HCL. The masses of the parent to daughter transitions of butylated glycine monitored were 135.2 to 79.0 (M + 3) and 132.2 to 76.0 (M).

The intra-assay precision of the triple quadrupole mass analyzer for measurements of erythrocyte free glycine enrichment was between 3 to 5 %. Accuracy for erythrocyte free glycine enrichment was measured using enrichment curves. Enrichment curves were linear within the ranges of expected sample enrichment. The results of the enrichment curve was $y = 0.732x + 0.042$, $r^2 = 0.99$ for glycine enrichment.

6.3.4.3 Urinary Sulphate

Urinary sulphate was measured using the method of Swaroop (Swaroop 1973). Briefly, a standard curve was made using known concentrations of Ba$^{2+}$ with sodium rhodizonate to form a red coloured complex which was measured at 520 nm against water. A known amount of sulphate was then added, to form a BaSO$_4$ precipitate, which resulted in a diminished colour and absorbance. The standard curve was obtained by plotting concentration of sulphate on the x-axis and differences in absorbance between blank (water) and corresponding standard on the y-axis. One ml of each urine sample was then diluted to 200 ml with distilled water from which 0.05 ml was removed and 2.0 ml of ethanol added and vortexed. To each tube, BaCl$_2$, sodium rhodizonate was added and the tubes vortexed. The tubes were then allowed to stand for 10 minutes in the dark after which the red color produced was measured at 520 nm against water. The difference of
absorbance between blank and sample was read on the graph and corrected for the dilution.

6.3.5 Calculations

6.3.5.1 Fractional synthesis rate of erythrocyte glutathione

The fractional synthesis rate (FSR) of erythrocyte glutathione (FSR$_{GSH}$) was calculated using the precursor-product method of Jahoor et al (Jahoor, Wykes et al. 1995):

$$\text{FSR}_{	ext{GSH}} \text{ (%) / d)} = \frac{(E_{t7} - E_{t5})/E_{RBC} \times (24 \times 100)}{(t7 - t5)} \quad (1)$$

Where $(E_{t7} - E_{t5})$ was the increase in the isotopic enrichment of erythrocyte glutathione between the fifth and seventh hours of infusion as a result of the incorporation of the labeled glycine, $E_{RBC}$ was the intracellular glycine enrichment at isotopic steady state, and $(t7 - t5)$ was the time interval between the fifth and seventh hour when the incorporation of glycine into glutathione is measured.

$$\text{ASR} = \text{GSH mass} \times \text{FSR}_{	ext{GSH}} \quad (2)$$

Where GSH mass = the product of the cell volume (or cell number or cell protein) and the concentration of GSH in the cell. Hematocrit was calculated using the formula;

$$\text{HCT (L/L)} = \frac{(\text{RBC} \times \text{MCV})}{1000}.$$
6.3.6 Statistical analysis

The data was analyzed by repeated measures ANOVA with the PROC MIXED procedure to assess the effects of cysteine intake on glutathione FRS, ASR and concentration. Other independent variables tested were cysteine intake, subject and order of cysteine intake as well as the interaction between cysteine intake and order.

Repeated measures ANOVA with the PROC MIXED and PROC GLM procedures were also used to assess the effect of cysteine intake on urinary sulphate excretion.

When significant differences were identified, individual differences were assessed by post hoc analysis with Bonferroni correction for multiple comparisons. Statistical significance was established at $p \leq 0.05$. Data was analyzed using SAS version 9.1 for Windows (SAS Institute Inc, Cary, NC).

6.4 Results

The age and physical characteristics of the 4 healthy male subjects who participated in the study are presented in Table 6.1. Isotopic steady state was achieved in the erythrocyte free intracellular glycine pool by 5 hours after the start of the isotope infusion. This was determined by the absence of a significant slope between data points from 5 to 7 hours using ANOVA (Figure 6.1). Therefore, FSR was calculated based on the linear incorporation of glycine into glutathione during the last two hours of the infusion.
Cysteine intake did not affect erythrocyte glutathione concentration (p = 0.9379) fractional synthesis rate (p = 0.4879), or absolute synthesis rate (p = 0.2245) Figure 6.2. The individual data for the FSR for each individual subject is presented in Table 6.4.

Urinary sulphate excretion normalized to creatinine excretion (Figure 6.3) increased with increasing cysteine intake (p = 0.002, r² 0.92). Order of cysteine intake did not affect urinary sulphate excretion (p = 0.57). Also, absolute urinary sulphate excretion (data not shown) increased with increasing cysteine intake (p = 0.0009, r² 0.95). Order of cysteine intake did not affect absolute urinary sulphate excretion.
Figure 6. 1 Mean tracer-to-tracee molar ratio (mol % above baseline) of erythrocyte free glycine

The mean net tracer-to-tracee molar ratio (mol% above baseline) of erythrocyte free glycine (A) and the net tracer-to-tracee enrichment of erythrocyte glutathione (B) during a 7-hour continuous infusion of [U-^{13}C_2-^{15}N]glycine in young healthy adult males. There was no difference among the erythrocyte free glycine enrichment at plateau at the
different time points from 5 to 7 hours determined by repeated measures ANOVA (p = 0.43).
Figure 6.2 Glutathione kinetics in response to graded intakes of cysteine

Mean (± SEM) effect of 5 levels of cysteine intake on glutathione concentration, fractional synthesis rates of glutathione, and the absolute synthesis rates of glutathionine for all 4 subjects who participated in the study at a set methionine intake of 14 mg.kg⁻¹.day⁻¹. Using repeated measures ANOVA with the PROC MIXED procedure, cysteine intake had no effect on GSH concentration (p = 0.9379), FRSGSH (p = 0.4879), or ASRGSH (p = 0.2245).
Table 6.4 Individual FSR at varying cysteine intake levels

Glutathione fractional synthesis rate (%/d) for each individual subject at each level of cysteine intake

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Cysteine intake (mg.kg⁻¹.day⁻¹)</th>
<th>GSH FSR %/d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>64.2</td>
<td>67.73</td>
</tr>
<tr>
<td>2</td>
<td>25.91</td>
<td>58.76</td>
</tr>
<tr>
<td>3</td>
<td>22.11</td>
<td>45.97</td>
</tr>
<tr>
<td>4</td>
<td>60.02</td>
<td>72.06</td>
</tr>
<tr>
<td>Mean</td>
<td>43.06</td>
<td>61.13</td>
</tr>
<tr>
<td>SD</td>
<td>22.11</td>
<td>11.52</td>
</tr>
</tbody>
</table>

By ANOVA, cysteine intake had no significant effect on glutathione synthesis, but subject did (p = 0.01)
Mean (± SEM) effect of varying cysteine intake on urinary sulphate excretion (normalized to creatinine) of all 4 subjects. Using repeated measures ANOVA, cysteine intake had a significant effect on sulphate excretion ($p = 0.002$, $r^2 0.92$). Means with different superscript are significantly different from each other (repeated-measures ANOVA followed by post hoc analysis with Bonferroni correction).
6.5 Discussion

This is the first study to our knowledge, to report on the erythrocyte glutathione kinetics in healthy adult males in response to varying cysteine intake levels in the presence of an adequate protein and energy intake and the mean methionine requirement of 14 mg.kg\(^{-1}\).day\(^{-1}\). These results suggest that in the presence of an adequate protein intake of 1 g.kg\(^{-1}\).day\(^{-1}\), and the mean methionine requirement of 14 mg.kg\(^{-1}\).day\(^{-1}\) (Di Buono, Wykes et al. 2001; Kurpad, Regan et al. 2003), further increases in the sulphur amino acid (SAA) intake in the form of cysteine, did not affect erythrocyte glutathione metabolism (Figure 6.2). The glutathione synthesis rates and concentrations observed in the current study were similar to those observed in previous studies of glutathione kinetics in healthy adults (Lyons, Rauh-Pfähler et al. 2000; Jackson, Gibson et al. 2004).

The inability to measure GSH kinetics in liver or muscle due to practical and ethical considerations is a potential limitation of in vivo studies with healthy subjects. However, erythrocyte GSH kinetics have been shown to respond to dietary changes in disease (Jahoor, Jackson et al. 1999; Reid, Badaloo et al. 2006), malnutrition (Badaloo, Reid et al. 2002) and even to small decreases in protein intake (Jackson, Gibson et al. 2004), demonstrating that erythrocyte GSH is a sensitive pool from which to detect changes in GSH metabolism. At a protein intake of 0.75 g.kg\(^{-1}\).day\(^{-1}\), set by WHO as the safe intake (FAO/WHO/UNU 1985), Jackson et al. (Jackson, Gibson et al. 2004) showed decreased erythrocyte GHS synthesis when compared to a habitual protein intake of 1.13 g.kg\(^{-1}\).day\(^{-1}\). In fact this higher protein requirement was recently confirmed by our group showing that a safe intake of protein is closer to 1 g.kg\(^{-1}\).day\(^{-1}\) (Humayun, Elango et al. 2004).
2007). In addition, cysteine supplementation at only 15 mg.kg\(^{-1}\).day\(^{-1}\) produced significantly increased GSH synthesis in symptom-free HIV individuals (Jahoor, Jackson et al. 1999) suggesting that at the cysteine intakes used in the current study significant changes in GSH metabolism should have been observed, had they occurred.

Although we did not observe changes in GSH metabolism in response to feeding graded intakes of cysteine to healthy adults receiving the previously derived mean methionine (TSAA) requirement (figure 6.2), we observed a significant linear increase in urinary sulphate production in response to graded cysteine intakes (figure 6.3). The precise mechanisms governing all aspects of sulphur amino acid metabolism are not yet completely understood. However the increases in urinary sulphate excretion observed in the current study can be partly explained by work conducted by Stipanuk et al. Because excess cysteine is considered toxic (Stipanuk, Dominy et al. 2006), the liver regulates cysteine concentration within a small range and maintains a plasma concentration within a 2.5-fold range (Stipanuk, Dominy et al. 2006). Cysteine concentration has been found to be the key regulator of its own metabolism (Stipanuk, Coloso et al. 1992; Bella, Hahn et al. 1999; Kwon and Stipanuk 2001; Stipanuk, Londono et al. 2002; Lee, Londono et al. 2004). When protein and/or SAA intake is low, \(\gamma\)-glutamylcysteine synthetase (GCS), the rate-limiting enzyme for GSH synthesis is upregulated resulting in a greater partitioning of SAA towards GSH synthesis. On the other hand, when protein and/or SAA intake is increased, cysteine dioxygenase (CDO) the enzyme which catalyzes cysteine to sulphate and taurine, is upregulated resulting in greater partitioning of cysteine towards sulphate production. Thus, increasing urinary sulphate observed in the present study appears to be
due to increased partitioning of dietary cysteine toward catabolism in response to graded intakes of cysteine.

However, a closer look at the pattern of the isotope results reveals a similar rate of erythrocyte GSH synthesis at cysteine intakes of 0, 20, 30 and 40 mg.kg^{-1}.day^{-1}, with an almost 45% increase from 0 at a cysteine intake of 10 mg.kg^{-1}.day^{-1}. This increase in GSH synthesis although not significant (p = 0.49), may be of biological importance especially because other investigators have shown significant results at lower changes in synthesis rates (Lyons, Rauh-Pfeiffer et al. 2000; Jackson, Gibson et al. 2004). The observed similar GSH synthesis rates at cysteine intakes of 20 mg.kg^{-1}.day^{-1} and above to that observed at a zero cysteine intake, suggest a return to baseline at the higher cysteine intakes and is supported by previous data which show that increasing the level of protein (soy and casein), as well as the SAA methionine and cysteine, in the diets of rats, or the addition of SAA to the culture medium of primary rat hepatocyte results in a rise in cysteine dioxygenase (CDO) and a decrease in \( \gamma \)-Glutamylcysteine (GCS) activity (Bella, Hahn et al. 1999; Bella, Hirschberger et al. 1999; Kwon and Stipanuk 2001). In healthy humans, a deficient protein and or sulphur amino acid intake has been shown to significantly decrease GSH synthesis (Lyons, Rauh-Pfeiffer et al. 2000; Jackson, Gibson et al. 2004). Although no comparison studies of graded protein or sulphur amino acid intake has been published, supplemental cysteine has been found to restore GSH synthesis to that of controls in diseased individuals (Jahoor, Jackson et al. 1999; Badaloo, Reid et al. 2002).

A potential limitation of our study is the number of subjects studied. There is a possibility that the small sample size could have given rise to a type II error, especially
with regard to the seeming biologically important increase in GSH synthesis at a cysteine intake of 10 mg.kg\(^{-1}\). Other investigators have found significant results with a considerably lower change in GSH synthesis rate (Lyons, Rauh-Pfeiffer et al. 2000; Jackson, Gibson et al. 2004) (15 to 27% compared to 45% in the current study). Because the change in GSH synthesis at the other intakes of cysteine was clearly not different, we think the results from the current study provided preliminary data to suggest that at the recommended daily allowance for SAA (24 mg.kg\(^{-1}\).day\(^{-1}\)), GSH synthesis may not be limiting. To prove a real biologically important effect at a cysteine intake of 10 mg.kg\(^{-1}\).day\(^{-1}\), a subsequent study is needed that uses cysteine intakes in the range of 5 to 15 mg.kg\(^{-1}\).day\(^{-1}\) in addition to a methionine intake of 14 mg.kg\(^{-1}\).day\(^{-1}\).

This is the first study to our knowledge, to report on GSH kinetics in response to varying cysteine intake in healthy adult men. These data provide a point of focus for the design of future experiments on adequate SAA requirement for the maintenance of whole-body antioxidant status. These data also provide preliminary evidence that the currently derived TSAA requirement for healthy adults, when provided in the presence of an adequate protein intake (Humayun, Elango et al. 2007) does not limit GSH synthesis in that population. Nevertheless, the need for TSAA may be higher in disease states where there is increased oxidative stress. Studies in rats have suggested an increase in cysteine requirement in septic rats compared to controls as evidenced by an increase in methionine transsulfuration and an increase in both methionine and cysteine flux compared with controls (Malmezat, Breuille et al. 2000). A later study showing increased GSH synthesis in septic compared with control rats was used as a possible explanation for the increased cysteine requirement in sepsis (Malmezat, Breuille et al. 2000). In
humans with HIV and malnutrition, decreased GSH metabolism was ameliorated by
cysteine supplementation which served to increase GSH synthesis and concentration to
that of control subjects. However, our current results show that typical cysteine intake is
not rate limiting for glutathione synthesis in healthy adult men, but rather that GSH
synthesis is maximized at a protein and TSAA intakes equivalent to those required for
adequate protein synthesis in healthy adult subjects.
7. DOES THE ADDITION OF CYSTEINE TO THE TOTAL SULPHUR AMINO ACID REQUIREMENT (METHIONINE ONLY) INCREASE ERYTHROCYTES GLUTATHIONE SYNTHESIS IN THE TPN-FED HUMAN NEONATE? A PILOT STUDY.

7.1 Abstract

Controversy exists as to whether the TPN-fed human neonate is capable of synthesizing adequate cysteine from methionine if the total sulphur amino acid is provided as methionine only. Our recent study, in which we determined the total sulphur amino acid requirement as methionine only, provided evidence that the TPN-fed human neonate is capable of synthesizing adequate cysteine from methionine at least for protein synthesis. However, there is evidence that protein synthesis takes precedence over glutathione synthesis when sulphur amino acid supply is limiting. The goal of this pilot study was to gather preliminary data on whether glutathione synthesis is maximized at a methionine intake adequate for protein synthesis in the TPN-fed human neonate. We measured glutathione concentration, fractional and absolute synthesis rate in 5 TPN-fed human neonates. Each neonate underwent two isotope infusion studies of 7 hour duration after a 2-day adaptation to the total sulphur amino acid requirement (methionine only) and again after further 2-day adaptation to the same methionine intake supplemented with cysteine at 10 mg.kg\(^{-1}\).day\(^{-1}\). The isotope used was \([U^{-13}C_2-{^{15}}N]\)glycine. Cysteine supplementation did not increase glutathione kinetics. Further studies with a larger sample size and a higher cysteine intake are needed in order to make a more definitive conclusion.
7.2 Introduction

The suggestion that cysteine might be an indispensable amino acid in preterm and term neonates was first introduced by Gaull and colleagues (Sturman, Gaull et al. 1970), who showed absence of cystathionase activity in the liver of premature foetuses and newborns. This report was later confirmed by the same investigators in subsequent experiments (Gaull, Sturman et al. 1972; Pascal, Gillam et al. 1972). Other investigators using differing endpoints have come to similar conclusions. For example, Vina et al. (Vina, Vento et al. 1995), using plasma cysteine concentrations and *in vitro* erythrocyte glutathione (GSH) synthesis concluded that neonates ≤ 32 weeks gestational age had lower plasma cysteine and slower GSH synthesis than neonates above 33 weeks gestation. In addition others have reported low plasma cysteine and high plasma methionine in neonates fed low to cysteine-free TPN (Pohlandt 1974; Winters 1977; Kanaya, Nose et al. 1984). These observations have been used by these authors as evidence that cysteine is an indispensable amino acid for the human neonate.

Contrary to the data from Gaull and colleagues, later studies evaluating cystathionase activity in liver, kidney, adrenals and pancreas of infants who died prior to 1 year of age concluded that cystathionase activity in the term and premature infant is considerably greater than previously appreciated and that if the total SAA was adequate and provided as methionine only, cysteine may not be a concern (Zlotkin and Anderson 1982). The same group (Zlotkin, Bryan et al. 1981) as well as others (Malloy, Rassin et al. 1984) also found that when cysteine was supplemented to cysteine free-TPN there was no improvement in growth and nitrogen balance in the supplemented group with both groups achieving similar to *in utero* nitrogen retention. More recent stable isotope data
showed a significant transfer of label from D-[U-\textsuperscript{13}C]glucose into (apo) B-100 derived cysteine of preterm infants fed cysteine free TPN (Shew, Keshen et al. 2005). This clearly showed that even preterm infants had the ability to synthesis cysteine \textit{de novo}. However, the synthetic capacity of these neonates was positively related to neonatal maturity. More recently, data in enterally fed neonates have provided additional convincing evidence that cysteine is not indispensable in the neonate (Riedijk, van Beek et al. 2007). All of these data however, relates to the neonates ability to synthesize cysteine for protein synthesis and does not extend to cysteine synthesis in amounts adequate for antioxidant status.

\textit{Vina et al.} (Vina, Vento et al. 1995) have shown that GSH synthesis is slow in the preterm neonate. \textit{Zlotkin et al.} (Zlotkin and Anderson 1982) were unable to account for all of the cysteine supplemented to neonates receiving cysteine-free TPN. \textit{Shew et al.} (Shew, Keshen et al. 2005) although able to show cysteine synthesis in preterm neonates also showed that there was a significant positive relationship between synthesis and neonatal age, providing evidence that the pathway is underdeveloped in the neonate especially those who are premature. The unanswered question therefore, is whether the minimum synthetic capacity for cysteine observed in the neonate is sufficient to maintain GSH status when sulphur amino acids are provided as methionine only. This question was partly answered in a recent study showing no difference in GSH synthesis between neonates receiving intravenous (IV) glucose or those receiving glucose plus amino acids (Te Braake, Schierbeek et al. 2008). The amino acid source used contained significant cysteine.
The goal of the current study therefore was to determine whether supplemental cysteine could further stimulate GSH synthesis above that observed when the total sulphur amino acids are provided at requirement (Courtney-Martin, Chapman et al. 2008) but as methionine only in the TPN-fed human neonates.
7.3 Subjects and Methods

7.3.1 Subjects

Five neonates treated during the months of November 2007 to June 2008, in the Neonatal Intensive Care Unit at The Hospital for Sick Children, Toronto, Canada were enrolled in this study. The following inclusion criteria were used to determine eligibility: lack of chromosomal anomalies, born at $\geq 34$ weeks gestation and $\leq 28$ days chronological age at the time of the study, birth weight and length appropriate for gestational age, medically stable as determined by normal blood results and lack of a fever or infection, at least 3 days post operatively, and on TPN providing adequate protein and calories as determined by attending physicians and dietitians. Exclusion criteria included: small for gestational age status, presence of disease or on medications known to affect protein and amino acid metabolism, documented infection, fever, unstable medical condition, and receiving enteral feeding providing greater than 10% of protein intake.

Ethical approval for the study was obtained from The Research Ethics Board at The Hospital for Sick Children. Permission was obtained from the attending physician before approaching parents, and written informed consent was obtained from at least one parent before enrolling subjects into the study. Study characteristics of the neonates included in the study is presented in Table 7.1.
Subject characteristics of TPN-fed neonates studied on TPN with and without cysteine

Subject Characteristics of parenterally fed neonates at entrance into the study for
determination of glutathione kinetics on TPN without and with cysteine but adequate
methionine.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>2.35</td>
<td>0.65</td>
</tr>
<tr>
<td>Birth Length (cm)</td>
<td>45</td>
<td>3.6</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>32</td>
<td>2.3</td>
</tr>
<tr>
<td>Gestational age (wk)</td>
<td>36.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Postnatal age (wk)</td>
<td>1.8</td>
<td>0.69</td>
</tr>
<tr>
<td>Postconceptional age (wk)</td>
<td>38.3</td>
<td>2.4</td>
</tr>
<tr>
<td>Gender (F:M)</td>
<td>2:3</td>
<td></td>
</tr>
<tr>
<td>Study weight (kg)-Day 1</td>
<td>2.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Diagnoses:

1 Jejunal atresias; n=1, gastroschisis; n=2, malrotation and duodenal web; n=1, post-
necrotizing enterocolitis (NEC) colonic stricture; n=1.
7.3.2 Experimental design

The precursor product model developed by Jahoor et al. (Jahoor, Wykes et al. 1995) was the method used to determine GSH kinetics in this study. Each neonate was studied over a period of 6 days. The study was divided in two parts. In part 1, subjects received a parenteral amino acid solution devoid of cysteine, with the total sulphur amino acid requirements provided as methionine only. In part 2, the solution was similar to that used in part 1 but contained a supplemental amount of cysteine at 10 mg.kg$^{-1}$. We did not randomize the subjects to the order of cysteine intake because of the length of the study. From our past experience, it was likely that the introduction of some form of enteral feeds would occur during the course of a 6-day study. In order to ensure the cysteine free part of the study remained as such, we chose not to use a random design.

During the first 3 days of the experiment, neonates received an amino acid solution (Bulk Solution #1) patterned after a commercial amino acid base solution (Primene, Baxter Laboratories, Mississauga, Ontario) (Table 7.2), plus dextrose, and a 20% lipid solution (Intralipid; Fresenius Kabi, Uppsala, Sweden) for provision of adequate protein, and non protein energy. Standard amounts of vitamin and minerals were provided in the form of a liquid supplement (Multi-12/K1, providing a mixture of fat and water-soluble vitamins) formulated for use in intravenous feeding. All vitamins and minerals met current DRI recommendations. The sulphur amino acid in this solution was provided as methionine only at the requirement (RDA = 58 mg.kg$^{-1}$) as previously derived from experiment 2 (Courtney-Martin, Chapman et al. 2008). On the third day, a primed, continuous 7-hour tracer infusion was carried out to measure GSH metabolism.
On days 4 to day 6, neonates were switched to a similar amino acid solution as that received on days 1 to 3 but the new solution contained cysteine in the form of cysteine.HCL and provide cysteine at an intake of 10 mg.kg.\(^{-1}\).day\(^{-1}\) (Bulk Solution #2) (Table 7.2). Intakes of dextrose, lipid, vitamins, minerals, total calories and protein were kept the same as on the previous three days. On the 6\(^{th}\) day, a second primed, continuous 7-hour tracer infusion study was conducted for the comparison of GSH kinetics in the presence of additional sulphur amino acid as cysteine compared to that obtained with adequate sulphur amino acids as methionine only without cysteine.

Given the complexity and length of this study, it was very difficult to recruit babies and in fact, it took almost one year to complete this study with only 5 subjects completing the full protocol. The task of enrolling babies that would be stable post-operatively for 6 days on TPN and not advance to significant amounts of enteral feeds (>10% of protein intake enterally) was enormous. Two additional babies had to be withdrawn from the study because of advancement to large volumes of enteral feeds.
Table 7.2  Amino acid composition of amino acid solutions administered to neonates studied on TPN with and without cysteine

Amino acid composition of the base commercial amino acid solution and the amino acid composition of the two bulk solutions used in the study of GHS kinetics in the TPN fed human neonate

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Commercial amino acid composition (g/100g)</th>
<th>Bulk #1 (g/100g)(^1)</th>
<th>Bulk #2 (g/100g)(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine</td>
<td>6.7</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.9</td>
<td>9.9</td>
<td>9.9</td>
</tr>
<tr>
<td>Valine</td>
<td>7.6</td>
<td>7.6</td>
<td>7.6</td>
</tr>
<tr>
<td>Lysine (lysine-HCL)</td>
<td>10.9</td>
<td><em>(13.62)</em></td>
<td><em>(13.62)</em></td>
</tr>
<tr>
<td>Methionine</td>
<td>2.4</td>
<td>1.93</td>
<td>1.93</td>
</tr>
<tr>
<td>Cysteine-HCL</td>
<td>1.9</td>
<td>0</td>
<td>0.33</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.2</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>tyrosine</td>
<td>0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Glycyl-tyrosine (GT)</td>
<td><em>(5.25)</em></td>
<td><em>(6.06)</em></td>
<td><em>(6.06)</em></td>
</tr>
<tr>
<td>Threonine</td>
<td>3.7</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.8</td>
<td>3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Arginine: base amino acid solution (increase of 20% based on piglet data)</td>
<td>8.4</td>
<td>9.66</td>
<td>9.66</td>
</tr>
<tr>
<td>Glycine (total) (From GT)</td>
<td>4.0</td>
<td>4.0 total</td>
<td>4.0 total</td>
</tr>
<tr>
<td>Glycine to be added</td>
<td></td>
<td>1.66 from GT</td>
<td>1.66 from GT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.34 from Glycine</td>
<td>2.34 from Glycine</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.9</td>
<td>7.9</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Additional Variable amt added (to make solution isonitrogenous)</td>
<td>Additional Variable amt added (to make solution isonitrogenous)</td>
</tr>
<tr>
<td>Aspartate</td>
<td>6.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Glutamate</td>
<td>9.9</td>
<td>9.9</td>
<td>9.9</td>
</tr>
<tr>
<td>Proline</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Serine</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Taurine</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Ornithine (excluded because decreases the PH of the solution)</td>
<td>2.2</td>
<td>(0)</td>
<td>(0)</td>
</tr>
<tr>
<td>Total AA (grams/100g)</td>
<td>100.00</td>
<td>98.38</td>
<td>98.71</td>
</tr>
</tbody>
</table>
1Bulk #1 represents 98.38% of the total protein/total AA
2Bulk #2 represents 98.71% of the total protein/total AA
7.3.3 Study Diets

The amino profile of the amino acid solution used in the study (Bulk solution 1 & 2) is presented in Table 7.2. The composition was patterned after a commercial amino acid solution with an amino acid composition of cord blood (base amino acid solution). The amino acid solutions used (Bulk 1 & 2) were prepared using human pharmacy grade amino acids (Ajinomoto Company Inc., Japan via L.V Lomas, Brampton, Ontario) in our research laboratory, under sterile conditions. The profile of the base amino acid, was followed with some modifications; methionine was altered to provide the requirement estimate from study 2 (56 mg.kg\(^{-1}\).day\(^{-1}\)) (Courtney-Martin, Chapman et al. 2008), cysteine was removed from Bulk #1 but added to Bulk #2 at 10 mg.kg\(^{-1}\), tyrosine was provided as the dipeptide glycyl-tyrosine at a level of 4 g.100g\(^{-1}\) (Roberts, Ball et al. 2001). Arginine was increased from 8.4 to 9.66 g.100g\(^{-1}\) based on our studies in piglets (Bertolo, Brunton et al. 2003; Wilkinson, Bertolo et al. 2004) and aspartate was decreased from 6.0 g.100g\(^{-1}\) to 5.0 g.100g\(^{-1}\) to accommodate for the increased amount of nitrogen provided by arginine. Alanine was used to balance the nitrogen and make the solutions isonitrogenous. Alanine was prepared as a separate test solution at a concentration of 50 mg.mL\(^{-1}\). All prepared solutions were filter sterilized in the Research Pharmacy at The Hospital for Sick Children by being passed though a 0.22 μm filter. Solutions were subsequently demonstrated to be sterile and free of bacterial growth over 7 days in culture and to be proven pyrogen-free by the limulus amebocyte lysate test (Pearson 1979). The chemical composition of the solutions was verified by amino acid
analysis using high performance liquid chromatography (HPLC) and analysis of total nitrogen.

On each study day, vitamins and minerals were added to the solutions before delivery to the baby. All vitamins were supplied in a commercial solution Multi-12K₁ (Baxter Corporation, Mississauga Ontario), which provides a combination of fat and water-soluble vitamins, formulated for use in pediatric parenteral solutions. The mineral solution provided calcium, phosphorus, magnesium, zinc, copper, manganese, iodine, chromium and selenium. Nutrient intake for each individual neonate was prescribed by the attending physician and dietitian. All subjects were receiving adequate protein and energy (Zlotkin, Bryan et al. 1981; Zlotkin 1984). Non-protein energy was provided as dextrose and a 20% lipid solution Table 7.3. All calculations of nutrient intake for phase 1 of the study (days 1 to 3) were done using body weight on day 1 of the study whereas calculation of nutrient intake for phase 2 (days 4 to 6) were done using weight on day 4 of the study (Table 7.3).

At the start of the study, each neonate was switched from the commercial amino acid solution to Bulk solution #1, which they each received for the first 3 days. On day 3 the first isotope infusion study was conducted then on day 4 subjects were switched to Bulk solution #2 which was continued until day 6 at which point the subjects underwent the second isotope infusion study. On the 7th day, subjects were returned to the commercial amino acid solution which they were receiving before the start of the study. All subjects were prescribed a methionine intake of 58 mg.kg⁻¹.day⁻¹ on days 1 to 3 and the same methionine intake with a supplemental cysteine intake of 10 mg.kg⁻¹.day⁻¹ on days 4 to 6. However, the actual intake of the subject differed slightly from the
prescription because the volume of solution prescribed is not always that which is delivered in a real clinical environment (Table 7.3). The mean nutrient intake of each subject during phase 1 and phase 2 along with the overall group mean is presented in Table 7.3.

Baseline blood work performed for clinical monitoring was reviewed on each subject before the start of the study. All subjects had normal sodium, potassium, calcium, phosphorous and pH.
### Table 7.3 Individual nutrient intakes of neonates studied on TPN with and without cysteine

Mean Individual subject’s nutrient intake during study of GSH kinetics for part 1 (no cysteine) and part 2 (cysteine supplementation)

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Wt (kg)</th>
<th>Methionine intake (mg/kg)</th>
<th>Cysteine intake (mg/kg)</th>
<th>Protein intake (g/kg)</th>
<th>Carbohydrate intake (g/kg/d)</th>
<th>Fat intake (g/kg)</th>
<th>Total energy intake (kcal/d)</th>
<th>Energy intake (kcal/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.18</td>
<td>58.4</td>
<td>0</td>
<td>3.03</td>
<td>13.2</td>
<td>2.98</td>
<td>276</td>
<td>87</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>66.6</td>
<td>0</td>
<td>3.4</td>
<td>12.5</td>
<td>2.94</td>
<td>214</td>
<td>85</td>
</tr>
<tr>
<td>3</td>
<td>1.9</td>
<td>57.3</td>
<td>0</td>
<td>2.98</td>
<td>12.1</td>
<td>2.72</td>
<td>152</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>1.71</td>
<td>60.2</td>
<td>0</td>
<td>3.14</td>
<td>11.1</td>
<td>3.01</td>
<td>137</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>58.8</td>
<td>0</td>
<td>3.0</td>
<td>14.0</td>
<td>2.85</td>
<td>220</td>
<td>88</td>
</tr>
<tr>
<td>Mean</td>
<td>2.36</td>
<td>60.26</td>
<td>0</td>
<td>3.11</td>
<td>12.6</td>
<td>2.9</td>
<td>200</td>
<td>84</td>
</tr>
<tr>
<td>SD</td>
<td>0.58</td>
<td>3.69</td>
<td>0</td>
<td>0.17</td>
<td>1.1</td>
<td>0.12</td>
<td>56</td>
<td>4.0</td>
</tr>
</tbody>
</table>
7.3.4 Tracer Protocol.

The tracer infusion studies were conducted on the 3rd and 6th day at the end of the first period without cysteine and at the end of the second period with cysteine supplementation. \([U-^{13}C_2-^{15}N]glycine (98\% {^{13}C_2, 98\% {^{15}N} )\), purchased from Cambridge Isotope Laboratories, Andover, MA was the isotope used for the measurement of erythrocyte GSH synthesis. Stock solutions were prepared in 0.9\% sodium chloride (10 mg/ml) by the Research Pharmacy at the Hospital for Sick Children, Toronto and were confirmed to be sterile and pyrogen free. Solution was aliquoted into sterile bottles and kept in the Research Pharmacy at the Hospital for Sick Children, Toronto, at 4 °C until use.

An intravenous priming dose of \([U-^{13}C_2-^{15}N]glycine\) was given at 50 \(\mu\text{mol.kg}^{-1}\) over 15 minutes followed by a continuous intravenous infusion of 40 \(\mu\text{mol.kg}^{-1}.\text{hr}^{-1}\) for the remainder of the 6.75 hours for a total of 7 hours isotope infusion time.

We chose to use an \(M+3\) glycine tracer as the GSH precursor to determine its incorporation into the GSH molecular ion by LCMS/MS. Details are as described in chapter 6.

7.3.5 Sample Collection

Blood samples were collected on day 3 and 6 at baseline before the start of the isotope infusion for analysis of background enrichment. A total of 0.7 ml of blood was collected at each time point; 0.5 ml for measurement of erythrocyte GSH concentration.
and enrichment and plasma amino acid concentration, and 0.2 ml for the measurement of erythrocyte intracellular glycine enrichment (the precursor pool from which glycine is drawn for GSH synthesis). Having previously established that isotopic steady state was reached in the precursor pool at 5 hours, two subsequent blood samples were taken at approximately 5 ½ and 6 ½ hours. Blood was drawn from a central line if the baby had central venous access, or from a heel prick if central access was not available. For heel prick sampling, the heel was warmed with a warm cloth before the blood was drawn. To minimize the amount of blood taken, hematocrit was obtained from blood samples collected for clinical monitoring on the same day of the isotope study.

7.3.6 Sample Analysis

7.3.6.1 Erythrocyte glutathione concentration and enrichment

All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, Ontario. A 0.5 mL aliquot of each blood sample collected in Na2EDTA was centrifuged for 2 minutes within 5 minutes of collection. Each tube was weighed pre and post blood collection to determine the volume of blood for normalization of the GSH concentration per litre of blood. After centrifugation the plasma was immediately removed. Two hundred µL of 100 mM N-ethylmaleimide, and 20 µL of 5 mM gamma-glutamyl-leucine (internal standard) were added to the separated red blood cells. The sample was then caped, vortex and left for 10 minutes at room temperature. Cells were then lysed with 50 µL 0.4 M ZnSO4, and the protein precipitated with 1 mL ice cold methanol. The sample was then vortexed, centrifuged for 2 minutes, the supernatant removed and stored at -80 °C until analyzed.
Glutathione concentration and enrichment were analyzed by using a triple quadrupole mass spectrometer API 4000 (Applied Biosystems/MDS SCIEX, Concord, Canada) operated in positive ionization mode with the Turbo Ion Spray ionization probe source (operated at 5.8KV). This was coupled to an Agilent 1100 HPLC system (Agilent, Mississauga, Canada). All aspects of system operation and data acquisition were controlled using The Analyst NT v 1.4.1 software. Glutathione concentration was measured using an external standard curve and the ratio of the analyte (GSH) to the internal standard (Gamma-glutamyl-leucine). Glutathione enrichment was calculated as a ratio of the (enriched) $M+3$ to (unenriched) $M$ peaks of the tripeptide molecule of glutathione and was expressed as mole percent excess and was calculated from peak area ratios at the isotopic steady state of glycine in the last 2 hours of isotope infusion.

### 7.3.6.2 Erythrocyte free glycine enrichment

Each sample was collected and centrifuged as above. Plasma was quickly removed and the cells washed twice with iced cold saline on each occasion. Samples were vortexed between each wash. Cells were then lysed and de-proteinated as above, vortexed, centrifuged for 2 minutes and the supernatant stored at -80 °C until analysis.

Fifty $\mu$L of each sample was then dried under nitrogen at 35 °C. One hundred $\mu$L of butanol.HCl (Sigma-Aldrich Canada Ltd., Oakville, Ontario), was then added and the sample vortexed, topped with nitrogen and heated for 20 minutes at 55 °C. The sample was again dried under nitrogen, and reconstituted in 0.1% formic acid (Sigma-Aldrich Canada Ltd., Oakville, Ontario). Samples were then analyzed using a triple quadrupole mass analyzer as described above. Glycine enrichment was calculated as a ratio of the
(enriched) $M+3$ to (unenriched) $M$ peaks and was expressed as mole percent excess above baseline and was calculated from peak area ratios at isotopic steady state.

### 7.3.6.3 Plasma amino acid concentration

Plasma total cysteine concentration was determined by LCMS/MS, using a bench top triple quadrupole mass spectrometer API 4000 (Applied Biosystems/MDS SCIEX) operated in positive ionization mode with the TurboIonSpray ionization probe source (operated at 5.8KV). This was coupled to an Agilent 1100 HPLC system (Rafii, Elango et al. 2007).

### 7.3.7 Calculations

#### 7.3.7.1 Fractional and absolute synthesis rate of erythrocyte glutathione

The fractional synthesis rate (FSR) of erythrocyte glutathione ($FSR_{GSH}$) was calculated using the precursor-product method of Jahoor et al. (Jahoor, Wykes et al. 1995).

$$FSR_{GSH} (%/d) = \frac{(E_{t7} - E_{t5})}{E_{RBC} \times (24 \times 100) / (t7 - t5)}$$

Where $(E_{t7} - E_{t5})$ was the increase in the isotopic enrichment of erythrocyte glutathione between the fifth and seventh hours of infusion as a result of the incorporation of the labeled glycine, $E_{RBC}$ was the intracellular glycine enrichment at isotopic steady state, and $(t7 - t5)$ was the time interval between the fifth and seventh hour when the incorporation of glycine into glutathione is measured.
\[
\text{ASR} = \text{GSH}_{\text{mass}} \times \text{FSR}_{\text{GSH}} \quad (2)
\]

Where \( \text{GSH}_{\text{mass}} \) = the product of the cell volume (or cell number or cell protein) and the concentration of GSH in the cell. Haematocrit was calculated using the formula;

\[
\text{HCT} \ (L/L) = \frac{(\text{RBC} \times \text{MCV})}{1000}.
\]

7.3.8 Statistical Analysis

7.3.8.1 Sample Size Consideration

Using glutathione FSR as the main outcome variable, a post-hoc sample size calculation was conducted using the mean and standard deviation of the difference between means obtained from the current study. The result obtained from the four subjects in whom the FSR was measured at isotopic steady state was used. Using \( p = 0.05 \), with a 80% power and two-tailed analysis, a sample size of 13 subjects with 2 studies per subject is required to detect a significant difference in FRS at a cysteine supplementation of 10 mg.kg\(^{-1}\).

The data was analyzed by repeated measures ANOVA with the PROC GLM procedure to assess the effect of cysteine intake on glutathione concentration, FSR, ASR and cysteine concentration. Independent variables tested were cysteine intake and subject. Since order of cysteine intake was not randomly assigned, order was not included in the model. Statistical significance was established at \( p \leq 0.05 \). Data was analyzed by SAS version 9.1 for Windows (SAS Institute Inc., Cary, NC).
7.4 Results

7.4.1 Clinical characteristics and nutrient intake

The clinical characteristics and diagnosis of the 5 subjects studied are presented in Table 7.1. Each subject was studied in a repeated measures design first without cysteine followed by a cysteine supplemental period. The methionine intake was based on the RDA as previously determined from experiment 2 and was prescribed at 58 mg.kg\(^{-1}\).day\(^{-1}\) for the both parts of the experiment. Cysteine was not provided in part 1 of the experiment but was prescribed at 10 mg.kg\(^{-1}\).day\(^{-1}\) during part 2 of the experiment (the cysteine supplementation part). The total nutrient intake was however dependent on the total volume of parenteral nutrition infused. The average methionine intake for phase 1 and 2 was 60.26 ± 3.69 and 60.52 ± 2.03 mg.kg\(^{-1}\).day\(^{-1}\) respectively while the cysteine intake in phase 1 was 0 mg.kg\(^{-1}\).day\(^{-1}\) by design and 10.32 mg.kg\(^{-1}\).day\(^{-1}\) in phase 2 Table 7.3. The average energy and protein intake for phase 1 was 84 ± 4.0 kcals.kg\(^{-1}\) and 3.11 ± 0.17 g.kg\(^{-1}\).day\(^{-1}\) respectively and was very similar for phase 2 with energy intake being 86 ± 4.0 kcals.kg\(^{-1}\).day\(^{-1}\) and protein being 3.14 ± 0.08 g.kg\(^{-1}\).day\(^{-1}\) Table 7.3.

7.4.2 Intracellular glycine enrichment

Isotopic steady state (plateau) in the intracellular glycine pool was achieved for all neonates by 5 hours after the start of the isotope infusion and was defined by the absence
of a significant slope between the data points at plateau. A typical study day isotopic enrichment in the intracellular glycine pool is presented in **Figure 7.1.**
Figure 7.1 Net tracer/tracer molar ratio of erythrocyte free glycine

Net tracer/tracer molar ratio (mol % above baseline) of free glycine from deproteinated extracts of erythrocytes
7.4.3 Glutathione kinetics

During the isotope infusion study on subject number 5, unfortunately the father refused to allow more than one blood sample to be taken at isotopic steady state. As a result, we were unable to calculate the linear incorporation of glycine into GSH for subject number 5 at isotopic steady. Rather, we calculated GSH fractional and absolute synthetic rate in two different ways; for the first 4 subjects at isotopic steady state and again for all 5 subjects as the incorporation over the first 5 ½ h of the infusion. Glutathione concentration was measured once for all 5 subjects.

There was no effect of cysteine supplementation on GSH concentration (n = 5, p = 0.19). The mean GSH concentration was 2.37 ± 0.52 and 2.14 ± 0.51 mmol/L without and with cysteine supplementation respectively (Figure 7.2). Similarly, cysteine supplementation did not affect GSH FSR (n = 4, p = 0.18) when FSR was measured at isotopic steady state, or when measured as incorporation over 5 ½ h (n = 5, p = 0.88) (Figure 7.2 & 7.3). Fractional synthesis rate when assessed at steady state in the 4 subjects was 35.2 ± 12.1 and 32.3 ± 13.2 %/d without and with cysteine supplementation and 16.3 ± 7.7 and 16.7 ± 3.2 %/d in all 5 subjects without and with cysteine supplementation respectively when assessed during the first 5 ½ hours of tracer infusion.

Cysteine supplementation did not affect GSH ASR whether measured in the 4 subjects at isotopic steady state (p = 0.16) or in all 5 subjects (p = 0.83) when measured over the first 5 ½ hours of tracer infusion (Figure 7.2 & 7.3). Absolute synthesis rate measured at steady state in the 4 subjects was 0.75 ± 0.18 and 0.6 ± 0.1 mmol/L/d and 0.36 ± 0.097 and 0.34 ± 0.046 mmol/l/d in all 5 subjects measured over the first 5 ½ hours of isotope
infusion. The individual data for FSR is presented in (Figure 7.4) for the 4 subjects measured at isotopic steady state 4A, and for all 5 subjects measured over 5 ½ hours 4B.
Figure 7.2 Glutathione kinetics in neonates fed TPN with and without cysteine

Mean (± SD) effect of cysteine supplementation on glutathione concentration (n = 5), fractional synthesis rate of glutathione and the absolute synthesis rates of glutathione for 4 of 5 subjects who participated in the study, measured at isotopic steady state. Using repeated measures two way ANOVA with PROC GLM procedure, cysteine supplementation had no effect on GSH concentration (0.19), FSR$_{GSH}$ (p = 0.18), or ASR$_{GSH}$ (p = 0.16).
Mean (± SD) effect of cysteine supplementation on glutathione concentration (n = 5), fractional synthesis rate of glutathione and the absolute synthesis rates of glutathione for 5 subjects who participated in the study, measured during the first 5 ½ hours of isotope infusion. Using repeated measures two way ANOVA with PROC GLM procedure, cysteine supplementation had not effect on GSH concentration (p = 0.19), $\text{FSR}_\text{GSH}$ (p = 0.88), or $\text{ASR}_\text{GSH}$ (p = 0.83).

Figure 7. 3 Glutathione FSR of neonates fed TPN with and without cysteine
Figure 7. 4 Individual glutathione synthesis rates of neonates fed TPN with and without cysteine

Individual data showing erythrocyte glutathione FSR for 4 subjects measured at isotopic steady state (4A), and for all 5 subjects measured over the first 5 ½ hours of isotope infusion (4B).
7.4.4 Amino acid concentration

Cysteine concentration increased numerically with cysteine concentration from 152.5 ± 43.5 (mean ± SD) to 173.7 ± 53.0 mmol/L but the increase was not statistically significant (Figure 7.5).

![Figure 7.5](image-url) Plasma cysteine concentration of neonates fed TPN with and without added cysteine

Mean (± SD) effect of cysteine supplementation on plasma cysteine concentration (n = 5), Using repeated measures two way ANOVA with PROC GLM procedure, cysteine supplementation had not effect on cysteine concentration (p = 0.14).
7.5 Discussion

This is the first study to our knowledge, conducted in the TPN-fed human neonate to determine whether the total sulphur amino acid requirement (Courtney-Martin, Chapman et al. 2008) (provided as methionine only) needed for adequate protein synthesis is also adequate for maintenance of antioxidant status. The main goal was to directly determine whether adding supplemental cysteine to a TPN solution providing the total sulphur amino acids requirement as methionine only will stimulate an increase in GSH synthesis and concentration. Indirectly, the secondary goal was to assess whether the transsulfuration pathway is capable of synthesizing adequate cysteine from methionine, thereby providing further clarification as to whether cysteine is an indispensable amino acid in the TPN-fed human neonate.

The results show that GSH concentration did not differ with cysteine supplementation from that observed when the sulphur amino acids were provided as methionine only (figure 7.2). Further it was comparable to that obtained in neonates receiving PN (Te Braake, Schierbeek et al. 2008) as well as that obtained from venous blood taken from the umbilical cord immediately after delivery in term babies, but was slightly lower than that observed in 20-month old enterally fed children treated for and recovered from malnutrition (Reid, Badaloo et al. 2000).

There was no data upon which to base a sample size calculation. Hence we chose a within subject repeated measures design as the best approach to show the effect of cysteine supplementation, should one occur. Because of the anticipated difficulty in conducting such a study, we chose to recruit 5 infants as a means of obtaining pilot data for a future supplemental trial. Unfortunately, we were unable to measure GSH synthesis
at steady state in one of the five subjects. As a result we measured GSH synthesis in two different ways; in 4 of the 5 subjects at steady state and in all 5 subjects as incorporation over 5 ½ hours. At steady state the GSH synthesis rates was higher than that measured over the course of the 5 ½ hours (Figures 7.2 and 7.3) but synthesis rates were not increased with cysteine supplementation regardless of the method used for measuring synthesis rates (figures 7.2 and 7.3). A more recent study suggested that a total of ≥ 3 subjects would be sufficient to detect a difference in GSH synthesis in the TPN-fed neonate (Te Braake, Schierbeek et al. 2008).

This data is supported by previous isotope data which showed that preterm neonates are capable of cysteine synthesis as evidenced by incorporation of $^{13}$C label form $[^{13}$C$_6$] glucose into apo B-100 cysteine (Shew, Keshen et al. 2005), and more recent isotope data showing that there is significant transsulphuration of methionine in the neonate (Thomas, Gruca et al. 2008) whether fed enterally or parenterally. From the results of the current study it seems likely that not only are they capable of cysteine synthesis for adequate growth and nitrogen balance (Zlotkin, Bryan et al. 1981; Malloy, Rassin et al. 1984), but that cysteine synthesis from methionine is also adequate for maintenance of antioxidant status.

One of the important limitations of this study is the small sample size, thereby limiting the confidence required to draw definitive conclusions. Because of the difficulty inherent in the current design, a future design in which babies are randomized to cysteine supplementation or no supplementation in the presence of the current methionine intake should be more feasible. However, a look at the individual data (figure 7.4) shows no sign of any trend towards an increase in GSH FSR. Another important consideration was
whether at 10 mg.kg\(^{-1}\).day\(^{-1}\) the level of cysteine supplementation was adequate to detect a significant change in GSH kinetics if one occurred. This level was chosen because of the concern for acid base balance in the babies with the form of cysteine being cysteine.HCL. A prior cysteine supplementation study was able to show a statistically significant 25% increase in GSH FSR when cysteine was supplemented at 15 mg.kg\(^{-1}\).day\(^{-1}\) (Jahoor, Jackson et al. 1999). With the total sulphur amino acid requirement provided at the RDA as methionine (Courtney-Martin, Chapman et al. 2008), and knowledge that babies are able to synthesize adequate cysteine for proteins synthesis from methionine (Zlotkin, Bryan et al. 1981; Malloy and Rassin 1984), the addition of cysteine as a supplement therefore, would only be required for GSH synthesis. Consequently we felt that a supplemental intake of 10 mg.kg\(^{-1}\).day\(^{-1}\) was adequate to detect a change in GSH FSR if one occurred.

An important finding in this study was the plasma cysteine concentration without cysteine and with cysteine supplementation. The plasma cysteine concentrations were 152.5 ± 43.7 mmol/L and 173.7 ± 53.0 mmol/L (figure 7.4) without and with cysteine supplementation respectively which was not a statistically significant difference. Further, plasma cysteine concentration without cysteine was higher than that observed in term breast fed babies (Wu, Edwards et al. 1986), and suggest that when the sulphur amino acids are provided as methionine only TPN-fed human neonates are able to synthesize adequate cysteine from methionine for cysteine homeostasis.

This is the first study to our knowledge, in which the definition of the requirement of an amino acid has been extended to include requirement for antioxidant status in addition to that required for protein synthesis. The results suggest that the total sulphur
amino acid requirement when provided as methionine only is adequate to meet the needs of the TPN-fed human neonate for antioxidant synthesis as well as protein synthesis. Further studies with larger numbers and an increased cysteine intake are needed for more conclusive results.

While the TPN-fed human neonate might be capable of synthesizing adequate cysteine for GSH synthesis, it has been shown that providing the total sulphur amino acid as methionine only leads to increased homocysteine concentration (Shoveller, House et al. 2004; Courtney-Martin, Chapman et al. 2008) in the neonate. Therefore, consideration should be given to providing the total sulphur amino acids as a balance between methionine and cysteine particularly since methionine has been implicated in TPN cholestasis (Moss, Haynes et al. 1999).

The neonates in this study were stable, post operative and did not have any clinical evidence of TPN cholestasis. They may not be representative of sicker neonates that are TPN-fed for prolonged periods. Further studies are needed to determine if neonates on long term TPN with clinical evidence of TPN cholestasis have an increased requirement for cysteine for GSH synthesis.
8 GENERAL DISCUSSION, CONCLUSION AND FUTURE DIRECTIONS

8.1 General Discussion and Conclusions

In the first study presented in this thesis (chapter 4), a safe and stable amino acid solution appropriate for use in the study of amino acid metabolism in humans was developed. The development of such a solution allows for the variation of any amino acid of interest so that its metabolism and requirement could be determined. This work was therefore crucial as it provided the foundation needed for subsequent studies in this thesis (chapters 5 and 7) as well as for other studies in our laboratory and the laboratories of others who study amino acid metabolism in TPN-fed humans.

In the second experiment in this thesis (chapter 5), the total sulphur amino acid requirement in the TPN-fed human neonate was determined directly using Indicator Amino Acid Oxidation technique. This is the first human study ever published using this novel approach. It is also the first of what we hope to be a series of amino acid requirement estimates in the neonate aimed at formulating an appropriate amino acid pattern for the TPN-fed human neonate.

Based on our previous piglet work (Shoveller, Brunton et al. 2003); we hypothesized that the mean requirement for methionine would be \(~52 \text{ mg.kg}^{-1}.\text{day}^{-1}\). The mean requirement estimate and a 95% confidence interval were 49.0 and 58.0 mg.kg\(^{-1}\).day\(^{-1}\) respectively. This remarkably similar estimate to that predicted from the piglet data serves to further validate the piglet model as a suitable model for estimation of amino acid requirement in the human neonate. We propose that the total sulphur amino
acid requirement be set at 58 mg.kg⁻¹.day⁻¹ (RDA) of methionine to avoid compromise in protein synthesis and growth in TPN-fed neonates.

Nevertheless, because methionine is the most toxic of all amino acids (Hardwick, Applegarth et al. 1970; Benevenga 1974), with implications in TPN-associated liver disease, we recommend that the minimum methionine requirement be determined and the sulphur amino acids be provided in TPN solutions as a balance between methionine and cysteine. The provision of a minimum methionine requirement with the balance of the sulphur amino acids supplied as cysteine is important for two reasons:

1. There is a direct relationship between methionine intake and plasma homocysteine concentrations (chapter 5) (Shoveller, House et al. 2004; Courtney-Martin, Chapman et al. 2008), which can be reduced by supplying a portion of the sulphur amino acids as cysteine (Di Buono, Wykes et al. 2003).

2. Intravenous methionine is associated with TPN-associated liver disease (Moss, Haynes et al. 1999).

Because the requirement estimate calculated from our piglet data (52 mg.kg⁻¹.day⁻¹) was almost identical to that estimated in our current work (49 mg.kg⁻¹.day⁻¹) (chapter 5), we propose that until a formal study is conducted to determine the minimum methionine requirement in the TPN-fed human neonates, that an estimate of 36 mg.kg⁻¹.day⁻¹ based on our piglet data (Shoveller, Brunton et al. 2003) be accepted as the tentative minimum methionine requirement to be provided in the presence of an excess of cysteine (58 mg.kg⁻¹.day⁻¹). Table 8.1 presents the methionine concentrations of current
pediatric amino acid solutions compared with the proposed concentration based on our current results and the predicted minimum methionine requirement based on the data from our piglet study (Shoveller, Brunton et al. 2003). The information presented in table 8.1 show that with the exception of Aminosyn-PF, current pediatric amino acid solutions have methionine content between 24 to 76% in excess of the true methionine requirement. Such high methionine concentrations could be a contributing factor in the etiology of TPN-associated liver disease in babies on TPN.

Current methods of determining TSAA requirement measures the requirement for net protein synthesis. It is unclear whether the requirement estimate includes a proportion needed for GSH synthesis. This is especially important in the human neonate in light of evidence which suggest that cysteine is conditionally indispensable in the human neonate, because the transsulphuration pathway is underdeveloped. Since most commercial amino acid solutions contain little to no cysteine, it is important to know whether the human neonate can synthesize adequate cysteine for both protein and GSH synthesis from methionine. The fourth study therefore (chapter 7), was a pilot study conducted to begin to address this gap in the literature. The results, although not conclusive because of the small sample size, strongly suggest that cysteine supplementation did not increase GSH synthesis, and that TPN-fed neonates are capable of synthesizing adequate cysteine from methionine for protein synthesis as well as antioxidant synthesis. The plasma cysteine concentration of the neonates on the cysteine-free TPN also suggest that they were capable of synthesizing adequate cysteine from methionine.
Table 8.1 Comparison between methionine concentrations in currently available commercial solutions and proposed concentration based on requirement estimate

A comparison of the methionine concentration in currently available pediatric amino acid solutions with the proposed concentration obtained for our current requirement estimate

<table>
<thead>
<tr>
<th>Commercial Pediatric Amino Acid Solutions</th>
<th>Methionine Concentration (g.100g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primene</td>
<td>2.4</td>
</tr>
<tr>
<td>TrophAmine</td>
<td>3.4</td>
</tr>
<tr>
<td>Aminosyn-PF</td>
<td>1.8</td>
</tr>
<tr>
<td><strong>Proposed Methionine Concentration¹</strong></td>
<td>1.93</td>
</tr>
<tr>
<td><strong>Proposed Methionine Concentration²</strong></td>
<td>1.2</td>
</tr>
</tbody>
</table>

¹ Total methionine requirement without dietary cysteine (TSAA requirement) (chapter 5)

² Minimum proposed methionine requirement in the presence of excess dietary cysteine, calculated from our piglet data (Shoveller, Brunton et al. 2003).
A post-hoc sample size calculation using FSR as the main outcome variable suggest an adequate sample size of 13 to detect a change in FSR with cysteine supplementation. Although a sample size of 13 was impractical for the purposes of this thesis, it may also be impractical as a future endeavor given the tremendous difficulty inherent in obtaining subjects on TPN for a 6-day study, or obtaining consent from parents when blood taking is required, not only on one but on two separate days. Therefore the possibility of conducting a trial, with babies randomly assigned to receive cysteine or no cysteine should also be considered. Such a study will allow each baby to be studied for only a 3 instead of a 6-day period. However, a larger sample size would be required. A theoretical sample size calculation using the means and standard deviation generated from the current study estimate for 80% power and p = 0.05, a total of 326 subject will be required in each group.

In the third study (chapter 6), a repeated-measures design was applied to determine whether the mean total sulphur amino acid requirement for protein synthesis was adequate for antioxidant synthesis in healthy adult males. We found that with 4 subjects and 5 different levels of cysteine intake a statistically significant result was not obtained. Rather, we saw no significant increase in GSH metabolism as we varied the cysteine intake from 0, 10, 20, 30 and 40 mg.kg⁻¹.day⁻¹. At an intake of 10 mg.kg⁻¹.day⁻¹ we saw a numerical increase in GSH FSR equivalent to about 45% higher than that observed at the zero cysteine intakes. At cysteine intakes above 10 mg.kg⁻¹.day⁻¹, we observed a return to baseline in GSH synthesis rates. The lack of statistical significance on the change in GSH synthesis rate at a cysteine intake of 10 mg.kg⁻¹.day⁻¹ might be partly but not fully explained by an inadequate sample size. The inability to use the
inadequate sample size as the whole explanation is mainly because of the return to baseline of the GSH synthesis rates at all cysteine intakes above 10 mg.kg$^{-1}$.day$^{-1}$. This return to baseline is an interesting phenomenon requiring further study, as it suggests a suppression of GSH synthesis at higher intakes of cysteine. It has been previously suggested that GSH has a strong inhibitory influence on the rate limiting enzyme $\gamma$-glutamylcysteine acting as an effective feedback control for the regulation of GSH synthesis (Beutler 1989). Further study into this potential mechanism is warranted in light of our current findings.

The current thesis has presented two studies in which cysteine supplementation failed to produce a response in GSH synthesis first in healthy adult males and second in TPN-fed neonates. The results are in opposition to results previously reported in adults and children in which variation in sulphur amino acid intake led to a significant change in GSH synthesis (Jahoor, Jackson et al. 1999; Lyons, Rauh-Pfeiffer et al. 2000; Badaloo, Reid et al. 2002; Jackson, Gibson et al. 2004). The results presented in the current adult study (chapter 6), may differ from that of other adult studies in a number of important ways;

1. Deficient dietary protein intake. Healthy subjects experienced a significant decrease in GSH kinetics in response to a switch from a habitual to a deficient protein intake (Jackson, Gibson et al. 2004).

2. Deficient sulphur amino acid intake. Healthy subjects experienced a significant decrease in GSH synthesis in response to a sulphur amino acid-free diet when compared to a complete diet (Lyons, Rauh-Pfeiffer et al. 2000).
3. The presence of disease. Subjects with sickle cell disease had decreased GSH concentration and increased synthesis. Decreased concentration is related to increased consumption (Reid, Badaloo et al. 2000). In HIV infection decreased GSH concentration and synthesis was ameliorated by cysteine supplementation (Jahoor, Jackson et al. 1999), with similar findings in children with protein energy malnutrition (Reid, Badaloo et al. 2000; Badaloo, Reid et al. 2002).

Contrary to the above characteristics, the subjects in our study were healthy, and fed a diet sufficient in protein, energy, sulphur amino acids as well as vitamins and minerals. Hence they were neither stressed nor deficient in sulphur amino acids. At a cysteine intake of 10 mg.kg\(^{-1}\).day\(^{-1}\), we observed a physiological increase in GSH synthesis that was not statistical significant. This would not be a particularly surprising result, since methionine was provided at the mean intake and an additional cysteine intake of 10 mg.kg\(^{-1}\).day\(^{-1}\) would be closer to the RDA for the total sulphur amino acids. The return to baseline at intakes of cysteine above 10 mg.kg\(^{-1}\).day\(^{-1}\), we believe is evidence that cysteine is not needed for GSH synthesis at intakes above the total sulphur amino acid requirement in a healthy population.

The neonates studies (chapter 7), also showed no evidence of increase in GSH synthesis with cysteine supplementation. It is possible that because subjects were not randomized to the cysteine supplementation, that there could have been an order effect. There was however no change in the infants clinical condition during the course of the study and we had previously reported similar amino nitrogen flux rates in post surgical
infants (1 to 4 days post surgery) (Duffy and Pencharz 1986) on TPN as that observed in a similar group of infants on TPN who had not had surgery (Duffy, Gunn et al. 1981).

It is possible that these stable neonates were not unduly stressed and that the previous studies which showed no change in nitrogen balance with cysteine supplementation can also be extended to include no change in GSH synthesis with cysteine supplementation.

8.2 Future Directions

As an extension of study two in this thesis, a future study should determine the minimum methionine requirement in the presence of excess cysteine, so that the total sulphur amino acid could be provided as a balance between methionine and cysteine.

Future studies are needed to conclusively determine if healthy adults need additional cysteine in addition to the mean methionine requirement to maintain adequate antioxidant status. A larger sample size and a focus on lower intake levels of cysteine that would allow a conclusive decision are necessary. This means that cysteine intakes should be studied between 5 and 20 mg.kg\(^{-1}\).day\(^{-1}\). In addition mechanistic studies are needed to determine if a true suppression of GSH at higher cysteine intakes actually occurs.

As a follow-up to the fourth study (chapter 7), a larger number of subjects (n = 13) need to be studied to determine if GSH synthesis is truly unaffected by cysteine supplementation. A clinical trial in which babies are randomized to receive cysteine or no cysteine supplementation on a base methionine intake should also be considered, but the required sample size may be prohibitive. In light of the possibility that GSH synthesis is suppressed at higher cysteine intakes, a cysteine supplementation of 10 mg.kg\(^{-1}\).day\(^{-1}\) should be repeated with the addition of 20 mg.kg\(^{-1}\).day\(^{-1}\) s in a larger number of subjects (n
Further studies into neonates on long term TPN, having clinical symptoms of TPN cholestasis is also required to determine if cysteine supplementation is beneficial in that population.
9 REFERENCES


nutrition is caused by infusate, not the route of administration." J Pediatr Surg

discussion 1274-5.


role of sarcosine." Metabolism 29(8): 707-20.

Mudd, S. H. and J. R. Poole (1975). "Labile methyl balances for normal humans on
various dietary regimens." Metabolism 24(6): 721-35.


Pascal, T. A., H. H. Tallan, et al. (1972). "Hepatic cystathionase: immunochemical and
electrophoretic studies of the human and rat forms." Biochim Biophys Acta


10.1: Mean plasma cysteine concentration of adult males who participated in study number 3 (chapter 6) in response to increasing cysteine intake.

**Figure 10.1:** Mean plasma cysteine concentration of adult males who participated in study number 3 (chapter 6) in response to increasing cysteine intake.
10.2: Mean plasma homocysteine intake of adult males in response to increasing cysteine intake

Figure 10.2: Mean plasma homocysteine concentration of adult males who participated in study number 3 (chapter 6) in response to increasing cysteine intake.
10.3 CONSENT FORMS

Title of Research Project:
THE METHIONINE REQUIREMENT OF THE TPN FED NEONATE.

<table>
<thead>
<tr>
<th>Investigators</th>
<th>Contact number</th>
<th>Contact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paul Pencharz</td>
<td>TPN Program, GI and Nutrition</td>
<td>416-813-7733</td>
</tr>
<tr>
<td>Glenda Courtney-Martin</td>
<td>Dietitian, PhD Student</td>
<td>416-813-8580</td>
</tr>
<tr>
<td>Dr. Aideen Moore</td>
<td>Neonatologist</td>
<td>416-813-7331</td>
</tr>
<tr>
<td>Dr. Jae Kim</td>
<td>Neonatologist</td>
<td></td>
</tr>
<tr>
<td>Dr. Jacob Langer</td>
<td>Surgeon</td>
<td>416-813-6405</td>
</tr>
</tbody>
</table>

Why are we doing this study?

This study is being performed to improve the nutrition of infants needing intravenous nutrition. Infants fed with intravenous nutrition receive their protein needs from a solution made up of a careful balance of amino acids (building blocks of protein). There are currently several solutions available that differ in both the quality and balance of their amino acids. The goal of this study is to determine the best level of one of the 20 amino acids, which will provide optimal growth for infants receiving intravenous nutrition.

What will happen during the study?

The purpose of this study is to test the optimal amount of the amino acid methionine needed by the body. For this study, six levels of methionine will be studied. Each level will be tested over a period of two days. It would be beneficial if your child can participate in two levels for a total of four days. If you choose for your child to participate in the study (s)he will receive a standard pediatric amino acid solution used routinely in the NICU for the first day. On the second day, (s)he will receive the same solution with a different amount of the amino acid under study. In order to see how your
baby uses this building block (specifically known as phenylalanine), we have specially marked a small amount of an amino acid in the IV solution to see how methionine (the test amino acid) is processed in your infant’s system. This marker is a naturally occurring substance and is completely harmless. We will then look for the appearance of the marker in the babies’ breath and urine. Urine will be collected by placing cotton swabs into the diaper or by using urine bags. Periodically, breath will be collected by placing a clear ventilated hood around your baby’s head. This will take approximately fifteen minutes. Past experience has shown that most babies sleep through this procedure. Throughout much of the study the individual conducting the research (a dietitian) will be helping with the extra attention your child will be receiving.

We will also need to take 0.75mL of blood at the start and at the end of the study to look at the amount of glutathione in your child’s body. Glutathione is a substance which protects the cells from damage and the amount in the blood can be decreased during infection or illness. We aim to coordinate the taking of blood with the regular TPN blood work.

**Are there good things and bad things about the study?**

There are no known harms associated with participation in this study.

**Potential Benefits:**

This study has no direct benefit to your infant, however it will provide information that should result in the design of better solutions for future infants in need of intravenous nutrition at The Hospital for Sick Children and in other children’s hospital centres.

**Who will know about what I did in the study?**

Confidentiality will be respected and no information that discloses the identity of the subject will be released or published without consent unless required by law. This legal obligation includes a number of circumstances, such as suspected child abuse and infectious disease, expression of suicidal ideas where research documents are ordered to be produced by a court of law and where researchers are obligated to report to the appropriate authorities.

For your information, the research consent form will be inserted in the patient health record.

**Can I decide if I want to be in the study?**

Nobody will be angry or upset if you do not want to be in the study. Participation in research must be voluntary. If you choose not to participate, you and your family will continue to have access to quality care at The Hospital for Sick Children. If you choose on behalf of your child to participate in this study you can withdraw your child from the study at any time. Again, you and your family will continue to have access to quality care at The Hospital for Sick Children.

**Consent:**

*By signing this form, I agree that:*
1) The study has been explained to me. All my questions were answered.
2) The possible harms and discomforts and the possible benefits (if any) of this study have been explained to me.
3) I know about the alternatives to having my child take part in this study. I understand that I have the right to refuse their participation and the right to stop at any time. My decision about whether or not to participate will not affect my child’s health care at The Hospital for Sick Children.
4) I am free now, and in the future, to ask any questions about the study.
5) I have been told that my child’s medical records will be kept confidential, except where release of information is required by law, e.g., suspected child abuse, public health.
6) I understand that no information that would identify my child, will be released or printed without asking me first.

I hereby consent for my child ________________________________ to participate.
I have read and understood pages 1 to _____ of this consent form.

__________________________________________________________
Name of Parent

__________________________________________________________
student.

__________________________________________________________
Signature & Date

The Person who may be contacted about the research is:

Glenda Courtney-Martin MSc RD, PhD

who may be reached at telephone #:

416-813-5744 or page 416-390-0701

__________________________________________________________
Name of person who obtained consent

__________________________________________________________
Signature & Date

The person whom may be contacted about the research is:

Glenda Courtney-Martin MSc. RD

She may be reached at: Tel: 416-813-5744 (HSC) or 416-284-9264 (Home)

Pager: 416-390-0701
Title of Research Project:
THE LEVEL OF CYSTEINE INTAKE TO OPTIMIZE GLUTATHIONE (GSH) SYNTHESIS IN THE HEALTHY ADULT MALE.

Investigators

<table>
<thead>
<tr>
<th>Name</th>
<th>Contact number</th>
<th>Pager</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glenda Courtney-Martin MSc. RD</td>
<td>(416) 813-5744</td>
<td>(416) 390-0701</td>
</tr>
<tr>
<td>Paul B. Pencharz, M.D., Ph.D.</td>
<td>(Principal Investigator)</td>
<td>(416) 813-7733</td>
</tr>
</tbody>
</table>

Purpose of the Research:
The purpose of this study is to modify existing literature methods to create a minimally invasive method for measuring glutathione (GSH) synthesis in response to graded intakes of cysteine. In our lab we have previously determined the cysteine requirement for net protein synthesis. However, we believe than the amount required for GSH synthesis is higher that that required for net protein synthesis alone. Our ultimate goal is to use the minimally invasive method derived from this study to measure the cysteine requirement for optimal GSH synthesis in the TPN fed human neonate.

Description of the Research:
We are asking you to consider taking part in a series of five studies. These studies will involve you to eat different levels of the amino acid cysteine for 3 day periods at one time.

Before the study begins, you are required to visit Clinical Investigation Unit (Room 5D12, The Hospital for Sick Children) for a pre-study assessment of your height, weight, fat mass, muscle mass, resting metabolic rate and medical history. These assessments will take about 1 hour to complete. You need to fast from 8 PM on the night before the pre-study assessment day. We need the pre-study assessment to calculate your dietary requirements for the study.

Prior to the first study day you will need to fast from 8.00pm the night before and arrive at the CIU (Rm 5D12, the hospital for sick children). On the first study day you will receive hourly liquid formula diet with crystalline amino acids starting at 8.00AM in the morning, for a period of 8 hours. Blood samples will be collected at regular intervals throughout the study day. A Registered Nurse will insert a small needle into a vein in your hand to enable blood collection. A 30- minute metabolic rate measurement will be measured once during a study day.
After four hours have gone, we will start to infuse a labeled amino acid into your IV site. This we will infuse until the end of the study. This amino acid occurs naturally and has been used in the past with no adverse effects. We will measure its incorporation into glutathione to determine how much cysteine is needed to optimize GSH synthesis.

For three days before the study you will be asked to collect all your urine into a container. Also, for two days prior to the study, we will provide the test diet separated into 4 meals to be consumed at 8.00AM, 12 noon, 4.00PM and 8.00PM. Each meal also contains two varieties of cookies (butterscotch and cornflake cherry flavor). The experimental meals are calculated and specific to meet your requirements for all nutrients including, energy and protein. Multi-vitamin tablets will be provided and you are requested to take one per day. You are required to consume all the experimental meals and cookies, and are not allowed to eat other foods or drinks. You are only allowed to drink water. Smoking and alcohol consumption is also prohibited during the study period. You are requested to keep your normal physical activity level but to refrain from participation in competitive sports. All 5 studies will be completed within a 3 month period.

Before the start of the study, during consumption of your habitual dietary intake you will be asked to record everything you eat and drink for 2 weekdays and 1 weekend day.

If changes are made to the study or new information that might affect your willingness to continue to participate in the research becomes available, you will be informed.

**Potential Harms:**

There are no known harms associated with participation in this study. There may be a small amount of bleeding when blood is taken from a vein and there may be slight discomfort and bruising or redness that will usually disappear in a few days.

**Potential Discomforts or Inconveniences:**

There is a significant time commitment associated with participation in this study as you will be required to be at the Hospital for Sick Children on two separate days (one for 1 hour and the other for 8 hours). There is an extra time commitment for getting to the hospital if you do not work at the Hospital for Sick Children. Other inconvenience is that of coming to the hospital fasting if you would normally eat breakfast at home.

**Potential Benefits:**

You will not benefit directly from participating in this study.

The benefit lies in being able to develop a method for measuring GSH synthesis that is quick, easy and non-invasive (if it is found that measuring in urine is an alternative to plasma) in the TPN fed neonate.
**Confidentiality:**

Confidentiality will be respected and no information that discloses the identity of the subject will be released or published without consent unless required by law. This legal obligation includes a number of circumstances, such as suspected child abuse and infectious disease, expression of suicidal ideas where research documents are ordered to be produced by a court of law and where researchers are obligated to report to the appropriate authorities.

**Reimbursement:**

Subjects will be reasonably reimbursed for all expenses related to participating in the study. The cost of travel, parking and loss of pay will be covered in the reimbursement as well as a suitable amount for the inconvenience incurred.

**Participation:**

Participation in research is voluntary. If you choose not to participate, you can withdraw from the study at any time and you and your family will continue to have access to quality care at HSC.

**Consent:**

The following must be the last section on the form and must be reprinted verbatim for participants who can consent for themselves.

"By signing this form, I agree that:
1) The study has been explained to me. All my questions were answered.
2) The possible harms and discomforts and the possible benefits (if any) of this study have been explained to me.
3) I know about the alternatives to taking part in this study. I understand that I have the right not to participate and the right to stop at any time. The decision about whether or not to participate will not affect my health care at The Hospital for Sick Children.
4) I am free now, and in the future, to ask any questions about the study.
5) I have been told that my medical records will be kept confidential, except where release of information is required by law, e.g., suspected child abuse, public health.
6) I understand that no information that would identify me will be released or printed without asking me first."

I hereby consent to participate.

Name of Patient and Age

The Person who may be contacted about the research is:
For answers to questions about research subjects’ rights and research-related injury, please contact the Research Ethics Board Manager at (416) 813-5718.
Title of Research Project:
THE EFFECT OF INCREASING CYSTEINE INTAKE ABOVE THE LEVEL OF THE TOTAL SULPHUR AMINO ACID REQUIREMENT ON GSH SYNTHESIS IN THE TPN FED HUMAN NEONATE.

Investigator(s): Contact Number Pager
Glenda Courtney-Martin MSc. RD 416-813-5744 416-390-0701
(Dietitain, PhD Student)
Paul Pencharz, Principal Investigator 416-813-7733
(Principal Investigator. TPN Program, GI Nutrition)
Dr. Jacob Langer 416-813-6405
(Surgeon)

Purpose of the Research:
This study is being performed to improve the nutrition of infants needing intravenous nutrition. Infants fed with intravenous nutrition receive their protein needs from a solution made up of a careful balance of amino acids (building blocks of protein). There are currently several solutions available that differ in both the quality and balance of their amino acids. The goal of this study is to determine if giving one of the 20 amino acid at a higher rate than the amount needed for protein synthesis will results in a better antioxidant status for infants receiving intravenous nutrition.

Description of the Research:
The purpose of this study is to test whether giving additional cysteine to neonates on TPN will result in an increase in the synthesis of glutathione. Glutathione is a substance which protects the cells from damage and the amount in the blood can be decreased during infection or illness. The amount of glutathione is also found to be low in new born babies. The amount of glutathione that the infant makes will be studied on two different days. If you choose for your child to participate in the study (s)he will receive a modification of a standard pediatric amino acid solution used routinely in the NICU (without cysteine) for the first three days. On the fourth to the sixth day, (s)he will receive the same solution to which cysteine will be added. In order to see how your baby uses cysteine to make the antioxidant glutathione, we have specially marked a small amount of an amino acid in the IV solution to see how cysteine is processed in your infant’s system. This marker is a naturally occurring substance and is completely harmless. We will then look for the appearance of the marker in the babies’ blood. Blood will be collected on two different days. 0.7mls of blood will be collected on each of the 2 days at 4 different time points, for a total of 2.8 mls/day. We aim to coordinate the taking of two blood samples on each of the two
days with the regular TPN blood work that is routinely taken for clinical purposes. Blood will be taken from your child’s central line to avoid him/her being poked. Urine will also be collected from cotton placed inside the diaper for 7 hours during the 3rd and 6th day of the study.

Throughout much of the study the individual conducting the research (a dietitian) will be helping with the extra attention your child will be receiving.

**Potential Harms:**
An extra 3mls of blood will be required from your child on 2 separate days within a 6 day period that required for routine clinical practice. This extra 3mls of blood of blood represents less than 2% of your child’s total blood volume and is well within the safety guidelines for blood taking. It is not expected to be harmful to your child.

It is possible that your child could develop and infection by accessing the line for an extra 2 blood samples. A Registered Nurse trained in sterile technique will be employed to draw the blood from your child’s line to minimize the potential risk of infection.

**Potential Discomforts or Inconvenience:**
There is no inconvenience to you or your child from participating in this study since your child is already an in-patient at The Hospital for Sick Children

**Potential Benefits:**
This study has no direct benefit to your infant, however it will provide information that should result in the design of better solutions for future infants in need of intravenous nutrition at The Hospital for Sick Children and in other children’s hospital centres.

**To individual subjects:**
Your child will not benefit directly from participating in this study.

**To society:**
The information from this study would be beneficial to design better TPN solutions for infants at The Hospital for Sick Children and especially for infants needing TPN for long periods of time. It will also benefit infants on TPN in other hospitals in Canada and around the world.

**Confidentiality:**
Confidentiality will be respected and no information that discloses the identity of the subject will be released or published without consent unless required by law. This legal obligation includes a number of circumstances, such as suspected child abuse and infectious disease, expression of suicidal ideas where research documents are ordered to be produced by a court of law and where researchers are obligated to report to the appropriate authorities.

For your information, the research consent form will be inserted in the patient health record.

**Reimbursement:**
There is no compensation for participation in this study since your child will be in hospital at the time of participation.

**Participation:**
Nobody will be angry or upset if you do not want to be in the study. Participation in research must be voluntary. If you choose not to participate, you and your family will continue to have access to quality care at The Hospital for Sick Children. If you choose on behalf of your child to participate in this study you can withdraw your child from the study at any time. Again, you and your family will continue to have access to quality care at The Hospital for Sick Children.

**Sponsorship:**
The sponsor/funder of this research is The Canadian Institute of Health Research (CIHR)

**Conflict of Interest:**
I, and the other research team members have no conflict of interest to declare.
**Consent:**

“By signing this form, I agree that:

1) You have explained this study to me. You have answered all my questions.
2) You have explained the possible harms and benefits (if any) of this study.
3) I know what I could do instead of having my child take part in this study. I understand that I have the right to refuse to let my child take part in the study. I also have the right to take my child out of the study at any time. My decision about my child taking part in the study will not affect my child’s health care at Sick Kids.
4) I am free now, and in the future, to ask questions about the study.
5) I have been told that my child’s medical records will be kept private except as described to me.
6) I understand that no information about my child will be given to anyone or be published without first asking my permission.
7) I agree, or consent, that my child___________________ may take part in this study.”

_________________________________
Printed Name of Parent/Legal Guardian  Parent/Legal Guardian’s signature & date

_________________________________
Printed Name of person who explained consent  Signature of Person who explained consent & date

Printed Witness’ name (if the parent/legal guardian does not read English)  Witness’ signature & date

If you have any questions about this study, please call __________________________ at __________

If you have questions about your rights as a subject in a study or injuries during a study, please call the Research Ethics Manager at 416-813-5718.”
10.4 STUDY DAY CALCULATIONS

CALCULATIONS FOR STUDY: TOTAL SULPHUR AMINO ACID AND REQUIREMENT IN THE TPN FED POST-SURGICAL HUMAN NEONATE.

Total Sulphur AA Requirement
Methionine Study With zero cysteine

Working out the N Content of the Test TPN Solution:
Bulk solution #1

Amino Acid Mix Bulk Solution # 1 (study day #2)
Study day includes 100% of the total intake. Study conducted over a periods of 2 x 24 hr periods

Total Protein (AA) = 2.5 to 3g/kg. x (wt) _______ kg = __________ g Prot/day[A]

Total Volume of AA solution needed
• 5% Solution: 50g/L _____ g [A] in ______ mLs[B] ÷ wt (kg) ________ mL/kg
• 3% Solution: 30g/L____ g [A] in ______ mLs[B] ÷ wt (kg) ________ mL/kg
• 2% Solution: 20g/L_____ g [A] in ______ mLs[B] ÷ wt (kg) ________ mL/kg

Total Nitrogen in Base Primene Solution:
• 5% solution (7534 mg of N/L) = 7.534mg/mL x ___mL[B] = __mg of N/day[C]
• 3% Solution (4520 mg of N/L) =4.52mg/mL x ______mL[B] = ________mg of N/day[C]
• 2% solution (3013.5mg of N/L)=3.013mg/mL x______mL[B] = _________mg of N/day[C]

Total Nitrogen in Bulk Solution #1:
• 5% solution (7250.035mg of N/L) = 7.250035mg/mL x ______mL[B] =_____mg of N/day[D]
• 3% solution (4350.02mg of N/L) = 4.35002 mg/mL x _____mL[B] =_______mg of N/day[D]
• 2% solution (2900.014mg of N/L)= 2.900014 mg/mL x_______mL[B] = ____mg of N/day[D]

Difference of Nitrogen needed. [C] – [D] ___________________________ mg/day[E]
To be made up by Phe (isotope), Meth. & Ala)

Phenylalanine Tracer(isotope)Intake: Calculating the N from the Isotope

Tracer Phe intake = [2.59 + (24 x 2.16)] = 54.43mg/kg Phe
Total Phe from tracer: 54.43mg x (wt) __________kg = __________ mg Phe/day[F]

Total N from Phe[E] : ([F] x 14000)/166190 = _________________ mg of N from tracer[G]

Methionine Intake: Calculating the N from the test methionine Intake
Test Methionine Intake Level (10, 30, 50, 80, 100, 120) mg/kg/d [H]
Total Meth intake: __________mg/kg/d [H] x (wt) _______kg = _______ mg of meth/day[I]
Total N from Meth[H]: ([I] x 14000)/149210 = _________________ mg of N from Meth [J]

Alanine Intake: Alanine to make up the balance of the N needed
Total N needed from Alanine: = [E] –(F + J) =_______________mg of N from Ala [K]
Total Alanine needed in mg: = ([K] x 89090)/14000 = _________ mg Ala/day [L]
Total Alanine in grams: [L]/1000 = _________ grams of Ala/day[M]

**Summary of Protein & AA To be Delivered to Patient**

1. Protein (g/day) __________________________ [A]
2. Volume of AA Solution (mL/day)________________________ [B]
3. Total Phenylalanine (isotope) mg/day _________________________[F]
4. Methionine intake mg/kg __________________________ [H]
   a. Total methionine (mg/day)________________________ [I]
5. Total alanine intake (mg/day)________________________ [L]

**Volume of Phe (isotope), Meth & Alanine to be added**

**Phenylalanine:** (Vial to be sent separately)

**Phe (isotope):** (Prime = 15.6μmol/kg). (CI = 13μmol/kg)

Prime = 2.59 mg/kg x ________ kg = ________________ mg
CI = 2.16 mg/kg/hr ___________ kg = ________________ mg/hr x 24h = _____________ mg

Total (P + CI) = ______________ mg phe = __________________ mg phe/day (isotope)

**Volume of Phe as Tracer (stock solution 15mg/mL)**

Prime = ______________ mg ÷ ______________ mg/mL = ______________ mL
CI = ______________ mg/24hr ÷ ______________ mg/mL = ______________ mL

**13Cphe Infusion Volumes:**
Prime __________ mL (infuse over 15 mins) = Prime volume x 60 mins ÷ 15 mins ________ mL/hr
CI __________ mL (infuse over 23.75 hrs) = CI ÷ 23.75 hrs __________ mL/hr x 23.75 hrs.

Add 0.9% NaCl to Prime:
Add 0.9% NaCl to CI:

**Methionine:** (To be Added to TPN Bulk Solution #1)

Test Methionine intake mg/kg __________________________ [H]
Total Methionine (mg/day)________________________ [I]

Volume of Methionine: (stock solution 20mg/mL)

Total Methionine__________mg[I] ÷ ______________ mg/mL = ______________ mL

Total Methionine in mg/L of TPN (Bulk solution #1) = ( [I] x 1000) ÷ [B] ______________ mg/L methionine

**Alanine:** (To be Added to TPN Bulk Solution #1)

**Alanine Intake: Alanine to make up the balance of the N needed**

Total Alanine needed (mg/day)________________________ [L]
Volume of Alanine: (stock solution 50mg/mL)
Total Alanine___________________mg[L] ÷ ______________ mg/mL = _________ mL

Total Alanine in mg/L of TPN (Bulk Solution #1) = ([L] x 1000) ÷ [B] ______________ mg/L alanine
### 10.5 Amino Acid Composition of PN Solution

Composition of crystalline L-amino acid mixture used to determine the total sulphur amino acid requirement of the TPN fed post-surgical human neonate

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Primene (g/100 g total AA)</th>
<th>Proposed Solution#1 (g/100 g total AA)</th>
<th>Final Solution (g/L total AA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ileucine</td>
<td>6.7</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.9</td>
<td>9.9</td>
<td>9.9</td>
</tr>
<tr>
<td>Valine</td>
<td>7.6</td>
<td>7.6</td>
<td>7.6</td>
</tr>
<tr>
<td>Lysine (lysine-HCL)</td>
<td>10.9</td>
<td>10.9 (13.62)</td>
<td>10.9 (13.62)</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.4</td>
<td>Variable</td>
<td>Variable</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phenylalanine(diet + isotope)</td>
<td>4.2</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.9</td>
<td>0</td>
<td>All supplied as GT</td>
</tr>
<tr>
<td>Tyrosine (total) (GT)</td>
<td>3.7</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.7</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.8</td>
<td>3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Arginine: base primene solution (increase of 20% based on piglet data)</td>
<td>8.4</td>
<td>8.4 (9.66)</td>
<td>9.66</td>
</tr>
<tr>
<td>Glycine (total)</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Glycine to be added (From GT)</td>
<td>2.34 (1.66)</td>
<td>2.34 (1.66)</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>7.9</td>
<td>7.9</td>
<td>Additional Variable amt added (to make solution isonitrogenous)</td>
</tr>
<tr>
<td>Aspartate</td>
<td>6.0</td>
<td>6.0 (5.0)</td>
<td>5.0</td>
</tr>
<tr>
<td>Glutamate</td>
<td>9.9</td>
<td>9.9</td>
<td>9.9</td>
</tr>
<tr>
<td>Proline</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Serine</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Taurine</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Ornathine (excluded because decreases the PH of the solution)</td>
<td>2.2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Total AA (grams/100g)</strong></td>
<td><strong>100.00</strong></td>
<td><strong>94.15</strong></td>
<td></td>
</tr>
</tbody>
</table>

*Proposed “Bulk Solution #1” represents 94.15% of the total protein/total AA.
10.6 Study day list of events

**Total sulphur amino acid requirement of the TPN fed post-surgical neonate: Study day List of Event by Time**

**Total Study Time:** 48 Hrs

**Pt Name:___________________________________________**

**Study #:_____________**

**Pt Weight:__________________________________________**

**Pt Height:__________________________________________**

**Dates of Study: Day 1          Day 2:**

**Pt DOB:**

**Pt Age:**

Phenylalanine Intake as Tracer: (prime = 15.6µmol/kg). (CI=13µmol/kg)

Prime = 2.59 mg/kg x _______ kg = _______ mg

CI = 2.16mg/kg/hr x _______ kg = _______ mg/ hr x 24h = _______ mg

Total (P + CI) = _________mg phe = ________________mg phe

Volume of phe as Tracer (stock solution 20mg/ml)

Prime =___________mg ÷ _________mg/ml = _________ml

CI = ________ mg/24hr ÷ __________mg/ml = __________ml

| Time (Hour:Min) | Breath Collection | List of Events | TPN, Isotope
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{13}$CO$_2$</td>
<td></td>
<td>Urine, calorimetry</td>
</tr>
<tr>
<td></td>
<td>VCO$_2$</td>
<td></td>
<td>Blood for AA, HcY, GSH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Blood for HCT within 24 hrs of starting the study</td>
</tr>
<tr>
<td>Pre-study</td>
<td>Written informed consent</td>
<td>Bulk TPN, Test Solution</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Patient set-up</td>
<td>Get Isotope Ready</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Equipment for breath collection</td>
<td>Isotope prime_______mls</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tubes, Epindorfs</td>
<td>CI _____________ mls/ hr</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Container for urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Machine for breath collection</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Container and chemicals for blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-study</td>
<td>Check TPN/Primene Order</td>
<td>Get doctor to sign order, Verify with dietitian correct TPN order</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Contact pharmacy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3:00pm – Dec 18/06</td>
<td>Obtain pt weight and length,</td>
<td>Start Primene TPN order (to be continued for 24 hrs)</td>
<td></td>
</tr>
<tr>
<td>7:00 -8:00 am – Dec 19/06</td>
<td>1st baseline urine collection</td>
<td>1st baseline breath collection</td>
<td></td>
</tr>
<tr>
<td>10:00 – 11:00am – Dec 1906</td>
<td>2nd baseline urine collection</td>
<td>2nd baseline breath</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>Action</td>
<td>Measurement</td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>------------------------------------------------------------------------</td>
<td>----------------------</td>
<td></td>
</tr>
<tr>
<td>1:00 – 2:00pm – Dec 19/06</td>
<td>3rd baseline urine collection</td>
<td>3rd baseline breath collection</td>
<td></td>
</tr>
<tr>
<td>3:00pm- Dec 19/06</td>
<td>Start Test TPN solution</td>
<td>Isotope Prime:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mls</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(infuse over 15 minutes)</td>
<td></td>
</tr>
<tr>
<td>3:15pm- Dec 19/06</td>
<td>Start CI isotope</td>
<td>CI mls/hr x</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.75hrs = mls</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(run at a constant rate/hr for the period of 23.75 hrs)</td>
<td></td>
</tr>
<tr>
<td>3:00am – Dec 20/06</td>
<td>1st plateau breath collection</td>
<td>1st plateau urine collection</td>
<td></td>
</tr>
<tr>
<td>7:00am – Dec 20/06</td>
<td>2nd plateau breath collection</td>
<td>2nd plateau urine collection</td>
<td></td>
</tr>
<tr>
<td>11:00p – Dec 20/06</td>
<td>3rd plateau breath collection</td>
<td>3rd plateau urine collection</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4th plateau breath collection</td>
<td>4th plateau urine collection</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5th plateau breath collection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:00pm – Dec 2006</td>
<td>VCO₂ measurement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2:00-3:00pm Dec 20/06</td>
<td>6th plateau breath collection Weight and Length</td>
<td>5th plateau urine collection</td>
<td></td>
</tr>
<tr>
<td>3:00pm Study Ends</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2nd blood collection</td>
<td></td>
</tr>
</tbody>
</table>
10.7 Order form for TPN Research Pharmacy: Total SAA requirement of the TPN fed post-surgical human neonate.

Investigation of Total Sulphur AA Requirements

Subject Parameters at time of study:
Patient #______________________
Methionine intake on Day 2: _________________
Study Dates. Day 1:_____________Day 2_____________
Diagnosis:___________________ Pt Length:_______ cm Pt Weight:_______ kg
D.O B:_____________ Pt Age:_______ mths/weeks Pt Head Circ:_______ cm

Birth Parameters:
Gest Age:_____Wks Birth Wt:_____kg Birth Lgth:_____cm Birth
Head Circ:_____ cm
Study is conducted over a period of 2 x 24 hr periods. Day 1 with a Primene amino acid (AA) based TPN & Day 2 with the special AA bulk base plus 3 individual AAs. Study gives the same total AA intake on both days.

Total AA Intake [A]
_______ (range 2.5 to 3)g/kg/day. x (wt) _________kg = __________g AA/day[A]

Total Volume of TPN solution needed [B]:
_________________________ g[A] x 1000 = __________mLs [B]. [B] ÷ wt (kg) = 
_________________________ mL/kg (range 100 – 150) 
g/L of AA in final TPN soln (from TPN order form Day 1)

Individual Study & Special Amino Acid Bulk Base Solution for Study Day #2

13C Phenylalanine Tracer (isotope) Intake
13C Phe intake = [2.59 + (24x2.16) = 54.43mg/kg/day].
Prime = 2.59 mg/kg x _______ kg = ________________mg
CI = 2.16 mg/kg/hr _______ kg = ________________mg/hr x 24h = ________________mg
Total (Prime + CI) = ________________mg/day of 13C phe (isotope) [F]

Volume of 13C Phe as Tracer (stock solution 15mg/mL)
Prime = ________________mg ÷ 15mg/mL = ________________mL
CI = ________________mg/24hr ÷ 15mg/mL = ________________mL
Total volume of 13C Phe = __________ mL. of 15mg/mL. (5 &10mL vials available). Send 
_________________________mL
vial(s) to be sent separately (not added to TPN bag).

**Methionine Intake:** (To be Added to Day 2 TPN Solution)
Test Methionine Intake Level as randomized (10, 30, 50, 80, 100, 120) mg/kg/day.
Column [H]
Total Methionine intake: ________mg/kg/d [H] x (wt)_______kg = ______mg of meth/day [I]
*Concentration of Methionine:*
Concentration of Methionine in Day 2 study TPN = __________mg/day [I] x 1000
Vol of TPN/day

= Methionine in Day 2 study TPN = ( [I] x 1000) ÷ [B] = __________ mg/L [C].

**Methionine TPN Study (with zero cysteine) - Investigation of Total Sulphur AA Requirements**

**Balance Alanine Intake:** (To be Added to Day 2 TPN Solution)
Balance Alanine Intake: Alanine to make up the balance of the N needed to keep the TPN iso-nitrogenous. As methionine increases, alanine will decrease as laid out in the randomization table, column [J].
Balance Alanine intake:______________mg/kg/day [J] x (wt)______kg = ________mg of alanine/day[K].
*Concentration of Balance Alanine:*
Balance Alanine in Day 2 study TPN = ([K] x 1000) ÷ [B] = ______________mg/L [D]

**Special Bulk Base AA Intake:** (To Make Day 2 TPN Solution)
Special Bulk Base AA intake = __________ [A] x 1000 – [_______F] + [______I] + [______K].= __________ mg/day.[M] [M] ÷ 1000 = _______g/day [N].
*Concentration of Special Bulk Base AA:*
Concentration of Special Bulk Base AA in Day 2 TPN (g/L) = [N] x 1000 ÷ .volume of TPN [B]
= __________ g/L [E]

---

Date: ________________ Signature ________________________________
(MD)  

Time: ________________ Print Name ________________________________
10.8 Study day calculations: Methionine-adequate cysteine-free diet does not limit glutathione synthesis in young healthy adult males.

**For Adaptation Days**

<table>
<thead>
<tr>
<th>Study #</th>
<th>Study Date</th>
<th>Subject</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Height: __________ m  Weight: __________ kg  Age: _________ yrs

---

**Energy Requirements**: kcal/day

RMR = __________ kcal/day

_________ kcal/day x 1.7 = ___________ kcal/day (2 Day Adaptation Diet) ) [A]

**Diet Constituents:**

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>= 10% total energy intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula</td>
<td>= 65% total energy intake</td>
</tr>
<tr>
<td>Butterscotch Cookies</td>
<td>= 12.5% total energy intake</td>
</tr>
<tr>
<td>Cornflake Cookies</td>
<td>= 12.5% total energy intake</td>
</tr>
</tbody>
</table>

Amino Acids = 1.0g Prot/kg/day x _______ kg = _______g Prot/day [B]

Formula = 0.427ml/kcal x 65% x [A] kcal/day = _______ml/day [C]

BS Cookies = 0.234g/kcal x 12.5% x [A] kcal/day = _______g/day [D]

CF Cookies = 0.229g/kcal x 12.5% x [A] kcal/day = _______g/day [E]

<table>
<thead>
<tr>
<th></th>
<th>Amount</th>
<th>Fat (g)</th>
<th>CHO (g)</th>
<th>Prot(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino Acids</td>
<td>[B]</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Formula</td>
<td>[C]</td>
<td>x 0.1</td>
<td>x 0.36</td>
<td>-</td>
</tr>
<tr>
<td>BS Cookies</td>
<td>[D]</td>
<td>x 0.223</td>
<td>x 0.565</td>
<td>-</td>
</tr>
<tr>
<td>CF Cookies</td>
<td>[E]</td>
<td>x 0.244</td>
<td>x 0.537</td>
<td>-</td>
</tr>
<tr>
<td>Total (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (kcal)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Actual Energy Intake = __________ kcal/day

Energy Distribution  = ________% Fat, ________% CHO, ________% Prot
Calculating the Nitrogen (N) Content of the four (4) Amino Acids Being Varied in the Study:

Using 1g/kg Protein Intake and Standard Egg Protein Pattern

Glycine:

Glycine Content in Egg Protein = 33.25mg/kg
Total glycine intake = 33.25mg/kg \times \frac{wt}{kg} = \text{[L]}
Total N from glycine = \frac{14000 \times [L]}{75070} = \text{mg N from glycine [M]}

Methionine:

Methionine content in egg protein = 29.67mg/kg
Total Met intake = 29.67mg/kg \times \frac{wt}{kg} = \text{[N]}
Total N from Met = \frac{14000 \times [N]}{149210} = \text{mg N from Met [O]}

Cysteine:

Cysteine content in egg protein = 22.11mg/kg
Total Cyst intake = 22.11mg/kg \times \frac{wt}{kg} = \text{[P]}
Total N from Cyst = \frac{14000 \times [P]}{121160} = \text{mg N from Cyst [Q]}

Alanine:

Alanine content in egg protein = 61.47mg/kg
Total alanine intake = 61.47mg/kg \times \frac{wt}{kg} = \text{[R]}
Total N from alanine = \frac{14000 \times [R]}{89090} = \text{mg N from alanine [S]}

Total N from gly, met, cys & alanine in standard egg protein = M+O+Q+S
\text{__________mg/d [T]}
Amino Acid Mix # 1 (study day)

Each meal represents 1/4 of daily intake (total of 4 meals = 100% of daily requirement)

_____ g Prot/day [B]

Mix # 1 represents 85.35% of the total AA reqt = _________ g Mix # 1 / study day [Bb]

______ g Mix # 1 / study day 4 meals = _______ g Amino acid mix # 1 / meal

The total of the other amino acids (gly, met, cys and Ala) = 14.65% of reqt = __________g [BB]

Glycine Intake

Glycine reqt/day = 66.50 mg/kg x ________ kg = ________ mg [F]

Dietary Gly/meal = [F] ÷ 4 = __________ mg glycine/meal [H]

N from dietary gly = 14000 x [F]/75070 = ________________ mg N (dietary glycine [V]
### Amino Acid Recipe

<table>
<thead>
<tr>
<th>Meal</th>
<th>AA Mix (g)</th>
<th>Gly (mg)</th>
<th>Met Intake (mg)</th>
<th>Cys Intake (mg)</th>
<th>Ala Intake (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>[Bb]</td>
<td></td>
<td></td>
<td></td>
<td>[10]</td>
</tr>
</tbody>
</table>

Total: AA mix + gly + met + cys + ala = ________________g/day
14.65% of reqment [BB] = ________________ g/day (Should be same as above)

Base Mix (AA mix #1 + Alanine) = A + B
Per Meal = A + B/4

---

\(^1\) 17/04/98 formula.plt
Methionine Intake:  = 14mg/kg/day (set level of methionine intake)

Met intake/day = _______ kg x 14mg/kg/d = ____________ mg/d [h]

Met intake/meal = [h]/4 = __________ mg/meal [i]

N from Met = 14000 x [h]/149210 = ____________ mg N (dietary Met) [W]

Cys Intake

Cys intake/day = ___ mg/kg/d x ______ kg = _____ mg [j]

Cys intake/meal = [j]/4= ___________mg [k]

N from Cyst = 14000 x [j]/121160 = ____________ mg N (dietary Cys) [X]

Balance Alanine Intake:

Alanine to be added to diet = add the N from glycine, met & cys

= [V] + [W] + [x] = _________________ ____ = ________________ mg N/day [Y]


Alanine/day = [Z] x 89090/14000 = _________________ mg alanine/day [Z1] ÷ 1000

____________grams alanine/day [10]
Formula Requisition (Study day)  Need 100% requirement i.e. 4 meals

Formula required per study day = [C] ml = ________ml/day [C]

Formula required per meal/d = [C] ml 4 = ________ml/meal [O]

Formula requisition = 4 x ________ml/meal [O] = ________ml/day [P]

Formula Recipe

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Conc'n (g/ml)</th>
<th>Study day vol(ml) [P]</th>
<th>Study day amt(g)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product #80056</td>
<td>0.281</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orange Tang</td>
<td>0.0832</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Koolaid Crystals</td>
<td>0.0832</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn Oil</td>
<td>0.033</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sterile water</td>
<td>0.64</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Study day amt (g) = Conc'n (g/ml) x study day vol [P]

Butterscotch Cookies (study day)

BS Cookies required per study day = [D] = ________g/day [D]

g BS Cookies required per meal = [D] 4 = ________g/meal

Cornflake Cookies (study day)

CF Cookies required per study day = [E] = ________g/day [E]

g CF Cookies required per meal = [E] 4 = ________g/meal

** Each meal is served in a separate plastic bag
Diet Requisition Form:

<table>
<thead>
<tr>
<th>Subject's Name:</th>
<th>Delivery Date:</th>
</tr>
</thead>
</table>

Formula Recipe: Study Day

__________ g Product #80056
__________ g Orange Tang
__________ g Koolaid Crystals  Flavour:__________
__________ g Corn Oil
__________ g Sterile Water

Total volume of formula: __________ ml

__4__ Bottles __________ml per bottle
Subject's Name:                       Delivery Date:

**Butterscotch Cookie:**  **Study Day**

\[
\text{Study Day} \quad \\
\text{g BS cookie per day} \\
\text{4 meals per day} \quad \text{g BS Cookie per meal}
\]

**Cornflake Cookie:**  **Study Day**

\[
\text{Study Day} \quad \\
\text{g CF cookie per day} \\
\text{4 meals per day} \quad \text{g CF Cookie per meal}
\]

*** All meals should be divided into separate plastic bags, each labeled as follows:
- Subject name
- Meal Number
- Type of Cookie
- Weight (grams) of cookie per bag
10.9 Study day calculations: Methionine-adequate cysteine-free diet does not limit glutathione synthesis in young healthy adult males.

For Study Day

Study #________________________
Study Date:_____________________
Subject _______________________
Height: ___________m
Weight: ____________kg
Age: _________yrs

Energy Requirements: kcal/day
RMR = __________kcal/day

_________ kcal/day x 1.5 = __________kcal/day (for Study Day) [A]

Diet Constituents:

Amino Acids = 10% total energy intake
Formula = 65% total energy intake
Butterscotch Cookies = 12.5% total energy intake
Cornflake Cookies = 12.5% total energy intake

Amino Acids = 1.0g Prot/kg/day x _______kg = ________g Prot/day [B]
Formula = 0.427ml/kcal x 65% x [A] kcal/day = ________ml/day [C]
BS Cookies = 0.234g/kcal x 12.5% x [A] kcal/day = ________g/day [D]
CF Cookies = 0.229g/kcal x 12.5% x [A] kcal/day = ________g/day [E]

<table>
<thead>
<tr>
<th></th>
<th>Amount</th>
<th>Fat (g)</th>
<th>CHO (g)</th>
<th>Prot(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino Acids</td>
<td>[B]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formula</td>
<td>[C]</td>
<td>x 0.1</td>
<td>x 0.36</td>
<td>-</td>
</tr>
<tr>
<td>BS Cookies</td>
<td>[D]</td>
<td>x 0.223</td>
<td>x 0.565</td>
<td>-</td>
</tr>
<tr>
<td>CF Cookies</td>
<td>[E]</td>
<td>x 0.244</td>
<td>x 0.537</td>
<td>-</td>
</tr>
<tr>
<td>Total (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (kcal)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Actual Energy Intake = __________ kcal/day

Energy Distribution = ________% Fat, ________% CHO, ________% Prot
Calculating the Nitrogen (N) Content of the four (4) Amino Acids Being Varied in the Study: Using 1g/kg Protein Intake and Standard Egg Protein Pattern

Glycine:

Glycine Content in Egg Protein = 33.25mg/kg
Total glycine intake = 33.25mg/kg x wt/kg = [L] mg
Total N from glycine = 14000 x [L]/75070 = ____________ mg N from glycine [M]

Methionine:

Methionine content in egg protein = 29.67mg/kg
Total Met intake = 29.67mg/kg x wt/kg = [N] mg
Total N from Met = 14000 x [N]/149210 = ____________ mg N from Met [O]

Cysteine:

Cysteine content in egg protein = 22.11mg/kg
Total Cyst intake = 22.11mg/kg x wt/kg = [P] mg
Total N from Cyst = 14000 x [P]/121160 = ____________ mg N from Cyst [Q]

Alanine:

Alanine content in egg protein = 61.47mg/kg
Total alanine intake = 61.47mg/kg X wt/kg = [R] mg
Total N from alanine = 14000 X [R]/89090 = ____________ mg N from alanine [S]

Total N from gly, met, cys & alanine in standard egg protein = M+O+Q+S ____________mg/d [T]
Amino Acid Mix # 1 (study day)

Each meal represents 1/12 of daily intake (total of 10 meals = 83.33% of daily requirement)

______g Prot/day [B]

Mix # 1 represents 85.35% of the total AA reqt = ________g Mix # 1 / study day

______g Mix # 1 / study day 12 meals = ________g Amino acid mix # 1 / meal

The total of the other amino acids (gly, met, cys and Ala) = 14.65% of reqt = __________g [BB]

Glycine Intake

Glycine reqt/day = 66.50 mg/kg x ______kg = ______ mg[F]

Tracer Gly intake = [3.1228 + (7 x 1.171)] x _____kg = ______mg Gly [G]
N from tracer = 14000 x [G]/78070 = ____________mg N (tracer) [U]

Dietary Gly = [F] - [G] = ________mg [H]
N from dietary gly = 14000 x [H]/75070 = ____________mg N (dietary glycine) [V]

The dietary Gly should be distributed between the meals to standardize the intake of each meal. Initially balance the intakes to the highest intake at meal #4.

Dietary Gly intake distributed over 12 meals [F] / 12 ____________mg glycine/meal [Ff]

Meal # 4 Gly isotope intake = [3.1228 + (1.171)] mg/kg x ______kg = ______mg Gly [a]
Meal #4 Dietary Gly = [Ff] – [a] ____________mg Gly [aa]
Meal #4 Isotope Prime = (3.1228) mg/kg x ______kg = __________mg [ab]
Meal #5 - #12 isotope CI = (1.171) mg/kg x ______kg = __________mg/h [b]
Meal #5 - #12 balance Gly = [Ff] – [b] = ____________ mg / meal #5 - #12 [c]

Meal #1 - #3 balance Gly = [Ff] = __________mg / meal #1 - #3 [d]

Dietary intake meal #1 - #3 = [d] = __________mg [1]
Isotope Intake meal #4 = [a] = __________mg [aa]
Isotope Prime = [ab] = __________mg
Isotope CI = [b] = __________mg
Dietary Intake meal # 4 = [aa]=__________ mg [2]
Dietary intake meal #5 - #12 = [c] = __________mg [3]
**Amino Acid Recipe**

First column = mg of amino acids

Second column = ml of isotope solution

<table>
<thead>
<tr>
<th>Meal</th>
<th>AA Mix #1 (g)</th>
<th>Gly Intake (mg)</th>
<th>Met Intake (mg)</th>
<th>Cys Intake (mg)</th>
<th>Ala Intake (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tracer Diet</td>
<td>Diet</td>
<td>Diet</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>[1] [i] [k] [10]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>P</td>
<td>[2]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CI</td>
<td>[aa]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>[bb] [3]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td>A</td>
<td></td>
<td>B</td>
<td></td>
</tr>
</tbody>
</table>

**Isotope solution concentrations**

15N 13C gly 10 mg/ml

Base Mix (AA mix #1 + Alanine) = A + B

Per Meal = A + B/10

2 17/04/98 formula.plt
**Methionine Intake:**  \[ \text{set level of methionine intake} \]

Met intake/day  \[ = \underline{\text{kg}} \times 14\text{mg/kg/d} = \underline{\text{mg/d}} \] [h]

Met intake/meal  \[ = \text{[h]/12} = \underline{\text{mg/meal}} \] [i]

N from Met  \[ = 14000 \times \text{[h]/149210} = \underline{\text{mg N (dietary Met)}} \] [W]

**Cys Intake**

Level  \[ \underline{\text{mg/kg/d}} \]

Cys intake/day  \[ = \underline{\text{mg/kg/d}} \times \underline{\text{kg}} = \underline{\text{mg}} \] [j]

Cys intake/meal  \[ = \text{[j]/12} = \underline{\text{mg}} \] [k]

N from Cyst  \[ = 14000 \times \text{[j]/121160} = \underline{\text{mg N (dietary Cys)}} \] [X]

**Balance Alanine Intake:**

Alanine to be added to diet  \[ = \text{add the N from glycine (isotope + diet), met & cys} \]

\[ = \text{[U]} + \text{[V]} + \text{[W]} + \text{[x]} = \underline{\text{mg N/day}} \] [Y]

Subtract [Y] from base egg protein N  \[ = \text{[T]} - \text{[Y]} = \underline{\text{mg N/day}} \] [Z]

Alanine/day  \[ = \text{[Z]} \times 89090/14000 = \underline{\text{mg alanine/day}} \] [Z1] ÷ 1000

\[ = \underline{\text{grams alanine/day}} \]

Alanine/meal  \[ = \text{[Z1]/12} = \underline{\text{mg alanine/meal}} \] [10]
**Formula Requisition (Study day)**  Need 83.33% requirement i.e. 10 meals

Formula required per study day = \[C\] ml = ________ml/day \[C\]

Formula required per meal/d = \[C\] ml 12 = ________ml/meal \[O\]

Formula requisition = 11 x ________ml/meal \[O\] = ________ml/day \[P\]

**Formula Recipe**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Conc'n (g/ml)</th>
<th>Study day vol(ml) [P]</th>
<th>Study day amt(g)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product #80056</td>
<td>0.281</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orange Tang</td>
<td>0.0832</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Koolaid Crystals</td>
<td>0.0832</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn Oil</td>
<td>0.033</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sterile water</td>
<td>0.64</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Study day amt (g) = Conc'n (g/ml) x study day vol [P]

**Butterscotch Cookies (study day)**

BS Cookies required per study day = \[D\] = ________g/day \[D\]

\[D\] g BS Cookies required per meal = \[D\] 12 = ________g/meal

**Cornflake Cookies (study day)**

CF Cookies required per study day = \[E\] = ________g/day \[E\]

\[E\] g CF Cookies required per meal = \[E\] 12 = ________g/meal

** Each meal is served in a separate plastic bag **
### Diet Requisition Form:

<table>
<thead>
<tr>
<th>Subject's Name:</th>
<th>Delivery Date:</th>
</tr>
</thead>
</table>

### Formula Recipe: Study Day

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product #80056</td>
<td>_______g</td>
</tr>
<tr>
<td>Orange Tang</td>
<td>_______g</td>
</tr>
<tr>
<td>Koolaid Crystals</td>
<td>_______g</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>_______g</td>
</tr>
<tr>
<td>Sterile Water</td>
<td>_______g</td>
</tr>
</tbody>
</table>

Flavour: _______

Total volume of formula: _________ ml

__11__ Bottles _________ ml per bottle
Subject's Name:  
Delivery Date:  

**Butterscotch Cookie:**  
**Study Day**

__________ g BS cookie per day

___10___ meals per day  
__________ g BS Cookie per meal

**Cornflake Cookie:**  
**Study Day**

__________ g CF cookie per day

___10___ meals per day  
__________ g CF Cookie per meal

*** All meals should be divided into separate plastic bags, each labeled as follows:

- Subject name
- Meal Number
- Type of Cookie
- Weight (grams) of cookie per bag
11. Composition of crystalline L-amino acid mixtures used to determine “Does the addition of cysteine to the total sulphur amino acid requirement (methionine only) increase erythrocyte glutathione synthesis in the TPN fed human neonate.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Primene</th>
<th>Proposed Solution#1 (g/100 g total AA)</th>
<th>Bulk #1 GSH Study</th>
<th>Bulk #2 GSH Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ileucine</td>
<td>6.7</td>
<td>6.7</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.9</td>
<td>9.9</td>
<td>9.9</td>
<td>9.9</td>
</tr>
<tr>
<td>Valine</td>
<td>7.6</td>
<td>7.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine (lysine-HCL)</td>
<td>10.9</td>
<td>10.9</td>
<td>10.9</td>
<td>10.9</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.4</td>
<td>Variable</td>
<td>1.93</td>
<td>1.93</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1.9</td>
<td>0</td>
<td>0</td>
<td>0.33</td>
</tr>
<tr>
<td>Cysteine-HCL</td>
<td></td>
<td></td>
<td></td>
<td>0.48</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.2</td>
<td>3.7</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tyrosine (total) (GT)</td>
<td>0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Glycyl-L-Tyrosine Dihydrate</td>
<td></td>
<td>(5.25)</td>
<td>(5.25)</td>
<td>(5.25)</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.7</td>
<td>3.7</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.8</td>
<td>3.8</td>
<td>3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Arginine: base primene</td>
<td>8.4</td>
<td>(9.66)</td>
<td>9.66</td>
<td>9.66</td>
</tr>
<tr>
<td>solution (increase of 20% based on piglet data)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine (total) Glycine to be added (From GT)</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0 total</td>
<td>4.0 total</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.34 (1.66)</td>
<td>1.66 from GT from Glycine</td>
<td>1.66 from GT from Glycine</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.9</td>
<td>7.9</td>
<td>7.9</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Additional Variable amt added (to make solution isonitrogenous)</td>
<td>Additional Variable amt added (to make solution isonitrogenous)</td>
<td></td>
</tr>
<tr>
<td>Aspartate</td>
<td>6.0</td>
<td>6.0 (5.0)</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Glutamate</td>
<td>9.9</td>
<td>9.9</td>
<td>9.9</td>
<td>9.9</td>
</tr>
<tr>
<td>Proline</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Serine</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Taurine</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Ornathine (excluded because decreases the PH of the solution)</td>
<td>2.2</td>
<td>(0)</td>
<td>(0)</td>
<td>(0)</td>
</tr>
<tr>
<td>Total AA (grams/100g)</td>
<td>100.00</td>
<td>94.15*</td>
<td>98.38</td>
<td>98.71</td>
</tr>
</tbody>
</table>
*Proposed "Bulk Solution #1" represents 94.15% of the total protein/total AA.
Bulk #1 GSH study represents 98.38% of the total protein/total AA
Bulk #2 GSH study represents 98.71% of the total protein/total AA
11.1 calculation form for study “Does the addition of cysteine to the total sulphur amino acid requirement (methionine only) increase erythrocyte glutathione synthesis in the TPN fed human neonate. Study days 1 & 2

**Cysteine GSH Study**

**Working out the N Content of the Test TPN Solution:**

**Bulk solution #1**

**Amino Acid Mix Bulk Solution # 1 (study days 1 & 2)**

*Study day includes 100% of the total intake. Study conducted over a periods of 6 x 24 hr periods*

**Total Protein** (AA) = 3g/kg. x (wt) _________kg = __________g Prot/day[A]

**Total Volume of AA solution needed:**

- 5% Solution: 50g/L _______g [A]in _______mLs[B] ÷ wt (kg) _______ mL/kg
- 3% Solution: 30g/L _______g [A]in _______mLs[B] ÷ wt (kg) _______ mL/kg
- 2.5% Solution: 25g/L _______g [A]in _______mLs[B] ÷ wt (kg) _______ mL/kg
- 2% Solution: 20g/L _______g [A]in _______mLs[B] ÷ wt (kg) _______ mL/kg

**Total Nitrogen in Base Primene Solution:**

- 5% solution (7534 mg of N/L) = 7.534mg/mL x ___________mL[B] = ____________mg of N/day[C]
- 3% Solution (4520 mg of N/L) =4.52mg/mL x ___________mL[B] = ____________mg of N/day[C]
- 2.5% Solution (3767 mg of N/L) = 3.767mg/mL x ___________mL[B] = ____________mg of N/day[C]
- 2% solution (3013.5mg of N/L) =3.013mg/mL x ___________mL[B] = ____________mg of N/day[C]

**Total Nitrogen in Bulk Solution #1:**

- 5% solution (7438.042mg of N/L) = 7.438042mg/mL x ___________mL[B] = ____________mg of N/day[D]
- 3% solution (4462.8252mg of N/L) = 4.4628252mg/mL x ___________mL[B] = ____________mg of N/day[D]
- 2.5% Solution (3719.021mg of N/L) = 3.719021mg/mL x ___________mL[B] = ____________mg of N/day[D]
- 2% solution (2975.2168mg of N/L) = 2.9752168mg/mL x ___________mL[B] = ____________mg of N/day[D]

Difference of Nitrogen needed. [C] – [D] ___________________________mg/day[E]

*To be made up by Alanine*

**Alanine Intake: Alanine to make up the balance of the N needed**

Total N needed from Alanine: = [E] = __________mg of N from Ala [F]

Total Alanine needed in mg: = ([F] x 89090)/14000 = __________mg Ala/day [G]
Summary of Protein & AA To be Delivered to Patient

6. Protein (g/day) __________________________ [A]

7. Volume of AA Solution (mL/day)________________________ [B]

8. Total alanine intake (mg/day)__________________________[G]

**Volume of Alanine to be added.**

*Alanine: (To be Added to TPN Bulk Solution #1)*

**Alanine Intake: Alanine to make up the balance of the N needed**

Total Alanine needed (mg/day) _________________ [G]

Volume of Alanine: (stock solution 50mg/mL)

Total Alanine ________ mg[L] ÷ __________ mg/mL

= _______ mL

*Total Alanine in mg/L of TPN (Bulk Solution #1) = ([G] x 1000) ÷ [B] __________ mg/L alanine*
11.2 calculation form for study “Does the addition of cysteine to the total sulphur amino acid requirement (methionine only) increase erythrocyte glutathione synthesis in the TPN fed human neonate. Study day 3.

Cysteine-GSH Study

Page 1 of 2     Today’s Date: ______________________

Subject Parameters at time of study:
Patient #______________________ Date:_____________
Study Day:___________________  Pt Weight: _____ kg  Pt Length: ____ cm
Diagnosis: ___________________   Pt Age: _______ mths/weeks  Pt Head Circ: ____ cm
D.O B:______________

Birth Parameters:
Gest Age: _______ weeks  Birth Wt: _____ kg  Birth Length: _____ cm
Birth Head Circ: ____ cm
Study is conducted over a period of 6 x 24 hr periods.
Day 3 “Special Bulk #1” Plus alanine and $^{15}$N$^{13}$C-Glycine

Total AA Intake for each day [A] Using Special Bulk #1
__________ g AA/kg/day (range 2.5 to 3) x _________kg (wt) = __________g AA per day [A](from current intakes)

Total Daily Volume of TPN solution needed [ii]:
__________g AA [A] x 1000 ÷ _________g/L of AA in final TPN soln= __________mL [B]
(from TPN order form Day 1 & 2)
__________mL [B] ÷ __________kg (wt) = ______________mL/kg (range 100 – 150)

Individual Study & Special Amino Acid Bulk Base Solution for Study Day #3 and 6 only

$^{15}$N$^{13}$C-Glycine Tracer (isotope) Intake for Day 3
$^{15}$N$^{13}$C-Glycine intake = [3.9035 + (6.75 x 3.1228) = 24.98 mg/kg/day].
$^{15}$N$^{13}$C-Glycine Prime = 3.9035 mg/kg x __________kg = __________mg [C]
$^{15}$N$^{13}$C-Glycine Continuous Infusion
(CI) = 3.1228mg/kg/hr x _________kg=___________mg/hr x 6.75h = ______ mg [D]
$^{15}$N$^{13}$C-Glycine
(Prime + CI) mg/d = __________mg [C] + __________mg [D] = ________mg/day of 13C Phe [E]

Volume of $^{15}$N$^{13}$C-Glycine as Tracer (stock solution 10mg/mL) for pharmacy to send
Total volume of $^{15}$N$^{13}$C-Glycine = ________mg/day [E] ÷ 10mg/mL = __________mL
5 & 10mL vials available
Send: _____ x 5mL vials and ______ x 10mL vials [vial(s)]
(to be sent separately and not added to TPN bag)

Pharmacy: Cysteine-GSH Study

Page 2 of 2        Today’s Date:________________________

Balance Alanine Intake: (To be added to TPN solution days 1 to 6)
Balance Alanine Intake: Alanine to make up the balance of the N needed to keep the
TPN iso-nitrogenous.

Balance Alanine intake: ________ mg/kg/day [J] (provided by Glenda) x _____ kg (wt)=
________ mg of alanine/day[K]
Concentration of Balance Alanine in Day 3 study TPN:
Balance Alanine in Day 3 study TPN mg/L = (_______ mg [K] x 1000) ÷ ______ mL
[B]

= __________ mg/L [L] (pharmacy to enter into BAXA

Special Bulk Base AA Intake: (To Make Day 3 TPN Solution)
Special Bulk Base AA intake:
= (Total AA intake ______ g/day [A] x 1000) – [15N13C-Glycine intake
_______ mg/day [E] +
Balance alanine intake ______ mg/day [K].
=_______________ mg/day [M]

Special Bulk Base AA g/day = ________ mg/day [M] ÷ 1000 = _______ g/day [N]
Concentration of Special Bulk Base AA in Day 3 study TPN:
Conc of Special Bulk Base AA in Day 3 TPN (g/L):
= (____________ g/day [N] x1000) ÷ _____________ mL [B]

= _____________ g/L [Q] (pharmacy to enter into BAXA computer)

(Pharmacy: once checked, please make photocopy of this MD order to keep on file,
return original to Glenda)

Date:_________________   Signature: ______________________________(MD)

Time:_________________   Print Name: ____________________________
11.3 calculation form for study “Does the addition of cysteine to the total sulphur amino acid requirement (methionine only) increase erythrocyte glutathione synthesis in the TPN fed human neonate. Study days 4 & 5.

Cysteine GSH Study: Days 4 & 5

Using Bulk Solution #2

Working out the N Content of the Test TPN Solution:

<table>
<thead>
<tr>
<th>Amino Acid Mix Bulk Solution # 2 (study days 4 &amp; 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study day includes 100% of the total intake. Study conducted over a periods of 6 x 24 hr periods</td>
</tr>
</tbody>
</table>

**Total Protein (AA)** = 3g/kg. x (wt) ________kg = ________g Prot/day[A]

**Total Volume of AA solution needed:**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration (g/L)</th>
<th>Volume needed (g)</th>
<th>Volume needed (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Solution</td>
<td>50</td>
<td>[A] x in mLs[B] ( \div ) wt (kg) = ________mL/kg</td>
<td></td>
</tr>
<tr>
<td>3% Solution</td>
<td>30</td>
<td>[A] x in mLs[B] ( \div ) wt (kg) = ________mL/kg</td>
<td></td>
</tr>
<tr>
<td>2.5% Solution</td>
<td>25</td>
<td>[A] x in mLs[B] ( \div ) wt (kg) = ________mL/kg</td>
<td></td>
</tr>
<tr>
<td>2% Solution</td>
<td>20</td>
<td>[A] x in mLs[B] ( \div ) wt (kg) = ________mL/kg</td>
<td></td>
</tr>
</tbody>
</table>

**Total Nitrogen in Base Primene Solution:**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration (mg of N/L)</th>
<th>Volume needed (mL)</th>
<th>Nitrogen needed (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% solution</td>
<td>7534</td>
<td>_________mL[B] = ________mg of N/day[C]</td>
<td></td>
</tr>
<tr>
<td>3% Solution</td>
<td>4520</td>
<td>_________mL[B] = ________mg of N/day[C]</td>
<td></td>
</tr>
<tr>
<td>2.5% Solution</td>
<td>3767</td>
<td>_________mL[B] = ________mg of N/day[C]</td>
<td></td>
</tr>
<tr>
<td>2% Solution</td>
<td>3013</td>
<td>_________mL[B] = ________mg of N/day[C]</td>
<td></td>
</tr>
</tbody>
</table>

**Total Nitrogen in Bulk Solution #2:**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration (mg of N/L)</th>
<th>Volume needed (mL)</th>
<th>Nitrogen needed (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% solution</td>
<td>7457.108</td>
<td>_________mL[B] = ________mg of N/day[D]</td>
<td></td>
</tr>
<tr>
<td>3% Solution</td>
<td>4474.2648</td>
<td>_________mL[B] = ________mg of N/day[D]</td>
<td></td>
</tr>
<tr>
<td>2.5% Solution</td>
<td>3728.554</td>
<td>_________mL[B] = ________mg of N/day[D]</td>
<td></td>
</tr>
<tr>
<td>2% Solution</td>
<td>2982.8432</td>
<td>_________mL[B] = ________mg of N/day[D]</td>
<td></td>
</tr>
</tbody>
</table>

Difference of Nitrogen needed. [C] – [D] ___________________________mg/day[E]  
*To be made up by Alanine*

Alanine Intake: Alanine to make up the balance of the N needed
Total N needed from Alanine: = [E] = ___________ mg of N from Ala [F]
Total Alanine needed in mg: = ([F] x 89090)/14000 = __________ mg Ala/day [G]

**Summary of Protein & AA To be Delivered to Patient**

9. Protein (g/day) _________________________ [A]

10. Volume of AA Solution (mL/day) _________________________ [B]

11. Total alanine intake (mg/day) _________________________ [G]

**Volume of Alanine to be added.**

*Alanine:* (To be Added to TPN Bulk Solution #2)

*Alanine Intake:* Alanine to make up the balance of the N needed

Total Alanine needed (mg/day) _________________________ [G]

Volume of Alanine: (stock solution 50mg/mL)

Total Alanine _________________________ mg[L] ÷ _________________________ mg/mL = ________ mL

*Total Alanine in mg/L of TPN (Bulk Solution #2) = ([G] x 1000) ÷ [B] _________________________ mg/L alanine*
11.4 calculation form for study “Does the addition of cysteine to the total sulphur amino acid requirement (methionine only) increase erythrocyte glutathione synthesis in the TPN fed human neonate. Study day 6.

Cysteine-GSH Study

Page 1 of 2     Today’s Date: ______________________

Subject Parameters at time of study:
Patient #______________________ Date:_____________
Study Day:___________________ Diagnosis: ___________________   Pt Weight: ________kg  Pt Length: _______cm
D.O B:______________            Pt Age: _______ mths/weeks  Pt Head Circ: ______cm

Birth Parameters:Gest Age: _____ weeks   Birth Wt: _________ kg Birth
Length: ______ cm   Birth Head Circ:____cm

Study is conducted over a period of 6 x 24 hr periods.
Day 6 “Special Bulk #2” Plus alanine and $^{15}$N$^{13}$C-Glycine

Total AA Intake for each day [A] Using Special Bulk #2
$\frac{\text{g AA/kg/day (range 2.5 to 3) x } \text{kg (wt)}}{\text{g AA per day [A]}}$ (from current intakes)

Total Daily Volume of TPN solution needed [ii]:
$\frac{\text{g AA} \times 1000}{\text{g/L of AA in final TPN soln}}$ mL [B]
(from TPN order form Day 1 & 2)

$\frac{\text{mL} [B]}{\text{kg (wt)}}$ mL/kg (range 100 – 150)

Individual Study & Special Amino Acid Bulk Base Solution for Study Day #3 and 6 only

$^{15}$N$^{13}$C-Glycine Tracer (isotope) Intake for Day 3
$^{15}$N$^{13}$C-Glycine intake = [3.9035 + (6.75 x 3.1228)] = 24.98 mg/kg/day].

$^{15}$N$^{13}$C-Glycine Prime = 3.9035 mg/kg x ________kg = __________mg [C]

$^{15}$N$^{13}$C-Glycine Continuous Infusion
(CT) = 3.1228mg/kg/hr x ________kg = __________ mg/hr x 6.75h = __________ mg [D]

$^{15}$N$^{13}$C-Glycine
(Prime + CT) mg/d = ________mg [C] + ________mg [D] = ________mg/day of 13C Phe [E]

Volume of $^{15}$N$^{13}$C-Glycine as Tracer (stock solution 10mg/mL) for pharmacy to send
Total volume of $^{15}$N$^{13}$C-Glycine = ________mg/day [E] ÷ 10mg/mL = __________mL

5 &10mL vials available
Send: ____x 5mL vials and ____x10mL vials [vial(s)]
(to be sent separately and not added to TPN bag)]
Pharmacy: Cysteine-GSH Study

Balance Alanine Intake: (To be added to TPN solution days 1 to 6)
Balance Alanine Intake: Alanine to make up the balance of the N needed to keep the TPN iso-nitrogenous.

Balance Alanine intake: _______mg/kg/day [J] (provided by Glenda) x _____ kg (wt)= _______mg of alanine/day[K]

Concentration of Balance Alanine in Day 6 study TPN:
Balance Alanine in Day 6 study TPN mg/L = (_______mg [K] x 1000) ÷ _______mL [B]

= __________mg/L [L] (pharmacy to enter into BAXA)

Special Bulk Base AA Intake: (To Make Day 6 TPN Solution)
Special Bulk Base AA intake:
= (Total AA intake _______g/day [A] x 1000) – \[^{15}\text{N}^{13}\text{C}\]-Glycine intake ________mg/day [E] + Balance alanine intake _______mg/day [K].

= _______________ mg/day [M]

Special Bulk Base AA g/day = _______mg/day [M] ÷ 1000 = _______ g/day [N]

Concentration of Special Bulk Base AA in Day 6 study TPN:
Conc of Special Bulk Base AA in Day 6 TPN (g/L):
= (____________g/day [N] x1000) ÷ _____________mL [B]

= ______________ g/L [Q] (pharmacy to enter into BAXA computer)

(Pharmacy: once checked, please make photocopy of this MD order to keep on file, return original to Glenda)

Date: _______________ Signature: __________________________(MD)

Time: _______________ Print Name: __________________________
11.5 Comparison of breakpoint

The mean requirement for methionine was determined for the PN fed human neonate using a two-phase linear regression crossover model. This model allows a partitioning of the data between two separate regression lines that minimize the residual error. This results in a crossover value that is termed the breakpoint. This breakpoint estimates the amino acid requirement of the sample population.

11.5.1 Breakpoint determination

All individual data points ($F^{13}$CO$_2$ or Oxidation) are plotted on the y-axis vs. levels of test amino acid intake (methionine) on the x-axis. Each point on the x-axis is representative of an individual subject. An estimation of the breakpoint is made by visual inspection in order to separate the test amino acid intakes into two regression lines. If the breakpoint cannot be visually determined all possibilities should be analyzed.

The analysis requires that there are at least three points on either side of the breakpoint to construct a regression line. Sometimes there is an exception and there are only two points on the right side of the breakpoint. There are four possible models programmed into SAS:

1. One line has a slope, unweighted regression
2. One line has a slope, weighted regression
3. Both lines have sloped, unweighted regression
4. Both lines have a slow, weighted regression

According to the IAAO concept, the second line should not have a slope or the slope should be minimal. The purpose of weighting the data is to reduce the numerical effect such that the points at the end of the line have a similar effect on the slope as the
points closer to the mean. Variances along a slope regression have proportionally equal
distance to the mean. Variance increases at the end of a line but it is proportionally
equal to the smaller variance of a point, which is nearer to the mean. When only one line
has a slope, weighting will only change the sloping line and the second line will have a
fixed slope of zero.

**Regression equations:**

Both lines sloping: \[ y = a_2 + (a_1 - a_2)C + b_2x + (b_1 - b_2)Cx + e \]

One line sloping: \[ y = a_2 + (a_1 - a_2)C + (b_1 - b_2)Cx + e \]

Where \( y = F^{13}CO_2 \) or Oxidation

\[ X = \text{test amino acid intake} \]

\( a_1 = \text{intercept of the first line} \)

\( a_2 = \text{intercept of the second line} \)

\( b_1 = \text{slope of the first line} \)

\( b_2 = \text{slope of the second line} \)

\( e = \text{the error associated with the model} \)

The parameter C (choose) is used to determine the equation of the individual regression
lines: \( C = 1 \) for observations before the breakpoint (first line) and \( C = 0 \) for observations
after the breakpoint (second line).

**Both lines sloping:** First line (\( C = 1 \)):

\[ y = a_2 + (a_1 - a_2) + b_2x + (b_1 - b_2) x \]

\[ y = a_1 + b_1x \]

Second line (\( C = 0 \)):

\[ y = a_2 + b_2 x \]

**One line sloping:** First line (\( C = 1 \)):

\[ y = a_2 + (a_1 - a_2) + (b_1 - b_2) x \]

\[ y = a_1 + (b_1 - b_2) x \]
Second line (C = 0): \( y = a_2 \)

\( b_2 = 0 \) since the second line does not have a slope

**Determine breakpoint:**

The breakpoint (estimated mean requirement) is where the two lines intersect.

*Both lines sloping:* \( a_1 + b_1x = a_2 + b_2x \) \[ \Rightarrow x = - \frac{(a_1 - a_2)}{(b_1 - b_2)} \]

*One line sloping:* \( a_1 + (b_1 - b_2)x = a_2 \) \[ \Rightarrow x = - \frac{(a_1 - a_2)}{(b_1 - b_2)} \]

\((a_1 - a_2)\) and \((b_1 - b_2)\) can be obtained directly from the SAS output as the parameters choose and met*choose (metchoose).

**Choosing the model:**

Once all four models are executed (all possibilities of the x-axis to the two regression lines), the model that best fit the data is chosen. The considered parameters are:

- The lowest standard error of the largest parameter (choose). The lowest error ensures that any estimation of the breakpoint will be the most accurate.
- The lowest root mean square error. The aim of linear regression is to minimize the sum of the squared distances of the points from the line
- The highest \( r^2 \) should be considered. It indicates that the slopes of the lines most closely match up to the mean.

**SAS program for breakpoint analysis:**

**Variables:**

Sub = subject
Met = test amino acid intake (methionine)

$\text{f13co2} = \text{individual rate of }^{13}\text{CO}_2\text{ released from}\ L-[1-^{13}\text{C}]\text{phenylalanine}$

wgt = weighting factor

**Parameters of the model are set:**

- intercept $= a_2$, intercept of the second line
- met $= b_2$, slope of the second line
- choose $= (a_1 - a_2)$, difference between the intercept
- metchoose $= (b_1 - b_2)$, difference between the slopes

The estimated position of the breakpoint is entered into the model by defining where choose = 0 or 1.

For example,

Choose = 0

```plaintext
options pagesize=60 nodate nonumber;
options formdlim="-";
data study1;
   input Subj met f13co2 ;
   wgt = 1/f13co2;
   cards;
1 9.5 2.5903
2 14.87 1.7494
3 19.16 1.3387
4 24.5 1.4200
5 29.95 1.2868
6 31.8 1.3250
7 33.9 0.9783
8 39.1 0.7152
9 43.7 0.9632
10 49.14 0.6054
11 55.6 0.6066
12 61.5 0.8486
13 61.51 0.3362
14 77.6 0.5368
15 86.3 0.4811
16 99.86 0.9419
17 107.0 0.7467
18 118.7 0.4107
;
proc print data = study1;
run;
```
```sas
proc glm data = study1;
class subj met;
model f13co2 = met subj;
means met/ duncan tukey;
run;
proc mixed data = study1;
class subj met;
model f13co2 = met/ddfm=kr outp=temp;
random subj;
lsmeans met /pdiff adjust=tukey;
run;
proc sort data=study1;
by subj;
run;

data bkpt;
set study1;
run;
proc print data=bkpt;
var met subj f13co2 ;
run;

data cutpt49_14; set bkpt; choose = 0;
if met le 49.9 then choose =1;
metchoose = met * choose;
run;
proc MIXED data=cutpt49_14;
title1 'Met intake 9.5, 14.87, 19.16, 24.5, 29.95, 31.8, 33.9, 39.1, 43.7, 49.14 VS 55.6, 61.5, 61.51, 77.6, 99.86, 107.0, 118.7';
title2 ' ';
title3 ' ';
model f13co2=choose metchoose;
random subj;
run;
proc reg data = cutpt49_14 outest = param1 covout outsscp =sse ;
title1 'Met intake 9.5, 14.87, 19.16, 24.5, 29.95, 31.8, 33.9, 39.1, 43.7, 49.14 VS 55.6, 61.5, 61.51, 77.6, 99.86, 107.0, 118.7';
title2 'One line has a Slope';
title3 'Unweighted Regression';
model f13co2 = choose metchoose;
run;
proc print data=param1;
run;
proc print data=sse;
run;
```

**Example of SAS output of breakpoint**

```
Example of SAS output of breakpoint

Met intake 9.5, 14.87, 19.16, 24.5, 29.95, 31.8, 33.9, 39.1, 43.7, 49.14 VS 55.6, 61.5, 61.51, 77
One line has a Slope
Unweighted Regression
```

The REG Procedure
Model: MODEL1
Dependent Variable: f13co2

Number of Observations Read 18

277
### Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>2</td>
<td>4.45973</td>
<td>2.22987</td>
<td>38.68</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>15</td>
<td>0.86472</td>
<td>0.05765</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>17</td>
<td>5.32445</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Root MSE: 0.24010  
R-Square: 0.8376  
Dependent Mean: 0.99338  
Adj R-Sq: 0.8159  
Coeff Var: 24.16987

### Parameter Estimates

| Variable      | DF | Estimate | Standard Error | t Value | Pr > |t| |
|---------------|----|----------|----------------|---------|------|---|
| Intercept     | 1  | 0.61357  | 0.08489        | 7.23    | <.0001|
| choose        | 1  | 1.88183  | 0.21842        | 8.62    | <.0001|
| metchoose     | 1  | -0.04053 | 0.00630        | -6.43   | <.0001|

Met intake 9.5, 14.87, 19.16, 24.5, 29.95, 31.8, 33.9, 39.1, 43.7, 49.14 VS 55.6, 61.5, 61.51, 77
One line has a Slope
Unweighted Regression

<table>
<thead>
<tr>
<th>Obs</th>
<th>MODEL</th>
<th><em>TYPE</em></th>
<th><em>NAME</em></th>
<th><em>DEPVAR</em></th>
<th><em>RMSE</em></th>
<th>Intercept</th>
<th>choose</th>
<th>metchoose</th>
<th>f13co2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MODEL1</td>
<td>PARMS</td>
<td>f13co2</td>
<td>0.24010</td>
<td>0.61357</td>
<td>1.88183</td>
<td>-0.04053</td>
<td>-1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>MODEL1</td>
<td>COV</td>
<td>Intercept</td>
<td>0.24010</td>
<td>0.00721</td>
<td>-0.00721</td>
<td>0.00000</td>
<td>.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>MODEL1</td>
<td>COV</td>
<td>choose</td>
<td>0.24010</td>
<td>-0.00721</td>
<td>0.04771</td>
<td>-0.00117</td>
<td>.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>MODEL1</td>
<td>COV</td>
<td>metchoose</td>
<td>0.24010</td>
<td>0.00000</td>
<td>-0.00118</td>
<td>0.000040</td>
<td>.</td>
<td></td>
</tr>
</tbody>
</table>

Met intake 9.5, 14.87, 19.16, 24.5, 29.95, 31.8, 33.9, 39.1, 43.7, 49.14 VS 55.6, 61.5, 61.51, 77
One line has a Slope
Unweighted Regression

<table>
<thead>
<tr>
<th>Obs</th>
<th><em>TYPE</em></th>
<th><em>NAME</em></th>
<th>Intercept</th>
<th>choose</th>
<th>metchoose</th>
<th>f13co2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SSCP</td>
<td>Intercept</td>
<td>18.000</td>
<td>10.000</td>
<td>295.62</td>
<td>17.881</td>
</tr>
<tr>
<td>2</td>
<td>SSCP</td>
<td>choose</td>
<td>10.000</td>
<td>10.000</td>
<td>295.62</td>
<td>12.972</td>
</tr>
<tr>
<td>3</td>
<td>SSCP</td>
<td>metchoose</td>
<td>295.620</td>
<td>295.620</td>
<td>10189.41</td>
<td>324.705</td>
</tr>
<tr>
<td>4</td>
<td>SSCP</td>
<td>f13co2</td>
<td>17.881</td>
<td>12.972</td>
<td>324.71</td>
<td>23.087</td>
</tr>
<tr>
<td>5</td>
<td>N</td>
<td></td>
<td>18.000</td>
<td>18.000</td>
<td>18.00</td>
<td>1</td>
</tr>
</tbody>
</table>